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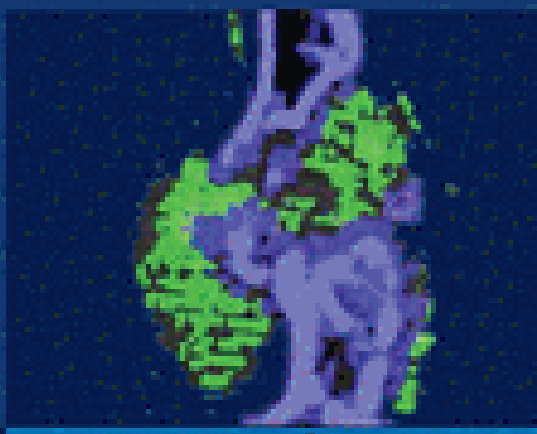

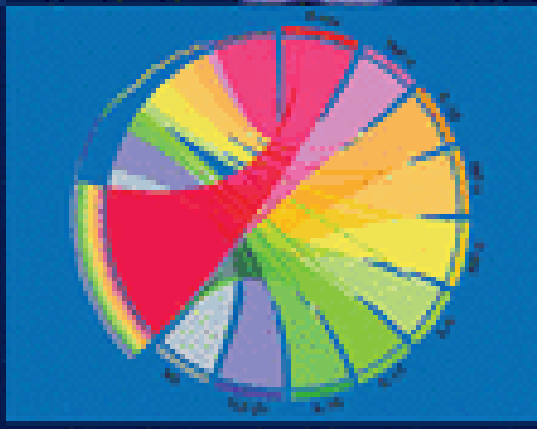

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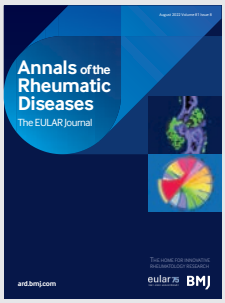

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Synovial fibroblasts as potential drug targets in rheumatoid arthritis, where do we stand and where shall we go?

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ABSTRACT

Fibroblast-like synoviocytes or synovial fibroblasts (FLS) are important cellular components of the inner layer of the joint capsule, referred to as the synovial membrane. They can be found in both layers of this synovial membrane and contribute to normal joint function by producing extracellular matrix components and lubricants. However, under inflammatory conditions like in rheumatoid arthritis (RA), they may start to proliferate, undergo phenotypical changes and become central elements in the perpetuation of inflammation through their direct and indirect destructive functions. Their importance in autoimmune joint disorders makes them attractive cellular targets, and as mesenchymal-derived cells, their inhibition may be carried out without immunosuppressive consequences. Here, we aim to give an overview of our current understanding of the target potential of these cells in RA.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disorder that affects 0.5%–1% of the Western world population.[1] There are several cell types that have been implicated in the pathogenesis of RA, among which both immune and non-immune cells can be found.[1] The prominent role of immune cells in autoimmune arthritis has been based on a plethora of experimental findings as well the successful use of immunosuppressive therapies in clinical practice. Particularly, novel targeted therapies that interfere with specific pathways of the inflammatory and immune response such as antibodies against inflammatory cytokines or against surface molecules on immune cells have revolutionised the therapy of RA. Nonetheless, still a considerable proportion of patients with RA do not respond adequately to available therapies.[2] In other words, although with the introduction of biological and targeted synthetic disease-modifying antirheumatic drugs (DMARDs), the treatment of RA improved significantly, a notable part of patients remains symptomatic. These patients might be considered as having 'difficult-to-treat' or 'refractory' RA, which categories represent great therapeutic challenges both to the medical teams and to the patients. In addition to new management strategies, the optimal care of these patients require new therapeutic drug targets. Meanwhile, there is an increasing body of evidence to suggest a crucial role for mesenchymal cell-derived cells, particularly fibroblast-like synoviocytes (FLS) in mediating both direct tissue injury and perpetuation of the complex disease process in autoimmune joint disorders like RA.[1]

FLS represent one key cell type of the synovial membrane and can be found in both the lining and the sublining layers of the membrane.[3] The normal function of FLS is essential for maintaining the homeostasis of the diarthrodial joints, where they are responsible for the production of the extracellular matrix, but also regulate their environment by releasing matrix metalloproteinases (MMPs).[3] Importantly, FLS in the lining layer cannot be considered as conventional barrier-forming cells at the border of the synovium and the synovial fluid, as they have a pronounced linking rather than a blocking feature and provide the joint cavity and the adjacent cartilage with lubricating molecules such as hyaluronic acid as well as with nutritive plasma. This is due to a peculiarity of the synovial membrane that unlike other border membranes lacks an organised basement membrane, and thus, the classical architecture of an epithelium. Several studies have demonstrated that although cellular contacts between the fibroblast-like synoviocytes lack tight junctions and desmosomes, there are specific adhesion molecules such as cadherin-11, which mediate a strong homophilic adhesion between synoviocytes through interaction with organisers of the cytoskeleton and that are largely responsible for their organisation into a tissue.[4 5]

Under inflammatory conditions such as in RA, FLS can undergo both morphological and phenotypical changes and transform into essential cellular component of the disease process.[1 6 7] These changes have been described in detail in a number of reviews and comprise a complex set of cellular changes that eventually result in the proliferation and differentiation into distinct subpopulations.[8 9] During this process, some FLS acquire invasive, tumour cell-like destructive characteristics through epigenetic and metabolic changes as well as through interaction with both themselves and different immune cells like lymphocytes or macrophages.[5 10] As a consequence of their transformation, some FLS subsets contribute prominently to the destruction of articular structures while others have been hypothesised to exert more regulatory functions.[6] These effector functions and orchestrating roles of FLS in autoimmune joint disorders, particularly in RA, make them attractive potential cellular targets with the possibility to promote disease control without immunosuppression in RA. In this review, we aim to give an overview of the different potential routes of intervention to influence the fate and function of RA-FLS (figures 1 and 2, table 1). Except for some selected cases, we do not discuss the effects of currently used conventional synthetic or biological DMARDs (cs-DMARDs or bDMARDs).

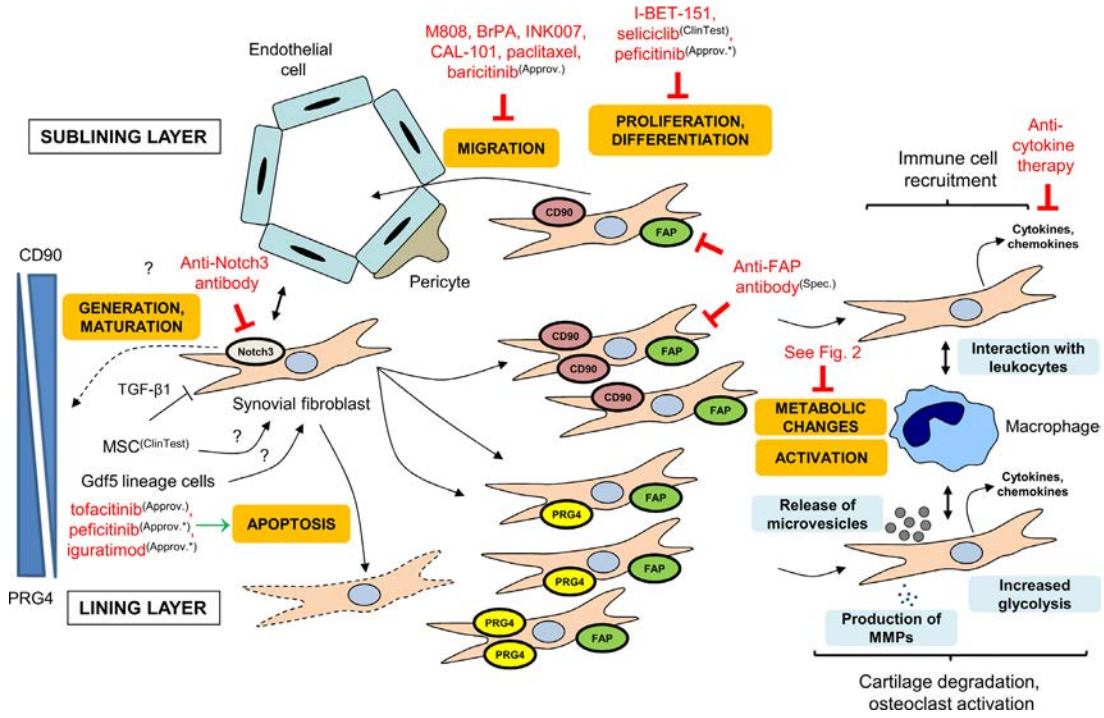

Figure 1 Overview of targeting RA-FLS. RA-FLS can be influenced at different points of their life cycle and activation. For example, the generation and proliferation, the migration, the direct destructive functions and the interactions with leukocytes can be targeted. Approv., approved in the European Union (EU); Approv.*, approved outside of the EU; BET, bromodomain and extraterminal protein; ClinTest, clinically tested; FAP, fibroblast activation protein; FLS, fibroblast-like synoviocytes; MMP, matrix metalloproteinase; MSC, mesenchymal stem cell; PRG4, proteoglycan 4; RA, rheumatoid arthritis; Spec., specific targeting molecule; TGF-β1, transforming growth factor β1.

TARGETING THE GENERATION AND DIFFERENTIATION OF FLS

The exact origin and pathways of transformation of RA-FLS is still a matter of debate, but understanding their origin is crucial for therapeutic strategies to interfere with their specific phenotypes. While it has been believed that RA-FLS are generated in the inflamed synovium through transformation or differentiation of existing synovial fibroblasts, recent interest has expanded towards the role of mesenchymal progenitors as well as potential transdifferentiation events.

There is recent evidence that the adult synovium harbours unique joint morphogenic cells that expand in response to injury and may give rise to FLS. It has been shown through lineage tracing experiments that Gdf5-lineage cells persist as mesenchymal stem cells (MSCs) in the adult synovium and that FLS can be differentiated from these Gdf5-positive interzone MSCs.[11] Based on the observation that these FLS are negative for nestin, leptin receptor and gremlin-1, it has further been hypothesised that Gdf5 lineage cells in the adult synovium differ and are distinct from skeletal stem cells found in other locations

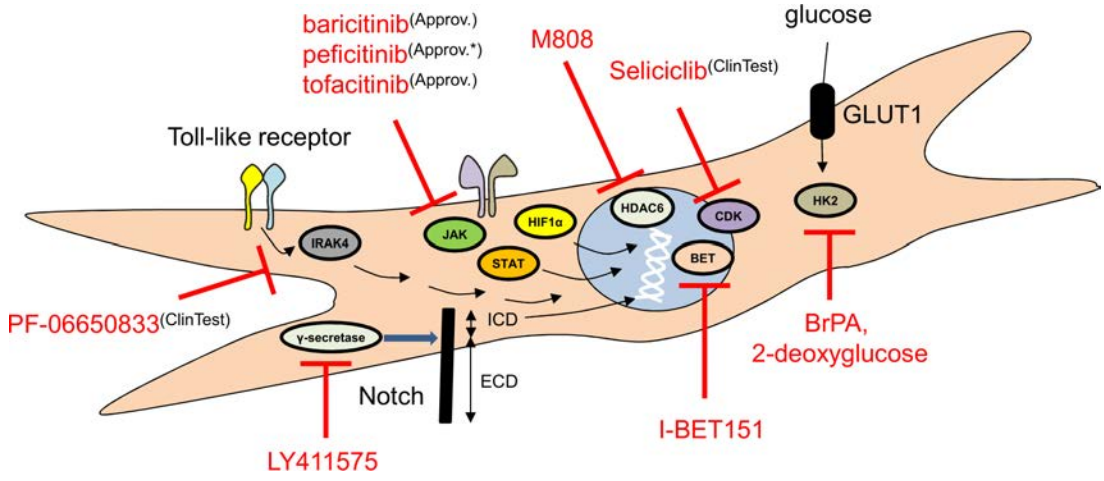

Figure 2 Potential intracellular molecular targets of RA-FLS. There are several attractive intracellular molecules that can be blocked in RA-FLS: inhibitors of proteins participating in epigenetic changes, in signal transduction or in proliferation may have beneficial effects in RA. Approv., approved in the European Union (EU); Approv.*, approved outside of the EU; BET, bromodomain and extraterminal protein; BrPA, 3-bromopyruvate; ClinTest, clinically tested; FLS, fibroblast-like synoviocytes; ICD, intracellular domain; RA, rheumatoid arthritis.

Table 1 Drugs and interventions in the pipeline aiming to control FLS in RA

Target/aim	Drug/intervention name	Company	Mouse (M) or human (H) data (clinical study phase)	Comments	Reference(s)
Notch3	Anti-Notch3 antibody	–	M	An anti-Notch3 antibody decreased inflammation in a mouse arthritis model.	10
SSAT-1	Diminazene aceturate	Sigma-Aldrich	H	The use of the SSAT-1 inhibitor diminazene aceturate decreased the expression of β_1 integrin, CXCL12 (stromal cell-derived factor 1, SDF-1) and MMP1 of RA-FLS; however, the reduction was more pronounced when the inhibitor was used with *S*-adenosyl methionine supplementation.	21
HDAC6	M808	?	M	M808 could decrease the MMP and cytokine release of RA-FLS, while attenuating their migration. Meanwhile, M808 had a positive effect in the rodent adjuvant-induced arthritis model.	23
BET proteins	I-BET151	GlaxoSmithKline	H	I-BET151 could significantly reduce the MMP1, MMP3, IL-6 and IL-8 production of TNFα-, IL-1ß- or Toll-like receptor agonist-activated synovial fibroblasts, while the proliferation rate was also affected.	25
Hexokinase 2	► BrPA ► 2-Deoxyglycose	Sigma-Aldrich	M, H	1. BrPA could reduce the activation of RA-FLS and attenuate joint inflammation in the K/BxN serum transfer arthritis. 2. The administration of 2-deoxyglucose, another inhibitor of hexokinase 2 could decrease the cytokine and MMP release and production of TNF-stimulated healthy and non-activated RA-FLS.	27 28
Notch	LY411575	Selleckchem	M	LY411575, an inhibitor of Notch cleavage, could effectively attenuate the inflammation, the cartilage and bone destruction in a rodent model of RA.	36
Cyclin-dependent kinases (eg, CDK2, CDK7, CDK9)	Seliciclib	Cyclacel Pharmaceuticals	H (phase 1b)	It is tested in TNF-inhibitor-refractory patients with RA (TRAFIC trial).	38
PDGFR, PI3K or GSK-3	PDGFR, PI3K or GSK-3 inhibitors	Merck Millipore, ChemScene, Active Biochem, Sigma-Aldrich	M	These inhibitors were shown to decrease the invasive characteristics of RA-FLS under in vitro circumstances.	47
IRAK4	PF-06650833	Pfizer	M, H (phase 2)	1. The inhibition of IRAK4 reduced the cytokine and MMP release of human RA-FLS. 2. The in vivo administration of PF-06650833 could attenuate the severity of collagen-induced arthritis. 3. PF-06650833 is being tested in a phase 2 trial in patients with RA (ClinicalTrials.gov identifier: NCT04413617).	55
FAPα	Depletion of FAPα-positive FLS by targeted photodynamic therapy	?	M	The conjugated antibody-treated animals showed a decreased inflammation score in the collagen-induced arthritis model.	59
Syndecan-4	Anti-syndecan-4 antibody	–	M	The antibody directed against the dimerisation domain of syndecan-4 could effectively decrease the expression of IL-1 receptor on FLS and reduced the pannus formation, the cartilage destruction and the MMP3 content of the affected joints in the TNF transgenic mouse arthritis model.	81
RA-FLS signalling and function	Mesenchymal stem cell therapy	Different sources	M, H (phase 1 and 2)	There are several ongoing clinical trials (see ClinicalTrials.gov identifiers NCT03618784, NCT03333681 or NCT03691909).	82 83

BET, bromodomain and extraterminal protein; BrPA, 3-bromopyruvate; FAPα, fibroblast activation protein-α; FLS, fibroblast-like synoviocytes; GSK-3, glycogen synthase kinase 3; HDAC6, histone deacetylase 6 ; IL-1, interleukin 1; MMP, matrix metalloproteinase; PDGFR, platelet-derived growth factor receptor; PI3K, phosphoinositide 3-kinase.; RA, rheumatoid arthritis; SSAT-1, spermidine/spermine-N^1-acetyltransferase; TGF-β1, transforming growth factor β1; TNF, tumour necrosis factor.

such as in the bone marrow. Moreover, joint surface injury by medial parapatellar arthrotomy led to a proliferative response in these Gdf5 lineage cells that clearly contributed to synovial hyperplasia. Most interestingly, conditional knockout of YAP1 in Gdf5-lineage cells prevented synovial hyperplasia after cartilage injury pointing to one targetable pathway involved in the specific response of these cells.[11]

These findings are of high interest for RA and related conditions because it has been hypothesised that cartilage damage is an early and central triggering factor for the recruitment and activation of RA-FLS.[12 13] Consequently, it may be speculated that during RA, quiescent Gdf5 lineage cells in response to inflammatory cartilage damage along with concomitant immune activation re-enter the cell cycle and differentiate into specific RA-FLS populations. Pathways relevant for initiating this differentiation, such as the YAP pathway along with a more precise description of these progenitors in the future may, therefore, serve as a basis for therapeutically reducing the number of RA-FLS in the diseased

synovium. In addition, platelet-derived growth factor receptor α-expressing Gdf5 lineage cells were found to expand during experimental arthritis and the deletion of YAP from these cells reduced arthritis severity.[14] However, it is important to emphasise that the role of these Gdf5 lineage cells in inflammatory arthritis needs further investigations.

The differentiation of FLS can be mediated by cells of the vessel wall through a JAG1/DLL4-Notch3 interaction.[10] In a recent paper, Notch3-positive and Notch-activated FLS have been found to be upregulated in the RA synovium compared with osteoarthritic (OA) tissues and Notch3 was identified as a central element of the differentiation of CD90-positive perivascular and sublining FLS.[10] It was shown that in the synovium, the Notch ligands JAG1 and DLL4 had higher expression in the arterial than in the venous endothelial cells and Notch3 mRNA/protein levels were elevated in mural cells (like pericytes and vascular smooth muscle cells) and sublining FLS.[10] These data may indicate that the positional identity of CD90-positive sublining FLS is orchestrated by arterial endothelial cells through a JAG1/DLL4-Notch3 interaction.[10] Consistent with this, elevated levels of Notch3 intracellular domain (Notch3-ICD) were detected in JAG1- or DLL4-activated FLS.[10] While Notch3-deficient mice exhibited normal joint structure, those animals showed a significantly reduced arthritis severity compared with wild type ones.[10] Moreover, the pharmacological intervention by a Notch3-targeting antibody was able to attenuate the extent of joint inflammation and the appearance of bone erosions pointing to the effect of FLS on osteoclast activation.[10]

However, gene regulation and signalling behind the differentiation of synovial fibroblasts remain mainly undiscovered in contrast to the fact that it could be a robust and effective area, where FLS may be targeted to block the development of an aggressive and destructive phenotype in RA. This is highlighted by the finding that FLS can account for a significant percentage of RA heritability, and the function of some putative causal genes may be modified in the future.[15]

CONTROLLING THE AGGRESSIVELY IMPRINTED PHENOTYPE

Under non-infectious inflammation, RA-FLS undergo phenotypical changes and acquire an aggressive phenotype. Their destructive behaviour (which affects cartilage and bone structures) has been suggested to be due at least partly to epigenetic and metabolic changes. The consecutive higher proliferation rate and invasive migratory characteristics make them important effectors and ideal cellular targets in RA.

Epigenetic changes of FLS in the focus of intervention

Epigenetic changes result in the modification of gene expression without the alteration of the original DNA base sequence. There are several mechanisms of epigenetics like DNA methylation, histone modifications (eg, acetylation, ubiquitination, phosphorylation or methylation) or the transcription of non-coding RNA molecules.[16] In RA-FLS, DNA hypomethylation and subsequently increased gene expression could be observed with pathways mediating cell migration or extracellular matrix interactions pointing to a possible role of these changes in the pathogenesis of RA.[17] In line with this, it has been found that RA-FLS have decreased levels of DNA methyltransferase 1 (Dnmt1) compared with OA synovial fibroblasts, which correlated with the hypomethylation of DNA in RA-FLS.[18] Polyamines are important regulators of cell growth and their metabolism is largely affected by the activity of spermidine/spermine-N^1-acetyltransferase (SSAT).[19] It has been shown that the expression of SSAT-1 in RA-FLS was higher than in OA synovial fibroblasts and has been proposed that the elevated polyamine metabolism could be linked to a decrease in the level of the methyl donor *S*-adenosyl methionine, thus contributing to DNA hypomethylation in RA-FLS.[20] The use of the SSAT-1 inhibitor diminazene aceturate decreased the expression of $ß_1$ integrin, CXCL12 (stromal cell-derived factor 1, SDF-1) and MMP1 of RA-FLS; however, the reduction was more pronounced when the inhibitor was used with *S*-adenosyl methionine supplementation.[21] Moreover, diminazene aceturate, *S*-adenosyl methionine or their combination could effectively decrease the invasion of FLS to the cartilage.[21] On the other hand, the inhibition of Dnmt1 by 5-azacytidine resulted in an RA-FLS-like phenotype of normal synovial fibroblasts, including elevated expression of cytokines, growth factors or MMPs.[18] However, both the hypothesis of a general hypomethylation and the question of how important DNA hypomethylation is in mediating the aggressive phenotype of FLS are still being discussed controversially.

Histone modifications are crucial molecular events of epigenetic changes, where acetylation is mediated by histone acetyltransferases (HATs), which make the histone-DNA interaction less tight and the transcription of the affected genes more intense.[22] On the other hand, the catalytic activity of histone deacetylases (HDACs) suppresses gene expression and an imbalance has been found between the expression and the activity of HATs and HDACs in RA with a HDAC predominance.[22] M808, a specific inhibitor of HDAC 6 (HDAC6), could inhibit the MMP and the cytokine release of interleukin 1ß (IL-1ß)-activated RA FLS.[23] HDAC6 inhibition also decreased the IL-1ß-induced migration of the cells, probably due to altering the reorganisation of F-actin.[23] Moreover, in the murine adjuvant-induced arthritis model, M808 could dose-dependently decrease the macroscopic signs of joint inflammation, while attenuating the extent of cartilage damage and bone erosions.[23] Bromodomain and extraterminal (BET) proteins recognise acetylated lysines in histones and transcription factors and regulate gene expression.[24] BET proteins can recruit HATs but can also contain HAT domains influencing the chromatin structure and transcription.[25] The BET family proteins BRD2, BRD3 and BRD4 have been shown to be upregulated in the RA synovium and the inhibitor of the molecules, I-BET151 could significantly reduce the MMP1, MMP3, IL-6 and IL-8 production of tumour necrosis factor α (TNFα)-, IL-1ß- or Toll-like receptor (TLR) agonist-activated FLS.[25] Moreover, the proliferation rate of RA-FLS was also decreased in the presence of the inhibitor.[25]

Metabolic changes as therapeutic targets

RA-FLS have an increased rate of metabolism in general including accelerated glucose metabolism, features that are common in tumour cells and which most likely help to support the augmented energy demand of proliferative FLS.[9] In line with this, in a recent metabolomic study, it was found that RA-FLS in terms of metabolism had a tumour cell-like phenotype with disturbances in glycolysis, glyconeogenesis or the pentose phosphate pathway, which resulted in a decrease in glycose and amino acid levels, while promoted the production of nucleotide precursors that are essential in proliferating cells.[26] Garcia-Carbonell and his colleagues found that the mRNA level of the main glycose transporter on synovial fibroblasts, glucose transporter 1 (GLUT1) and the expression of hexokinase 2 (HK2) was upregulated in RA-FLS compared with OA-FLS, and the former was correlated with a higher baseline MMP3 production.[27] The authors also detected that the attenuation of glycolysis by 3-bromopyruvate

(BrPA), a HK2 inhibitor resulted in a reduced PDGF-induced RA-FLS migration, lipopolysaccharide-triggered IL-6 production and MMP3 production, while the in vivo administration of BrPA led to the attenuation of arthritis severity in a mouse model of RA, similarly when given at the time of induction or later when the joint inflammation already developed.[27] Moreover, the administration of 2-deoxyglucose, another inhibitor of HK2 could decrease the cytokine and MMP release and production of TNF-stimulated healthy and non-activated RA-FLS.[28] Interestingly, treatment with the JAK (Janus kinase) inhibitor tofacitinib decreased the extent of glycolysis partially by downregulating the expression of GLUT1 and HK2 in the RA synovium.[29] Interestingly, primed synovial fibroblasts seem to require the complement factor 3 and its receptor to mediate metabolic changes in response to repeated inflammatory stimuli.[30] These results point towards the target potential of the molecules that are involved in the metabolic changes of FLS in RA.

Modulating hypoxia-induced factors

Hypoxia is well established in the RA synovium, and it was observed that both macroscopic and microscopic inflammation showed a correlation with the severity of hypoxia, which raised the possibility that reduced oxygen tension may alter the inflammatory process.[31] In line with these findings, FLS migration was enhanced at lower pO_2 levels and hypoxia induced the production of the chemotactic and angiogenic cytokine CXCL12 and vascular endothelial growth factor (VEGF) by FLS.[31 32] Under hypoxic conditions, the transcription modulator hypoxia-inducible factor 1α (HIF-1α) escapes the effects of hydroxylation of a proline residue by prolyl hydroxylases (which normally leads to the ubiquitination and degradation of the molecule), makes a heterodimer with the constitutively expressed HIF-1β and translocates to the nucleus, where it binds to the DNA.[33] On binding, HIF-1α promotes the production of different molecules from the epigenetic modificating histone demethylases to VEGF, p53, Notch or MMPs, while it can also interact with non-coding RNA molecules, as well.[33 34] Moreover, HIF-1α has influences on the cytokine production of RA synovial fibroblasts, for example, the expression of IL-6 and IL-8 was decreased in HIF-1α small interfering RNA- (siRNA-)treated RA-FLS, while the overexpression of HIF-1α resulted in the enhancement of cytokine production.[35] The modulation of HIF-1α expression also had effects on RA-FLS-mediated Th1 and Th17 responses, as well as on B-cell activation and antibody production.[35] In a recent study, both Notch1 and Notch3 ICD were found to be overexpressed in the RA synovium compared with OA samples and under hypoxia, HIF-1α could bind to the Notch1 and Notch3 promoter sites, suggesting that under hypoxic conditions, HIF-1α could regulate the expression of Notch1 and Notch3.[36] The authors found that Notch1 and Notch3 augmented the invasion and Notch1 promoted the migration of RA-FLS under hypoxia, while Notch3 had antiapoptotic effect at lower oxygen tensions.[36] In line with these findings, LY411575, an inhibitor of the cleavage of Notch ICD, substantially suppressed the inflammatory and structural signs of collagen-induced arthritis in rats.[36]

Blocking proliferation and induction of apoptosis

RA-FLS show a strong tendency to proliferate and in the affected RA synovium, some populations of FLS have been shown to expand.[10 37] Seliciclib (R-roscovitine), a cyclin-dependent kinase inhibitor blocking cell proliferation and partially acting on FLS has been found to be safe in a phase 1b clinical trial in TNF-inhibitor-refractory RA patients, giving way to further studies.[38] In line with the importance of JAK-STAT signalling in arthritic FLS functions (see below), the pan-JAK inhibitor peficitinib has been shown to modulate synovial fibroblast proliferation in a concentration close to the in vivo well-tolerated values.[39]

There are several ways to induce apoptosis in FLS. MicroRNAs (miRNAs) are small, non-coding gene silencing RNA molecules. A regulatory molecule, miR-140-5 p had decreased expression in the rheumatoid synovium in contrast to healthy synovial tissue and RA-FLS had a lower miR-140-5 p level compared with healthy FLS.[40] The overexpression of miR-140-5 p in RA-FLS decreased survival and proliferation, while downregulating proinflammatory cytokine production, which all seemed to be dependent on the effect of miR-140-5 p on the expression of signal transducer and activator of transcription 3 (STAT3).[40] Similarly, the level of miR-431-5 p was lower in the RA synovium and in RA-FLS, and while the overexpression of mIR-431-5 p induced apoptosis, its inhibition resulted in a prolonged survival.[41] Interestingly, treatment with iguratimod, a novel antirheumatic agent approved in Japan and China, increased the level of another miRNA, miRNA-146a and induced apoptosis in the arthritic synovium, which could be reversed by miR-146a inhibition in the collagen-induced arthritis model.[42] Moreover, Fas expression was found to be elevated in FLS and apoptosis could be triggered by an anti-Fas antibody.[43] Furthermore, JAK inhibitors like tofacitinib or peficitinib effectively promoted RA-FLS apoptosis.[44] In a recent article, methotrexate and theaflavin-3-3′-digallate decreased the production of proinflammatory and angiogenic markers, while the combination of the two drugs led to the restoration of the balance between autophagy and apoptosis in RA-FLS.[45]

These results indicate that blocking the proliferation and/or the apoptosis of synovial fibroblasts could help to control autoimmune joint inflammation.

Interference with migration

RA-FLS have aggressive migratory characteristics that contribute to bone and cartilage destruction in RA. In an RA-FLS-cartilage implantation model, where the sponge-cartilage was implanted with fibroblasts to SCID (severe combined immunodeficient) mice subcutaneously and a cell-free cartilage-sponge complex contralaterally, the cartilage destruction could not only be observed in the coimplantation (ipsilateral) site but also on the synovial fibroblast-free contralateral area, indicating that the RA-FLS were able to migrate actively from one site to the other.[46] The phoyphorylation rate of the receptor tyrosine kinase PDGFR has been observed to be elevated in the rheumatoid synovium and PDGFR inhibitors were found to dose-dependently inhibit the in vitro migration of human FLS.[47] Moreover, the inhibition of PI3Kδ (by INK007) could decrease PDGF-induced migration of RA-FLS (both when investigated in the wound-healing assay or in the Boyden chamber), while invasion of the cells was also affected by the presence of the PI3K inhibitors INK007 and CAL-101.[48] Paclitaxel, a known anticancer drug was shown to inhibit the in vitro migration of RA-FLS and could reduce the severity of joint inflammation in mice in the collagen-induced arthritis model.[49] However, the paclitaxel-induced decrease of the production of inflammatory mediators by RA-FLS can also contribute to the in vivo phenotype, while the inhibitor itself has a considerable toxicity, which requires further investigation.[49] Meanwhile, there are more and more data on the altered metabolism of RA-FLS (see above) and its effects on the aggressive behaviour of these cells, which is nicely highlighted with the massive inhibitory effect of glycogen

synthase kinase 3 (GSK-3) inhibitors on the migratory capacity of RA-FLS.[47] In a recent paper, Orange and colleagues found elevated levels of a special mesenchymal cell type with shared features of inflammatory synovial fibroblasts, namely the CD45/CD31/Podoplanin triple positive preinflammatory mesenchymal (PRIME) cells in the blood by transcriptional analysis just before RA flares (and after B-cell activation in the circulation).[50] These PRIME cells have been proposed to migrate to the affected joints and participate in the initiation of the inflammatory process.[50] The potential blockade of these cells may serve as a beneficial target option in the prevention of RA flares.

Focusing on signal transduction

Targeting signal transduction pathways is an emerging therapeutic strategy in RA. Type I and II cytokine receptors (eg, the IL-6, the granulocyte-macrophage colony-stimulating factor (GM-CSF) or the interferon-γ (IFN-γ) receptors) signal through the tyrosine kinase JAK and the transcription factor STAT (JAK-STAT pathway). JAK1 is a central molecule in autoimmune arthritis, and it seems that inhibitors of JAK1 partially exert their effects by modulating synovial fibroblast function.[51] Tofacitinib, a JAK inhibitor mainly targeting JAK1 and JAK3, was found to attenuate the production of IL-6 and monocyte chemoattractant protein-1 in oncostatin M-treated RA-FLS.[29] Moreover, tofacitinib reduced the level of IL-1β, IL-6, IL-8 and soluble intercellular adhesion molecule (sICAM) in ex vivo synovial explants.[29] JAK2 has been shown to participate in the IFN-γ-induced migration of FLS, and baricitinib was able to decrease the invasion of these cells.[52] Tofacitinib has been also shown to influence the invasion of RA-FLS and the outgrowth of RA-FLS from RA explants in Matrigel.[29] However, JAK inhibitors are not selective for FLS and have immunosuppressive effects in patients with RA due to the inhibition of different immune cell functions.

FLS express several types of the pattern recognition receptor TLRs, some of which signal through IRAK4.[53 54] The inhibition of IRAK4 by a specific molecule called PF-06650833 reduced the cytokine and MMP release of human RA-FLS when stimulated by TLR1/2, TLR4 or TLR5 agonists under in vitro conditions.[55] Moreover, the in vivo administration of PF-06650833 could attenuate the severity of collagen-induced arthritis in rats.[55] Based on the effectiveness of the molecule in preclinical models, PF-06650833 was and is being tested in phase 2 trials in patients with RA (ClinicalTrials.gov Identifiers: NCT02996500 and NCT04413617).

FLS express different types of heterodimer integrins (like β_1 and β_3 integrins) on their cell surface that mainly mediate cell-extracellular matrix (eg, FLS-collagen, FLS-laminin and FLS-fibronectin) interactions in RA.[56] β_1 integrins were shown to signal through the Syk tyrosine kinase in airway epithelial cells that raises the possibility of a similar signalling machinery in FLS.[57] However, while the administration of R406, a so-called Syk (but rather a non-selective tyrosine kinase) inhibitor had effects on RA-FLS activity, in the absence of data regarding the impact of a specific Syk inhibitor, the role of Syk in RA-FLS is still largely unknown.[58]

A real-time cell analysis system mainly based on a wound-healing assay and intracellular phosphorylation of human FLS pointed towards the effectiveness of the inhibitors of PDGFR, Akt, PI3K or GSK-3 in controlling the invasive capacity of RA-FLS.[47] However, more detailed studies are needed to test these inhibitors in the control of experimental and human autoimmune arthritis.

ATTEMPTS TO DEPLETE SYNOVIAL FIBROBLASTS

The depletion of FLS would be an ideal selective therapy in RA without immunosuppressive consequences. In RA, the synovial hyperplasia does not affect the different layers equally: the sublining area expands much more than the lining layer, which suggests that different types of FLS exist. Fibroblast activation protein-α (FAPα), a cell surface marker of activated stromal fibroblasts was upregulated in the RA synovium, suggesting that it may be linked to an inflammatory behaviour.[6] Similar density augmentation could be observed in a murine model of arthritis, where the deletion of FAPα reduced the severity of joint inflammation in both the acute and the chronic arthritis forms and attenuated the development of bone erosions, cartilage destruction and immune cell infiltration.[6] Within the FAPα-positive group, arthritic FLS seemed to form various subpopulations with dedicated functions: while lining layer-resident FAPα single positive cells were found to be essential in mediating cartilage and bone destruction, FAPα/CD90 double-positive FLS recruited immune cells to the inflamed joints.[6] In line with these findings, arthritic synovium-derived CD90-negative fibroblasts expressed higher amounts of RANKL and MMPs, while cytokine and chemokine production was much more robust in the CD90-expressing cells.[6] Intra-articular injection of CD90-positive FLS resulted in a significant increase of ankle thickness and recruitment of leukocytes like neutrophils to the synovial area.[6] Another group developed a new therapeutic strategy by the selective ablation of FAPα-positive cells by targeted photodynamic therapy, where an anti-FAPα antibody was conjugated to a photosensitiser.[59] The authors found that the conjugated antibody-treated animals showed a decreased inflammation score in the collagen-induced arthritis model, further highlighting the importance of FAPα in the pathogenesis of autoimmune joint disease and providing a selective pharmacological method to block the proinflammatory and tissue destructive functions of FLS by depleting them.[59]

However, the picture seems to be even more complex as in another work, Mizoguchi and his colleagues identified seven different synovial fibroblast groups in the RA synovium by bulk and single-cell transcriptomics.[37] They found that CD90, podoplanin and cadherin-11 triple-positive FLS — which localise in the perivascular zone, secrete cytokines and show a proliferative and invasive potential — are upregulated in the joints of patients with RA, which may lead to the development and use of bispecific antibodies against this harmful group of cells.[37]

BLOCKING CELLULAR AND CELL-MATRIX INTERACTIONS OF SYNOVIAL FIBROBLASTS

Interference with endothelial cells in the focus

E-selectin is an adhesion molecule expressed by activated endothelial cells that mediate cellular interactions mainly with leukocytes.[60] In a recent study, the authors showed that E-selectin could mediate the adhesion and rolling of FLS under flow conditions and RA-FLS had increased binding capacity towards TNFα-stimulated endothelial cells.[61] It was also detected that the E-selectin ligand CD15s was expressed in RA-FLS in the human synovium, while E-selectin and P-selectin-deficient mice showed a decreased invasion score in the contralateral site in the cartilage implantation model pointing at the importance of selectins in mediating synovial fibroblast migration in the circulation.[61] Interestingly, different adipokines (like visfatin or resistin) were found to be able to increase the adherence of RA-FLS to endothelial cells under flow conditions, which could be attenuated by the application of corticosteroids.[60] These data raise the possibility to target molecules that participate in the

adhesion and migration of FLS in RA by blocking endothelial cell-RA-FLS interactions.

Targeting interactions with leukocytes and platelets

Several articles have been published on how FLS communicate with recruited leukocytes — especially with lymphocytes or macrophages — in the synovial area. The levels of the Th1 cell-recruiting CXCR3 ligands (CXCL9, CXCL10 and CXCL11) were found to be elevated in the synovial fluid of patients with RA, and inflammatory cytokines like TNFα or IFN-γ were shown to stimulate the expression and production of these chemokines from RA synovial fibroblasts, pointing at an important interaction between the two cell types.[62] Furthermore, the fractalkine receptor CX3CR1 was found to be upregulated on peripheral T cells of patients with RA and the synovium contained significant amount of CX3CR1-positive T cells, while fractalkine is thought to be produced by RA-FLS and endothelial cells.[63] However, the treatment with a monoclonal anti-fractalkine antibody (E6011) did not show efficacy in a phase 2 clinical trial in patients with RA.[64] FLS can also upregulate major histocompatibility complex class II molecules on their cell surface when stimulated by Th1 or Th17 cells and are thought to be able to present antigens to T cells.[65] FLS can interact with B cells in the rheumatoid synovium: they may promote maturation, differentiation or antibody class-switching through the production of IL-6 or BAFF/APRIL.[66 67]

One important source of IL-6 in RA is the FLS and the production of IL-6 is greatly enhanced by TNFα that is mainly derived from macrophages.[68] On the other hand, FLS produce macrophage colony-stimulating factor and GM-CSF, which are important in the local expansion of macrophages.[69] In a recent study, the inflammatory heparin binding EGF-like growth factor-(HBEGF-)positive macrophages were found to be shaped by FLS and in turn, these macrophages were able to promote the invasive characteristics of synovial fibroblasts, while several currently used drugs could reduce the HBEGF-positive macrophage cluster with a potential alteration of the macrophage-FLS axis.[70] In addition to communicating with macrophages, RA-FLS also contribute to osteoclastogenesis as synovial fibroblast-specific receptor activator of nuclear factor κB ligand (RANKL) expression was found to mediate bone erosions through promoting osteoclast differentiation under experimental conditions.[71] Here, using the Cre-Lox system, the authors showed that the deletion of the RANKL gene from FLS and chondrocytes did not influence the arthritis severity in the collagen antibody-induced arthritis model, but decreased the number of osteoclasts and bone erosions in contrast to animals with chondrocyte- and T-cell specific deletion of the molecule, suggesting that bone destruction can be independent of the macroscopic inflammatory phenotype and targeting synovial fibroblast functions can result in the preservation of the original bone structure.[71]

Podoplanin (gp38) has been shown to be upregulated in the RA synovium, especially in cases with lymphoid neogenesis or seropositivity, and was sensitive to anti-TNF therapy.[72] Podoplanin on FLS was further analysed in fibroblast-platelet cocultures, where its binding to CLEC2 was associated with increased production of IL-6 and IL-8.[72] It is an interesting finding that podoplanin-positive FLS express the transcription factor autoimmune regulator (Aire) in a cytokine-dependent manner, which seems to mediate the enhanced production of various proinflammatory cytokines and an IFN-γ signature after TNFα- and/or IL-1ß stimulation, without mediating the transcription of tissue restricted antigens, further highlighting the immune reaction-orchestrating role of RA-FLS.[73]

Targeting other cell-cell and cell-matrix interactions of FLS

FLS express several molecules that mediate their adhesion to other cells, to themselves or the extracellular matrix. Identifying important unique molecules in these processes could lead to the selective targeting of FLS. Cadherin-11, one of the major adhesion molecules of synovial fibroblasts, was found to be essential in regulating synovial architecture as its absence resulted in a hypoplastic synovium with the loss of an evident lining layer, meanwhile cadherin-11-deleted mice showed a decreased arthritis severity compared with the wild-type controls in an experimental mouse model.[4] Cadherin-11 was also found to be an important molecule in mediating the invasiveness of FLS and participating in the development of cartilage erosions in vivo.[4] In line with its proposed role in RA, cadherin-11 mRNA transcripts were detected in the majority of patients with RA in comparison with control subjects and cadherin-11-positivity correlated with polyarthritis.[74] In accordance with the above findings, treatment with a cadherin-11-Fc fusion protein or an anti-cadherin-11 antibody resulted in a decreased arthritis severity in a mouse model of autoimmune joint inflammation.[4] However, the treatment with a humanized monoclonal antibody against cadherin-11 (RG6125) failed to show efficacy in patients with RA inadequately responding to anti-TNFα therapy in a phase 2 clinical trial and led to the withdrawal of this synovial fibroblast-selective intervention from the list of potential future therapies of RA.[75] Meanwhile, in a recent article, the LIM and SH3 domain protein 1 (Lasp1) was found to be upregulated in the RA synovium and was described as an important mediator of the cell responses (eg, migration) of arthritic FLS.[5] Further studies revealed that the loss of Lasp1 resulted in abrogated cadherin-11-containing cell-cell contacts and decreased the severity of experimental arthritis in mice, pointing at its target potential in the therapy of RA.[5]

As mentioned before, integrins are heterodimer adhesion molecules, which are crucial in several processes from embryogenesis to immune functions. It has been shown that the α9ß1 integrin had higher levels in the rheumatoid synovium compared with the synovial tissue of patients with OA and stimulation of RA-FLS with tenascin-C, an α9ß1 ligand resulted in elevated MMP and IL-6 expression, which could be downregulated in the presence of an anti-α9 monoclonal antibody.[76] Moreover, anti-α9 treatment could decrease arthritis severity in the collagen-induced arthritis model without a major influence on systemic immunomodulation.[77 78] Interestingly, ASP5094, a humanized monoclonal antibody against integrin α9, did not show efficacy in patients with RA with a therapeutic refractory to methotrexate.[79]

Syndecan-4 is a cell surface molecule that can interact with extracellular matrix components and can mediate various cell responses in synovial fibroblasts. In a recently published paper, the authors found that syndecan-4 was an essential regulator molecule in the pathogenesis of RA and was important in mediating nitric oxide, reactive oxygen species, IL-1ß, IL-6 and TNFα production in RA-FLS, while contributing to the resistance to apoptosis.[80] In line with these findings, an antibody directed against the dimerisation domain of syndecan-4 could effectively decrease the expression of the IL-1 receptor on FLS and reduced the pannus formation, the cartilage destruction and the MMP3 content of the affected joints in the TNF transgenic mouse arthritis model.[81]

MESENCHYMAL STEM CELLS AS THERAPEUTIC MEDIATORS

MSC-based therapeutic approaches have gained significant attention in the past decade for the control of autoimmune

arthritis. The administration of bone marrow-derived MSCs has been shown to ameliorate the signs of collagen-induced arthritis, to decrease histological scores, the levels of proinflammatory cytokines and to upregulate anti-inflammatory molecules like IL-10 or transforming growth factor β1 (TGF-ß1).[82] Mechanistic studies revealed that nuclear factor kappa B (NF-κB) activity was downregulated in synovial fibroblasts, perhaps due to the lowered miR-548e levels provoked by TGFß1.[82] In line with these findings, the cotransplantation of MSCs and miR-548e viruses to arthritic mice abolished the effect of MSC transplantation alone, pointing at a possible scenario, where MSCs exert their therapeutic effect by increasing TGFß1 levels and attenuating NF-κB signalling by decreasing miR-548e expression in FLS.[82] In another study, umbilical cord-derived MSCs were found to decrease the cadherin-11 upregulation of RA-FLS (and of FLS of the synovium of collagen-induced arthritis in rats), mainly through the production of the anti-inflammatory cytokine IL-10.[83] The promising results with the administration of MSCs in autoimmune joint inflammation led to the launch of several clinical trials in RA, for instance the effect of intravenous administration of allogeneic umbilical cord-derived MSCs is investigated in a phase 1/2 trial (NCT03618784; for further studies, visit ClinicalTrials.gov (identifiers NCT03333681 or NCT03691909).

DISCUSSION AND CONCLUDING REMARKS

FLS are crucial cellular elements in RA and are promising targets of arthritis therapy in the future. There are many ways where these mesenchymal cells could be influenced: their origin and differentiation, their epigenetics and metabolism, their proliferation and apoptosis, their signal transduction and their interactions with other cells (eg, with immune cells) or the extracellular matrix can be targeted resulting in the decrease of different cell responses like migration and invasive tendencies or the production of MMPs.

The specificity of RA synovial fibroblast therapy can be a real challenge as altering the fate or functions of regular fibroblasts at other sites may cause side effects (eg, reduced wound healing). Buechler and colleagues—based on the development of fibroblast atlases by integrating single-cell transcriptomic data—showed that there are only two universal fibroblast transcriptional subtypes in mice, which serve as sources for different fibroblast variants in various organs and diseases, with high similarity to human fibroblasts.[84] In addition, in a recent study, the expansion of two clusters of FLS (a $CXCL10^{+}CCL19^{+}$ 'immune interacting' and a $SPARC^{+}COL3A1^{+}$ 'vascular interacting') were identified in patients with inflamed tissues of RA, inflammatory bowel disease, interstitial lung disease and Sjögren's syndrome by single-cell RNA sequencing (Korsunsky *et al*, bioRxiv, 2021). However, several FLS subsets have been described in the last years (eg, by integrating single cell transcriptomics and mass spectometry) and the recognition of this heterogeneity may support the development of novel therapies (perhaps by aiming selective depletion, etc.), which could significantly decrease the off-target effects of FLS-based treatments.[85] In a recently published paper, the different FLS subsets have been associated with distinct clinical and laboratory alterations, raising the possibility of FLS-based personalized treatments in the future.[86] So far, however, there are no well-working FLS-specific therapies in RA and several of the above-mentioned therapeutic approaches can modify the function of other cell types, as well.

The development and successful use of drugs acting on RA synovial fibroblasts, which can decrease the imprinted aggressive behaviour, can potentiate the use of novel treatments in other fibroblast-mediated diseases like systemic autoimmune disease-associated pulmonary fibrosis or in skin and internal organ involvement in systemic sclerosis. Meanwhile, the mesenchymal cell origin could be the basis of the development of promising non-immunosuppressive future therapies in RA, which is not a negligible factor in these years of a global pandemic.

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EPIDEMIOLOGICAL SCIENCE

2022 EULAR points to consider for remote care in rheumatic and musculoskeletal diseases

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ABSTRACT

Background Remote care and telehealth have the potential to expand healthcare access, and the COVID-19 pandemic has called for alternative solutions to conventional face-to-face follow-up and monitoring. However, guidance is needed on the integration of telehealth into clinical care of people with rheumatic and musculoskeletal diseases (RMD).

Objective To develop EULAR points to consider (PtC) for the development, prioritisation and implementation of telehealth for people with RMD.

Methods A multidisciplinary EULAR task force (TF) of 30 members from 14 European countries was established, and the EULAR standardised operating procedures for development of PtC were followed. A systematic literature review was conducted to support the TF in formulating the PtC. The level of agreement among the TF was established by anonymous online voting.

Results Four overarching principles and nine PtC were formulated. The use of telehealth should be tailored to patient's needs and preferences. The healthcare team should have adequate equipment and training and have telecommunication skills. Telehealth can be used in screening for RMD as preassessment in the referral process, for disease monitoring and regulation of medication dosages and in some non-pharmacological interventions. People with RMD should be offered training in using telehealth, and barriers should be resolved whenever possible.
The level of agreement to each statement ranged from 8.5 to 9.8/10.

Conclusion The PtC have identified areas where telehealth could improve quality of care and increase healthcare access. Knowing about drivers and barriers of telehealth is a prerequisite to successfully establish remote care approaches in rheumatologic clinical practice.

Key messages

What is already known on this subject?
⇒ Remote care and telehealth can improve healthcare access and outcomes, particularly in the treatment of chronic diseases.
⇒ The COVID-19 pandemic made the use of telehealth even more frequent in rheumatology, with ad hoc implementation of remote care services in several centres.
⇒ Guidance is needed on how remote care and telehealth should be developed and integrated into long-term rheumatology clinical care.

What does this study add?
⇒ These points to consider indicate how telehealth should be developed and implemented in routine clinical care of people with rheumatic and musculoskeletal diseases (RMD).
⇒ They cover several aspects including screening for RMD, preassessment in the referral process, disease monitoring and modification of medication dosages and non-pharmacological interventions.
⇒ The task force identified drivers and barriers to telehealth, which may support a timely implementation in clinical practice.
⇒ These points to consider can be used to tailor telehealth to needs and preferences of people with RMD.

How might this impact on clinical practice or future developments?
⇒ These points to consider can guide the development of national and local telehealth strategies to support best clinical practice.

INTRODUCTION

The prevalence of rheumatic and musculoskeletal diseases (RMD[1]) ('A diverse group of diseases that commonly affect the joints, but can also affect the muscles, other tissues and internal organs') in developed countries has increased by 60% from 1990 to 2010 and is expected to continue rising. An ageing population, earlier diagnosis and improved survival among people with RMD are the main reasons for the increased prevalence.[2 3] Compounded by

a relative drop in the number of rheumatologists[4] and other healthcare providers (HCPs), the pressure on the healthcare system has increased.[5 6] Waiting times for a new or follow-up rheumatology appointment have grown, hampering implementation of guidelines for good clinical care.[7] Alternative forms of care using telehealth for follow-up of people with RMD and for self-management interventions may preserve and even improve quality of care.

Remote care makes use of digital technologies—so-called 'telehealth' interventions.[8] It is used in all parts of the patient pathway, including communication with patients/caregivers, disease screening or monitoring of different aspects of the disease (eg, disease activity, damage, quality of life, adherence, etc). It can be delivered synchronously (HCP and patient being present at the same time) or asynchronously and be divided into three main types of modalities: *live video* (synchronous) and the asynchronous modalities: *store and forward* (transmission of recorded health history) and *remote patient monitoring*.[9]

Telehealth can improve healthcare access and outcomes, particularly in the treatment of chronic diseases.[10] It can reduce demands on overstretched facilities and make the health sector more resilient[8] and has become even more relevant during the COVID-19 pandemic when it has been difficult to deliver face-to-face care and investigations as usual.[11 12] Furthermore, the COVID-19 pandemic has resulted in wider use of telehealth services, and remote care has become much more socially acceptable.[13]

However, guidance is needed on how telehealth should be integrated into routine clinical care.

The aim of this EULAR task force (TF) was to formulate points to consider (PtC) for the development, prioritisation and implementation of remote care and telehealth for people with RMD. The target users are people with RMD and their relatives, physicians and other HCPs involved in the care of people with RMD, regulators and policy makers.

In the context of these PtC and following the definition of the WHO, telehealth was defined as: 'the use of telecommunications and virtual technology to deliver healthcare outside of traditional healthcare facilities'.[8] Remote care was defined as 'the provision of care using telehealth and virtual technology allowing patients to be evaluated, monitored and possibly treated while the patient and HCP are physically remote from each other'.

METHODS

This work was conducted using the 2014 updated EULAR standardised operating procedures for developing PtC/recommendations.[14] After approval from the EULAR Executive Committee, the conveners (AdT and CD) and fellows (PB and AM) formed an international TF representing 14 European countries. TF members included one methodologist and two comethodologists (TAS, CBM and YM), rheumatologists (including one representative from EMEUNET), one epidemiologist (also representing EMEUNET), health professionals in rheumatology (nurses, occupational therapists, physiotherapists and one psychologist) and four people with RMD. In preparation for the application of this project to EULAR, a broad scoping review (online supplemental appendix 1) was undertaken to map the current research and knowledge gaps within remote care interventions in rheumatology. A scoping review does not aim to produce a critically appraised and synthesised result nor to answer a particular question, but rather to provide an overview of the contents of effect studies on this topic.[15] As such, the scoping review was presented at the first TC meeting and informed the outlined research questions and the search strategy in the systematic literature review (SLR): what is the efficacy, safety, cost-effectiveness, user perception and adherence of remote care or blended care as compared with standard care in people with RMD? How is remote care delivered/tailored to people with RMD and integrated into clinical practice? What are the drivers and barriers for implementation of remote care in clinical practice? These questions were transformed into the PICO (Population, Intervention, Comparator, Outcome) format, driving the development of the search strategy for the SLR. The SLR was conducted by the two fellows under the guidance of the methodologist and two comethodologists in accordance with the Cochrane Handbook.[16] The results of the SLR were reported in accordance with the **P**referred **R**eporting **I**tems for **S**ystematic Reviews and **M**eta-**A**nalyses guidelines[17] and have been published separately.

The two TF meetings were held via a virtual online platform. During the first meeting in November 2020, definitions for remote care and telehealth were discussed and the key questions were phrased.

During the second virtual meeting, held in April 2021, the TF members formulated the overarching principles and PtC based on evidence from the SLR and expert opinion, through a nominal group process. Consensus was accepted in the first round if >75% of the members voted in favour of a statement. As all statements were accepted in the first round, no additional rounds were necessary. Finally, each TF member anonymously indicated their level of agreement (LoA) to each statement using Survey Monkey (LoA, 0–10 numeric rating scale ranging from 0 ='completely disagree' to 10 = 'completely agree'). The mean and SD of the LoA as well as the percentage of TF members with an agreement ≥8 are presented. The level of evidence was assigned to each statement based on the standards of the Oxford Centre for Evidence Based Medicine.[18]

Finally, a research agenda was formulated based on evidence gaps and controversial points. The final manuscript was reviewed and approved by all TF members and the EULAR Council.

RESULTS

An overview of the overarching principels and PtC can be found in table 1.

The TF identified key themes considered to apply across all PtC, formulated and agreed on them as four overarching principles. They are not necessarily a direct result of the SLR, but considered to be fundamental aspects of the specific area and form the framework for the PtC.

Overarching principels

Tailored care combining remote and face-to-face attendance should be based on shared decision-making as well as the needs and preferences of people with RMD

The decision on using remote care should be tailored to the patient's needs and preferences including demographic, social situation, geographical access to healthcare, employment status, specific diagnosis, comorbidities, disease phase and status, that is, in a newly onset rheumatoid arthritis (RA), a face-to-face visit should be performed, but telehealth may be optimal for education about disease symptoms, disease activity, therapy and self-management. Patients with a well-established diagnosis, stable disease activity and less complex diseases can be offered the use of telehealth solutions.[19] Patients with long-standing, stable RA might require less education and training and their treatment might not need modification. Some of these patients may prefer telehealth consultations rather than hospital visits. Similarly, a

Table 1 EULAR points to consider for the use of remote care in people with RMD

Overarching principles	LoE	LoA*
A. Tailored care combining remote and face-to-face attendance should be based on shared decision-making as well as the needs and preferences of people with RMD.	n.a.	9.7 (0.7) 96.6%>8
B. Remote care† for people with RMD can be delivered by all members of the healthcare team using a variety of telehealth techniques.	n.a.	9.1 (1.3) 86.2%>8
C. Telehealth‡ interventions should be developed in collaboration with all stakeholders including the healthcare team, caregivers and people with RMD.	n.a.	9.7 (0.7) 100%>8
D. Members of the healthcare team involved in remote care interventions should have adequate equipment and training, as well as telecommunication skills.	n.a	9.7 (0.7) 96.6%>8
Specific points to consider		
1. Pre-assessment by telehealth may be considered to improve the referral process to rheumatology and help prioritisation of people with suspected RMD.	2b	8.5 (2.1) 82.1%>8
2. Telehealth may assist pre-diagnostic processes for RMD; however, diagnosis should be established in a face-to-face visit.	2b	8.7 (2.0) 71.4%>8
3. The decision to initiate disease-modifying drugs should be made in a face-to-face visit. Telehealth may be used for drug education, monitoring and facilitating adherence.	2b	9.1 (1.4) 89.3%>8
4. Dose modifications or suspension of disease-modifying drugs, as well as addition of analgesics, NSAIDs or glucocorticoids can be discussed with people with RMD using telehealth.	2b	9.3 (1.3) 92.9%>8
5. Telehealth can be used to monitor symptoms, disease activity and other outcomes.	2b	9.6 (0.8) 96.4%>8
6. Telehealth may be used to discuss the need for a face-to-face consultation or other interventions.	2b	9.8 (0.7) 96.4%>8
7. Telehealth should be considered for non-pharmacological interventions including, but not limited to, disease education, advice on physical activity and exercise, self-management strategies and psychological treatment.	2b	9.4 (1.1) 92.9%>8
8. Barriers to remote care should be evaluated and resolved wherever possible.	5	9.7 (0.8) 96.4%>8
9. People with RMD using remote care should be offered training in using telehealth.	5	9.5 (1.0) 96.4%>8

*LoA, level of agreement (mean (SD)).
†Remote care: the provision of care using telehealth and virtual technology allowing patients to be evaluated, monitored and possibly treated while the patient and HCP are physically remote from each other.
‡Telehealth: the use of telecommunications and virtual technology to deliver healthcare outside of traditional healthcare facilities.
HCP, healthcare provider; LoA, level of agreement; LoE, level of evidence; NSAIDs, nonsteroidal anti-inflammatory drugs; RMD, rheumatic and musculoskeletal disease.

younger patient in full-time employment living far away from the hospital might prefer telehealth, whereas an elderly patient living nearby and with limited access to technology might opt for a face-to-face visit.

Remote care for people with RMD can be delivered by all members of the healthcare team using a variety of telehealth techniques

As is seen in online supplemental table 1, telehealth makes use of different types of technology and modalities. Not all services in the RMD care pathway can be delivered remotely; but allowing for security, feasibility and need, all members of the healthcare team can deliver them. Different services are offered by different HCPs. Interventions within diagnostics, for example, are usually delivered by physicians; whereas interventions on training and exercise are mostly managed by physiotherapists. Monitoring of disease activity, rehabilitation and self-management interventions may involve different HCPs (online supplemental table 1).

Telehealth interventions should be developed in collaboration with all stakeholders including the healthcare team, caregivers and people with RMD

User involvement by all stakeholders (eg, patients, carers, HCPs and decision-makers) are believed to be important in order to overcome usability issues of telehealth solutions.[20 21] Patients are at the centre of this process, and the goal is to develop user-friendly, intuitive and effective technology that helps to improve healthcare services from a patient's perspective. It is essential to include HCPs in order to reflect daily clinical practice, that is, by letting them propose, which intervention can be delivered remotely and how technology could be incorporated into the overall clinical evaluation and workflow. The involvement of administration personnel and funding bodies is required to guarantee reimbursement of services and to prevent additional bureaucracy to HCPs and patients.

Members of the healthcare team involved in remote care interventions should have adequate equipment and training as well as telecommunication skills

Successful telehealth interventions require that the healthcare team receives training in telehealth communication, interaction, legacy and clinical assessment.[22] Despite great interest, HCPs often are unaware of available telehealth tools[12] and should take responsibility for their ongoing professional development,[23] but healthcare organisations are also responsible for preparing the workforce for telehealth-based clinical practice.[22] In future, telehealth should be incorporated into the existing curricula at universities and other healthcare educational institutions, so that HCPs can develop the skills to provide safe and competent telehealth care.

PtC 1: Preassessment by telehealth may be considered to improve the referral process to rheumatology and help prioritisation of people with suspected RMD

Waiting lists within rheumatology are forecasted to become longer in the future.[24] Effective prioritisation is, therefore, key to guarantee rapid access to those patients with the most severe and active diseases. A short preassessment via telehealth may help to decide on this priority, advise patients and other HCPs on which tests should be done and/or whether another specialist should be involved first. The SLR identified one study showing that referrals could be triaged by a nurse practitioner, with a rheumatologist participating in the encounter via a tele-link.[25] Agreement to this statement was lower than to other PtC, mainly because the TF was of the opinion that more evidence is needed about which preassessment methods are most appropriate as well as in which patients and at what level of the referral process they should be applied.

PtC 2: Telehealth may assist prediagnostic processes for RMD; however, diagnosis should be established in a face-to-face visit.

During a face-to-face visit, additional information (resulting from personal interaction and clinical and physical examinations) helps to make a diagnosis. For that reason, face-to-face visits are indispensable to rheumatology. The final diagnosis may certainly be made after a face-to-face visit, as well, and discussed with the patient remotely, for example, when the clinician needs to wait for blood tests or images.

Patients with risk factors for developing an inflammatory rheumatic disease (eg, patients with psoriasis without arthritis, people with positive autoantibodies but no inflammatory symptoms and people with positive family medical history for systemic autoimmune disease) would benefit from screening using telehealth techniques. Hence, regular monitoring via telehealth could help to facilitate a face-to-face visit at the appropriate time. In other situations, where diagnosis largely depends on history and imaging (eg, for axial spondyloarthritis), several parts of the prediagnostic process could be handled by telehealth, and a face-to-face visit could be scheduled when treatment is initiated. Evidence indicates that such telehealth interventions may save unnecessary visits, time and resources for patients, the healthcare system and society.[24 26]

PtC 3: The decision to initiate disease-modifying drugs should be made in a face-to-face visit. Telehealth may be used for drug education, monitoring and facilitating adherence

The TF agreed that the decision to initiate or change disease-modifying antirheumatic drugs (DMARDs) would usually take place on the background of active disease, requiring a face-to-face consultation. However, a telehealth appointment may be more optimal to reinforce information and education to improve adherence to treatment, especially when the patient is in familiar surroundings and possibly with relatives. The SLR identified evidence that telehealth could be used for drug education, monitoring and facilitating adherence to drugs,[27–29] and that patients believe they can benefit from telehealth-provided drug information, but prefer it to complement face-to-face information rather than replacing it.[30]

PtC 4: Dose modifications or suspension of DMARDs as well as addition of analgesics, NSAIDs or glucocorticoids can be discussed with people with RMD using telehealth

In chronic inflammatory arthritis such as RA, optimal sequencing of DMARDs is important as the disease often fluctuates between active disease and remission.[31 32] The SLR identified some evidence that an intensive treatment strategy based on telehealth led to increased remission rates and a decrease in functional impairment.[33] The TF was also of the opinion that telehealth could be used in cases of infection, adverse events or abnormal lab results, where temporal or permanent discontinuation of DMARDs is needed. Furthermore, addition of analgesics, non-steroidal antiinflammatory drugs (NSAIDs) and glucocorticoids could be prescribed remotely as bridging therapies or to treat minor flares, residual disease activity and comorbidities until patients receive face-to-face assessment.

PtC 5: Telehealth can be used to monitor symptoms, disease activity and other outcomes

According to the EULAR treat-to-target (T2T) recommendation, disease activity in inflammatory arthritis should be evaluated every 1–6 months depending on disease activity and severity.[34] Due to resource constraints, a full implementation of T2T in rheumatology practice is still scarce.[35] In patients with low, stable disease activity, telehealth follow-up may be a valid alternative to face-to-face visits, given that this approach can make room for new patients or patients with more complex disease presentation. It may also help to monitor changes or emerging trends during long-term follow-up.

The SLR identified two randomised controlled trials (RCTs) indicating that in patients with sustained remission, telehealth follow-up resulted in similar outcomes, including disease activity, physical function and quality of life compared with regular face-to-face visits.[36 37]

PtC6: Telehealth may be used to discuss the need for a face-to-face consultation or other interventions

Telehealth can be a low-barrier opportunity to get in contact with the healthcare system either by a telephone helpline, a chat function or a secure email service. By doing so, patients can be referred for a face-to-face visit, a specific examination (eg, blood test or imaging) or to another specialist. The SLR identified one RCT showing that telehealth is a good platform for reaching a shared decision between the patient and the HCP.[38] Another study concluded that telehealth can be used to decide whether patients require a face-to-face consultation.[36]

PtC 7: Telehealth should be considered for non-pharmacological interventions including but not limited to disease education, advice on physical exercise, self-management strategies and psychological intervention

The SLR identified several studies that supported the use of telehealth as an intervention to promote physical activity and exercise.[39–44] The TF debated the mechanism of delivery of any non-pharmacological intervention considering the possibilities of delivering this entirely face-to-face, remotely or combinations thereof. The TF agreed that it depends on patient factors such as previous experience with the intervention and the intervention itself. Disease education, for example, may not require face-to-face visits, whereas complex physical exercises should preferably be instructed and checked face-to-face.

PtC 8: Barriers to telehealth care should be evaluated and resolved wherever possible

Telehealth has the potential to provide access to resources and care, increase flexibility and reduce waiting lists and patient travel time. However, some barriers that might obstruct successful implementation of telehealth must be assessed systematically.[30 41 45–52]

In table 2, we depict a list of possible barriers identified in the SLR and by the TF members and provide suggestions on how these barriers could be resolved. This list is not exhaustive but may form the basis for the development of local checklists enabling implementation of telehealth into clinical practice.

PtC 9: People with RMD using remote care should be offered training in using telehealth

Training of members of the healthcare team in telehealth techniques and communication skills was seen as an overarching principle; however, the TF agreed that a separate statement was needed on training people with RMD in using telehealth. Many of the included surveys and qualitative studies refer to problems with digital literacy.[46 52–54] People with RMD should be offered training in using telehealth solutions and should be informed how to prepare for a telehealth consultation (eg, by having questions prepared, sitting in a quiet place, etc). Any member of the healthcare team, depending on the local setting, can offer this training.

Based on the discussions and the areas of uncertainty, a research agenda has been proposed, which is depicted in box 1.

DISCUSSION

The current paper presents the first EULAR PtC on the use of telehealth in daily clinical rheumatology practice. These PtC can be used to inform and guide the development of national

Table 2 Identified barriers to telehealth and suggested interventions to overcome them

Factors	Identified barriers	Suggested interventions
Patient factors	Patient reluctance[30 45 47 51 62] Varying digital and health literacy skills[46 47 51 53] Lack of access to necessary equipment[46 54]	► Integration of digital support into routine patient education ► Assessment of the patient's health literacy before referral to telehealth care ► Assessment of the patient's digital skills before referral to telehealth careAssessment of access to required equipment before referral to telehealth
Clinical factors	No possibility of face-to-face clinical and instrumental examination[45 47 49 63] Disease burden, medical and psychological comorbidity[45 62]	► Assessment before referral: Will telehealth provide all necessary information needed to make a clinical decision? ► Assessment before referral: Will telehealth be safe for this patient?
Healthcare provider factors	Lack of training[48 49]	► Ensure necessary competencies by providing training on telehealth communication, interaction, legacy and clinical assessment
Organisational factors	Lack of data security[51 54] Lack of approval for reimbursement from insurance companies[44]	► Ensure that the telehealth interventions follow national and local obligations on legacy such as privacy and security requirements ► Involve payers and administration from the beginning in the development of telehealth interventions

recommendations and local telehealth solution and complement previous EULAR PtC for the development, evaluation and implementation of mobile health application aiding self-management of people with RMD.[55]

Within rheumatology, telehealth has been discussed in the context of the increasing prevalence of RMD and workforce limitations.[2 6] Recently, the COVID-19 pandemic has made telehealth even more relevant with 78% of patients finding it acceptable.[56] A recent EULAR survey conducted in 35 countries showed that during the pandemic, the majority of European face-to-face consultations were converted into telehealth consultations.[46] However, that study also pointed out that more research within tele-rheumatology is needed.[46]

As this is a relatively new research area, the present, PtC are only partially supported by evidence. Furthermore, few of the studies addressed disease monitoring in inflammatory arthritis.[33 36 37 57] PtC 8 (barriers) and 9 (training of people with RMD) are mainly based on qualitative research that is considered low quality of evidence by the Oxford hierarchy.[18] This does, however, not indicate a lower importance of these PtC.

Box 1 Research agenda

Remote care in rheumatic and musculoskeletal diseases—identified unmet needs and suggested focus for future research:

⇒ To conduct randomised non-inferiority and superiority trials to test the efficacy and patient satisfaction of telehealth interventions as compared with conventional care.
⇒ To perform longitudinal studies to test if telehealth leads to more or less treatment changes.
⇒ To evaluate methods of preassessment and prioritisation within different settings and diseases.
⇒ To evaluate the cost-effectiveness of telehealth interventions.
⇒ To explore factors associated with digital health literacy (for both, people with RMD and HCPs).
⇒ To explore barriers to the implementation of telehealth and how they can be solved.
⇒ To explore how artificial intelligence can be integrated into telehealth interventions in order to support the development of knowledge of clinical processes.
⇒ To evaluate patient safety and data security when using telehealth in daily clinical practice and in an extended follow-up.

Telehealth has been promoted as a means to increase cost-effectiveness, but this was only addressed in two studies on remote physiotherapy,[58 59] revealing conflicting results.

Furthermore, in some studies, telehealth interventions were applied as an add-on to and not as a replacement of face-to-face contact.[41 42 60 61] This makes a direct comparison between telehealth and face-to-face interventions difficult.

None of the included studies addressed security and potential adverse effects of telehealth interventions. Also, the follow-up time was generally short (mostly ≤1 year); therefore, it is not possible to make any conclusions about the long-term effects or potential harms of telehealth interventions. It is possible that more longitudinal studies looking at the effects of telehealth as opposed to routine care will become available in the next few years because of the greater shift to remote working during the COVID-19 pandemic.

In conclusion, these PtC identified several areas where telehealth may potentially improve quality of care and increase healthcare access within rheumatology. Although our SLR did not reveal any evidence on how to implement telehealth solutions, we identified barriers and facilitators that may potentially play a role for the implementation of telehealth interventions into clinical practice.

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Learning from chess engines: how reinforcement learning could redefine clinical decision-making in rheumatology

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It is the year 2035. For many years now, the concept of 'shared decision making' has looked nothing like it did in earlier times. Many clinical decisions, such as dose adjustments of methotrexate or certain biologics, are made neither by the rheumatologist nor by the patient, but by computer systems which are more or less autonomous. These consist of digital biomarkers, implanted or skin-integrated sensors and drug delivery systems based on microtechnology and nanotechnology, which have been used for some time in diabetes care. In the meantime, it has been shown that for rheumatoid arthritis and other rheumatological disorders, the disease activity and quality of life can be better controlled with these self-learning systems (formerly called artificial intelligence) than by the rheumatologist alone. Even in the case of non-drug treatments, such as physiotherapy or diet, the patient now receives personalised support through various algorithms. In any desired situation, the options are systematically assessed for their effectiveness and the best ones are suggested. If the treating rheumatologist retires, many years of experience about the individual course of the patient's disease are not lost, but the model continues to improve. It combines existing and new data, enabling it to treat more accurately with every passing day. Non-individual treatment recommendations for diseases no longer exist and treat-to-target strategies are not reviewed every 3–6 months, but daily to hourly. Of course, rheumatologists still exist. But their role has changed, especially when it comes to treating patients with common diseases and uncomplicated disease courses.

How did this development happen? As is often the case, such knowledge was initially developed outside of medicine. Learning systems initially came from the gaming industry, robotics and autonomous driving. In each of these fields, simulators are available that can be used to generate enormous amounts of data in order to test and improve machine-generated decisions. Chess is an excellent example of this.

To understand this better, let us return to the present. In the following, 10 theses are developed to underly the vision described above:

In December 2021, the World Chess Championship took place. Magnus Carlsen won again, retaining his status as World Chess Champion. He made fewer mistakes than his opponent Ian Nepomniachtchi and repeatedly generated surprise with unexpected moves that the chess computer had not predicted. During the live broadcast and in countless YouTube videos, renowned grandmasters commented on every move, every decision of the opponents, and discussed possible better alternatives. The 'gold standard', the best possible move, always comes from a chess computer such as Stockfish.[1] After all, since Deep Blue's victory over Garry Kasparov in 1997, chess computers have been considered invincible due to their computing power.

As another milestone, in 2017, Google's chess computer AlphaZero again defeated Stockfish.[2] AlphaZero, which also consists of neural networks (a subform of machine learning), took a whole 4 hours to learn chess. Unlike Stockfish, AlphaZero was not given any tactical instructions or human chess games from the past. It just knew the basic chess rules, and thus acted completely autonomously through reinforcement learning (RL). In RL, the so-called 'agent' determines which action offers the best decision in a certain situation and at different points in time (figure 1). A reward function is used to determine the best strategy to achieve a medium-to-long-term goal. In this case, to be able to checkmate the opponent at a later point in time. In doing so, the agent may accept sacrifices, even if this generates a worse position in the meantime. The underlying Action-Value formula in RL is the Q-function (Q stands for quality): $Q^{\pi}(s_t, a_t) = E\left[(R_{t+1} + \gamma R_{t+2} + \gamma^2 R_{t+3} + \ldots | s_t, a_t)\right]$. The Q-value for state (s) and a given action (a) is calculated by the expected discounted cumulative reward E, given that state and certain actions. Of note, thanks to the simulator, the chess computer is able to make use of an almost infinite amount of data. To understand this dimension better, there are up to 10^{120} different game courses in chess which can be simulated by the computer.

- *Reinforcement learning (RL), a subtype of machine learning, specialises in making the best possible decisions in a given environment and can far surpass human abilities through simulators.*

This is a different type of machine learning than that which is currently most used in medicine; classical supervised learning. If you look at the list of current FDA-approved machine learning algorithms, there are already over 100 applications.[3] These are mainly used for automated image recognition in radiology or for the detection of cardiac arrhythmias, for example. In most cases, these are automations to support non-complex medical tasks rather than genuine clinical decision-making aids. Almost always, these models have been trained and validated through supervised learning on labelled data sets such as X-ray images (table 1).

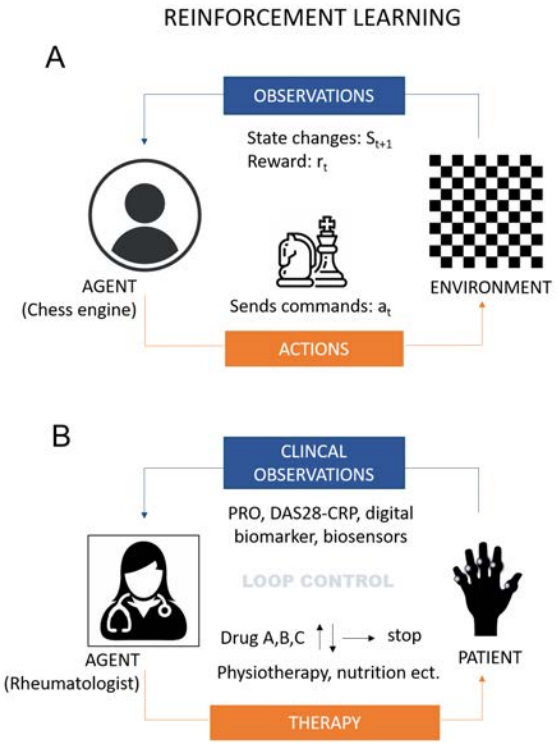

Figure 1 State-action pairs in the reinforcement learning concept using the example of chess (A) with transfer to rheumatology (B). An agent recognises the current situation (state) and independently takes an action. A reward function evaluates the respective decisions with regard to a certain goal, for example, remission. By this loop control, the system constantly improves its decisions. This could be a closed loop in the case of a drug pump and reliable biosensors and digital biomarkers, respectively. PRO, patient-reported outcomes.

Almost always, human labels are the ground truth that cannot be surpassed by the machine. There are exceptions when, for example, subsequent biopsy results are used to train the recognition of tumours on radiographic images. Notwithstanding, these data sets are (and remain) incomparably smaller than data sets from simulators such as in chess or autonomous driving. So, a substantial problem in medicine is that there is no realistic disease simulator in which treatments can be tried out and thus the amount of data cannot be increased while still maintain the quality.

- *In classical supervised learning, models are trained from existing fixed data sets that have been labelled by humans. Such algorithms therefore are supportive and time-saving, but they can never outperform the human performance.*

RL, on the other hand, also recognises and promotes prospectively raised actions through reward functions that lead to a sustainable, good result in the medium and long term. This is what we also expect from medical decisions. However, to a certain extent this means trial and error, which is medically and ethically problematic. On the other hand, we often try new experimental treatments in clinical trials, although under strictly defined conditions and in a fairly controlled manner. RL is therefore exciting because it is a granular decision-making aid for small steps at any desired time. In rheumatology, this could support smaller and less 'invasive' first-line interventions, such as an adjustment of the methotrexate or cortisone dose or non-drug interventions (physiotherapy, dietary changes, etc).

- *RL in the clinical setting will initially take over smaller, ethically justifiable interventions, where there is greater leniency for wrong decisions.*

In the future, we might have to allow the machine to make mistakes, at least to a certain extent, when necessary. After all, we make wrong decisions in the clinic every day. Why should not we allow the computer to do that if it learns to do better next time and the decision is made within certain rules? This approach differs substantially from the supervised machine learning that is currently applied in medicine. In supervised learning, clinical decision support consists primarily of predictions of specific events, such as the future disease status (eg, remission or flares).[4] Through regression analyses with deep neural networks, for example, algorithms trained on clinical data are already used to predict numerical values such as the DAS28-BSR at next visit.[5] Predictions are therefore decision-making aids by providing a more or less concrete look into the future. Potentially, this could improve the quality of therapy through a treat-to-predicted-target concept.

- *Clinical predictions of future disease states using supervised and unsupervised learning are already possible for rheumatoid arthritis, but always refer to previous observations.*

RL algorithms can also be trained retrospectively on large, existing data sets. This has been investigated, for example, for mechanical ventilation or fluid management in over 60 000 Intensive Care Unit stays.[6 7] However, RL becomes really exciting when it is no longer a human person who monitors

Table 1 Explanation of terms and concepts

Artificial intelligence (AI)	General term when computer systems take over tasks that are typically assigned to human attributes such as learning, recognising, planning and so on. Can also be robots or cars that move independently in their environment.
Algorithm	Set of steps for a computer program to accomplish a task or to solve a problem.
Machine learning (ML)	Subform of AI. Computer systems that learn and adapt independently from *data* without following explicit instructions. Can be prediction models or image recognition.
Supervised learning (SL)	Subform of ML. Models are trained and validated in existing, labelled data sets. These are typically used for classification tasks, for example, to predict future disease states or to detect pathologies on images.
Unsupervised learning (UL)	Subform of ML. Models are created from unlabelled data, for example, for clustering or outlier detection in electronic medical records.
Reinforcement learning (RL)	Subform of ML. Models that can make prospective decisions on their own and constantly improve them depending on the results. Works through a reward function (trial and error). Only good actions continue.
Q-learning	Subform of RL. A model-free, flexible RL algorithm to learn the value of a certain action. Random actions outside a specific system can be learnt, for example, by imitating and improving expert actions.
Artificial neural networks	A set of algorithms, modelled loosely after the human brain, in the form of different layers similar to neurons. A powerful tool which can be used for supervised, unsupervised or RL.

the reward function, but the environment is checked by the machine itself and actions are carried out independently. This is already possible in diabetes.[8] Through constant blood glucose measurement as a biomarker, the situation is assessed, and the micropump automatically injects an appropriate dose of insulin. Of note, the algorithm was trained beforehand to know how much insulin is approximately necessary in each situation and has strict constraints on the maximum amount of insulin that can be injected to avoid hypoglycaemia. This could potentially be carried out in rheumatology through the analysis of patient-reported outcomes (PROs), digital biomarkers and skin-integrated biosensor patches for the continuous measurement of inflammatory markers such as C reactive protein or cytokines. Altogether, this could assess the 'state' of the patient. A methotrexate pump or another implanted drug-delivery system could then carry out an 'action' and according to the response, this action will either be corrected next time or not (figure 1B). However, in clear contrast to diabetes, there are multiple possible biomarkers in inflammatory arthritis (not just glucose) and drugs have a much longer duration of action (weeks to months) compared with insulin. Furthermore, multiple antirheumatic drugs are often used at the same time and comorbidities such as fibromyalgia or depression or even side effects might confound digital biomarkers or PROs.

- *Disease-specific digital biomarkers and biosensors detecting inflammation are needed to make RL models more applicable to rheumatic diseases.*

It is essential that rules are imposed on such algorithms. In the case of the chess computer, these are the basic chess rules. Within these rules, anything is possible, even a queen sacrifice. In medicine, no patients or joints can be sacrificed and no regulatory rules can be disregarded. Compared with the regulation of blood glucose, inflammation as a 'system' seems more complex and algorithms must underly even more constraints and imposed rules (eg, in infection). At least for the time being, these rules still concern treatment recommendations, labels for reimbursement, contraindications or allergies.[9] Another important point is that an RL model must recognise when it is *not* in a position to make a decision. Quantitatively or qualitatively insufficient data must be recognised before taking action. This corresponds to situations in everyday clinical practice, where a doctor cannot make a decision without further diagnostics, for example.

- *In medicine, RL models must underly constraints based on expert knowledge and regulatory issues. An algorithm must be able to reject decisions, for example, due to low data quality or the lack of diagnostic information.*

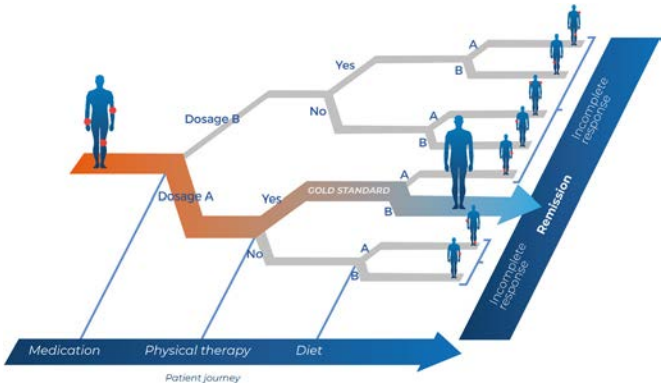

Figure 2 Multimodal decision making by reinforcement learning algorithms at different time points. Adapted from Ref. 15.

The attractive thing about RL as a decision-making professional is without doubt that every small therapy step and every situation can be re-evaluated by the algorithm (figure 2). Treatment recommendations in the form of hierarchical lines of therapy (first line, second line, etc) will no longer exist. Rather, the machine will create situation-dependent, highly granular standards, which include not only drug interventions but also lifestyle interventions. Thus, there will no longer be a rigid treat-to-target concept that is reviewed after 3–6 months. Through RL, it will be possible to achieve long-term targets through smaller and more regular treatment decisions.

- *RL makes treatment recommendations more flexible and granular. On the other hand, treatment targets become more long term.*

Due to the increasing availability of real-world data, such as PROs via apps, information on subjective symptoms, physical activity, nutrition and more can be incorporated into the algorithm more easily. Mental state and work ability can also be recorded regularly. In addition to the classic disease activity measures, other disease outcomes selected by the patient can be optimised. This is also necessary, because a minority of patients with rheumatoid arthritis do not achieve full remission despite targeted therapies. With RL algorithms, the 'point of care' of treatment may shift towards the empowered patient, who can better monitor and control his or her own treatment. Of course, this is restricted to patients appreciating such a computer support.

- *Therapy recommendations by RL will not only refer to medication, but also include physical activity, lifestyle modifications and diet, if this has a positive impact on quality of life.*

Back to chess, Magnus Carlsen stood out with unconventional moves and won. In fact, in chess one can distinguish computer-assisted decisions from human moves, or at least express a suspicion.[10] AlphaZero was a gamechanger. Through pure RL, AlphaZero did not win against Stockfish because it calculated faster. It only examined 60 thousand items per second compared with Stockfish's 60 million. AlphaZero played more creatively, closer to reality. It knew what it was thinking about and what it was ignoring. AlphaZero understood chess better than the calculating machine Stockfish.

Accordingly, RL rather than pure computational power may support human clinical decision-making in future. Computational simulations for rheumatic diseases both on a molecular and clinical level would leverage the performance of such algorithms immensely, although this still seems unthinkable today due to the complexity.[11]

A new approach to RL comes even closer to human reasoning. Originating from robotics, inverse Q-learning with constraints was developed. Here, the Q-function described above follows an expert policy. The machine trains itself to copy the action of an expert as best as possible and improve further while adhering to certain rules.[12] While the perfect rheumatologist to be copied probably does not exist, it could be a safer, smoother way to use artificial intelligence as a clinical decision support tool.

- *New algorithms are consciously oriented towards improving existing human actions while respecting certain limitations.*

So, computer algorithms are becoming more human and, to some extent, will be integrated into the shared clinical decision process within the next few years.[13] Of course, there are also several dangers and challenges involved, such as equity and the access to technology, especially for vulnerable groups. And yet, human decisions will always be necessary in medicine due to the complex interrelationships and the fact that

decisions are not always logical. The role of the doctor is not just to make decisions, but to listen to, inform and deal with emotions. Especially with multimorbid or elderly patients, it is not always about bringing the patient in remission, but also about evaluating factors such as polypharmacy or certain side effects that may influence the quality of life. Clinicians fortunately continue to act not only in a data-driven way, but also through experience, empathy and intuition. Those are features that are unlikely to be taken into account by RL-systems in near future. Notwithstanding, future rheumatologists will have to acquire a certain technical understanding of the quality and function of such algorithms, their data sources and medical devices such as sensors or autoinjectors that already exist for methotrexate.[14] In any case, new advances in medicine such as new drugs or biomarkers are first applied by humans and, if necessary and appropriate, can later be made accessible to automated or semiautomated systems as described here. Therefore, it can be concluded that:

- *Neither RL nor other types of artificial intelligence will ever replace a rheumatologist*.

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CLINICAL SCIENCE

Safety and efficacy of the miR-124 upregulator ABX464 (obefazimod, 50 and 100 mg per day) in patients with active rheumatoid arthritis and inadequate response to methotrexate and/or anti-TNFα therapy: a placebo-controlled phase II study

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ABSTRACT

Objective This phase 2a randomised, double blind, placebo controlled, parallel group study evaluated the safety and efficacy of a first-in-class drug candidate ABX464 (obefazimod, 50 mg and 100 mg per day), which upregulates the biogenesis of the mRNA inhibitor micro-RNA (miR)-124, in combination with methotrexate (MTX) in 60 patients (1:1:1 ratio) with moderate-to-severe active rheumatoid arthritis (RA) who have inadequate response to MTX or/and to an anti-tumour necrosis factor alpha (TNFα) therapy.

Methods The primary end point was the safety of ABX464; efficacy endpoints included the proportion of patients achieving American College of Rheumatology (ACR)20/50/70 responses, disease activity scores (DAS) 28, simplified disease activity score, clinical disease activity score), European League Against Rheumatism response, DAS28 low disease activity or remission.

Results ABX464 50 mg was safe and well tolerated. Two serious adverse events were reported (one on placebo group and one on ABX464 100 mg). Eleven patients were withdrawn for AEs (9 patients on 100 mg, 1 on 50 mg and 1 on placebo). Drug discontinuation was mainly due to gastrointestinal disorders. No cases of opportunistic infection, no malignancies and no death were reported. Compared with placebo, ABX464 50 mg showed significantly higher proportions of patients achieving ACR20 and ACR50 responses at week 12. DAS28-C reactive protein (CRP) and DAS28-erythrocyte sedimentation rate decreased significantly and rates of categorical DAS28-CRP response or CDAI remission increased significantly on ABX464 at week 12. A significant upregulation of miR-124 was observed in blood for every patient dosed with ABX464.

Conclusion ABX464 50 mg was safe, well tolerated and showed a promising efficacy. Mild-to-moderate gastrointestinal AEs led to a high drop-out rate of patients on ABX464 100 mg, which may not be a relevant dose to use. These findings warrant exploration of ABX464 at 50 mg per day or less for treating patients with RA.

Trial registration name Phase IIa randomised, double blind, placebo controlled, parallel group, multiple dose study on ABX464 in combination with MTX, in patients with moderate to severe active RA who have inadequate response to MTX or/and to an anti- TNFα therapy or intolerance to anti-TNFα therapy.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ ABX464 (obefazimod) is a first-in-class drug candidate for treating patients with active rheumatoid arthritis. ABX464 upregulates the biogenesis of the mRNA inhibitor micro-RNA-124 and can act as a natural brake on the production of various inflammatory mediators involved in inflammatory diseases.

WHAT THIS STUDY ADDS

⇒ This randomised, double-blind, placebo-controlled proof-of-concept study showed that at an oral daily dose of ABX464 50 mg was safe and well tolerated by patients.

⇒ An increased incidence of largely mild-to-moderate gastrointestinal adverse events in the ABX464 100 mg group led to a high drop-out rate of patients.

⇒ Several early efficacy endpoints showed promising results with ABX464 50 mg.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE AND/OR POLICY

⇒ Findings warrant further exploration of ABX464 as a rheumatoid arthritis treatment, using 50 mg per day or lower doses.

EUDRACT number: 2018-004677-27
Trial registration number NCT03813199.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterised by persistent synovitis and systemic inflammation. In the absence of disease control, RA ultimately results in severe progressive joint damage, disability, decreased quality of life, comorbidities and higher risk of mortality.[1 2] Conventional therapy with synthetic disease-modifying antirheumatic drugs (DMARDs) should be started as soon as the diagnosis of RA is made and methotrexate (MTX) should be part of the first treatment strategy.[2] If treatment target is not achieved and if poor prognosis factors are present, a targeted

therapy (biological (b) or targeted synthetic (ts) DMARD) is added. Although ts- and bDMARD drugs have revolutionised RA prognosis, safety concerns and difficult-to-treat RA[3 4] leave a room for novel, safe and effective oral targeted treatments, which may act with a different mechanism of action and better control the course of the disease.

ABX464 (obefazimod) upregulates the biogenesis of the mRNA inhibitor micro-RNA (miR)-124, which in turn modulates monocyte and macrophage activations[5–8] and can act as a natural brake on the production of various inflammatory mediators involved in inflammatory diseases such as ulcerative colitis (UC) and RA.[5–10] Among targets that miR-124 represses, CCL2 (monocyte chemoattractant protein-1, MCP-1), Signal transducer and activator of transcription 3 (STAT3), interleukine 6 receptor and tumour necrosis factor alpha (TNFα) converting enzyme (TACE) are important actors in RA.[11–13] ABX464 has demonstrated durable efficacy in treating patients suffering from UC, a chronic inflammatory disorder of the colonic mucosa.[14] The efficacy and safety profiles of this compound are likely related to its unique mechanism of action.[15] Recently, non-clinical in vivo data provided evidence that ABX464 (40 mg/Kg, per os) strongly reduced the incidence of collagen-induced arthritis in DBA-1 male mice.[10] These encouraging data have led to design the present 12-week randomised double-blind, placebo-controlled proof of concept study aimed to investigate the safety of ABX464 administered daily (50 mg or 100 mg) in patients with RA with active disease not responding to MTX.

METHOD

The study was conducted in 21 centres (eight in France, three in Belgium, seven in Poland and three in Hungary) in accordance with the standard operating procedures of the sponsor, which were designed to ensure adherence with the Declaration of Helsinki and Good Clinical Practice.

Patients

Eligible patients (ages 18–75 years) had a confirmed and documented diagnosis of RA, for at least 12 weeks, according to the revised 2010 American College of Rheumatology (ACR)-European League Against Rheumatism (EULAR) classification criteria, in addition to at least one positive criteria among rheumatoid factor, anticitrullinated peptide antibody or bone erosion. Inclusion criteria comprised swollen joint count (SJC) of ≥4 (28-joint count) and tender joint count of ≥4 (28-joint count), disease activity score (DAS)28 C reactive protein (CRP) of ≥3.2 and CRP ≥5 mg/L. Patients either had an inadequate response or failed either MTX (≥10 mg/week) or/and anti-TNFα therapy or were intolerant to anti-TNFα therapy for ≥12 weeks before trial entry. Exclusion criteria comprised a confirmed diagnosis of systemic lupus erythematosus or active or history of serious or opportunistic infections (OIs). Patients with a history of immunodeficiency or with history of malignancy were also excluded. In addition, patients were excluded if previously treated with non-anti-TNF bDMARDs, tsDMARDs, systemic corticosteroids at a dose >10 mg/day within 2–4 weeks prior to the study; or immunosuppressive drugs.

All participants provided written informed consent before participation. First patient was randomised on 1 August 2019 and the last patient was randomised on 2 February 2021; the last patient completed on 27 April 2021.

Randomisation and procedures

Randomisation was performed via a centralised treatment allocation system. Eligible patients were randomised according to a 1:1:1 ratio and were treated for 12 weeks followed by a 21-day follow-up period. During the treatment phase, patients were receiving either capsules containing ABX464 50 mg (n=21), ABX464 100 mg (n=19) or placebo (n=20) given orally once per day in a fed condition. Patients who completed the induction study at week 12 could roll over into a 52-week open-label maintenance study to evaluate the long-term safety and efficacy of 50 mg per day oral ABX464 in RA (trial ABX464-302; NCT04049448). Here, we present the results of the 12-week treatment induction phase of this clinical trial.

Assessments

Safety

The primary end point was the safety and tolerability of two doses of ABX464 versus placebo. The primary safety endpoint was the rate of all treatment-emergent adverse event (TEAEs). Safety was evaluated based on adverse events (AEs), clinical laboratory parameters, vital signs, physical examination, standard 12-lead ECG results. AEs were coded using the Medical Dictionary for Regulatory Activities (V.23.0). Severity of AEs was graded on a 5-point scale according to the Common Terminology Criteria for Adverse Events (V.5.0). The causal relationship between the study treatment and the occurrence of each AE was assessed by each investigator using clinical judgement.

Efficacy

Efficacy endpoints included an evaluation of the effects of ABX464 versus placebo on (1) the ACR 20/50/70 responses (≥20/50/70% improvement in tender/painful joint count (TJC) (28-joint count) and SJC (28-joint count) plus ≥20/50/70% improvement in three of the five remaining ACR core set measures) and all, components of the ACR response (CRP, TJC, SJC, pain-patient assessment of joint pain, patient global assessment of disease (PtGA), physician's global assessment of disease, disability index of the healthy assessment questionnaire (HAQ-DI), (2) DAS28 scores, simplified disease activity score (SDAI) and clinical disease activity score (CDAI), (3) clinical response (DAS28 EULAR good and moderate responses), (4) low disease activity (LDA; DAS28-erythrocyte sedimentation rate (ESR) ≤3.2) or remission (DAS28-ESR remission (DAS28 <2.6), ACR/EULAR Boolean remission (TJC(28), SJC(28), CRP (mg/dL) and PtGA, all ≤1), SDAI remission (SDAI ≤3.3) and CDAI remission (CDAI ≤2.8), (5) Functional Assessment of Chronic Illness Therapy (FACIT)-Fatigue score.

Laboratory tests

Blood samples from patients in each group were used to measure the expression of miR−124 at baseline and week 8. For each sample, RNA and DNA were extracted (AllPrep DNA/RNA/miRNA Universal kit, Qiagen) and their concentrations were determined (Nanodrop 2000 and Qubit V.2.0 fluorometer, ThermoFisher). The miRNAs were retro transcribed from 70 ng of the extracted RNA (TaqMan Advanced miRNA cDNA Synthesis Kit, ThermoFisher), then, after preamplification, cDNA was subjected to duplicate droplet digital PCR measurements using two singleplex assays targeting miR-16 and miR-124 at specified dilutions (1:10 for miR-124; 112 000 for miR-16).

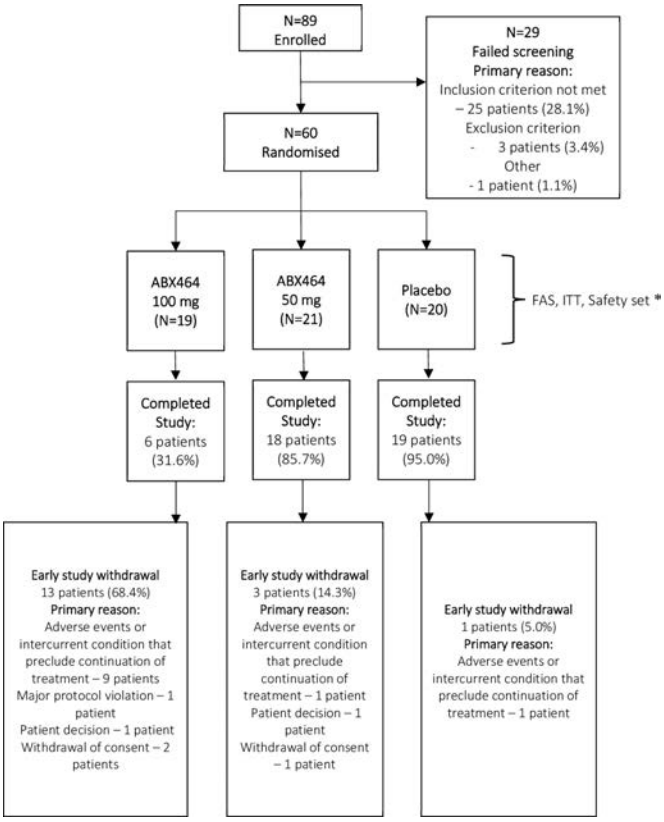

Figure 1 Patient disposition. *: For PP set, there were 18, 20 and 20 patients in ABX464 100 mg, 50 mg and placebo groups, respectively. To derive the PP set, one patient was excluded in the ABX464 100 mg group, one patient was excluded in the ABX464 50 mg and no patient was excluded in the placebo group. FAS, Full Analysis dataset; ITT, intent-to-treat.

Statistical methods

The rate of all TEAEs, categorical DAS28-CRP response, DAS28-ESR remission, SDAI remission, CDAI remission, ACR/EULAR Boolean remission, LDA and ACR 20/50/70 response rates were compared in patients who received a dose of ABX464 or placebo by likelihood ratio χ^2 test on a 10% two-sided significance level. A mixed model analysis of covariance was conducted for change from baseline in SDAI and CDAI scores, DAS28-CRP, DAS28-ESR, ESR, CRP and all ACR components.

The Full Analysis dataset (FAS population) was defined as those patients included in the study, who had received at least one dose of the study treatment, and who had at least one baseline data. *Post hoc* efficacy analyses were performed for the intent-to-treat (ITT, ie, patients randomised, regardless of whether the patient received a dose of study treatment or completed the study) and per-protocol (PP) (patients of the FAS population without any major protocol deviation) populations. Additional *post hoc* efficacy and safety analyses were also performed on subgroups of patients with or without previous exposure to anti TNFα therapy.

Statistical analyses were carried out using SAS, V.9.4 or later (SAS Institute, Cary, North Carolina). For miR-124 analyses, non-parametric Kruskal-Wallis test followed by a Dunn's multiple comparisons test was performed using GraphPad Prism software (V.9.3.0).

RESULTS

Demographics and baseline characteristics

Patient disposition is summarised in figure 1. For FAS, ITT and Safety sets, there were 19, 21 and 20 patients in ABX464 100 mg, 50 mg and placebo groups, respectively. For PP set, there were 18, 20 and 20 patients in ABX464 100 mg, 50 mg and placebo groups, respectively. Baseline demographic characteristics were similar among the three groups (table 1). Most patients (61.7%) were women, with a mean age of 57.0 years and a mean RA duration of 6.4 years. Thirty per cent of patients were previously exposed to anti-TNF therapies and 53.3% received corticosteroids at baseline. At baseline, mean DAS28-CRP and DAS28-ESR were, respectively, 5.4 and 5.8. History of MXT dose was comparable across treatment groups.

Safety

A total of 220 AEs were reported by 50 subjects (83.3%) during the study, and among them, 211 AEs were treatment emergent. Treatment with ABX464 was associated with the occurrence of mainly mild to moderate AEs. AEs occurred more frequently in the ABX464 treatment groups than in the placebo group (AEs, 94.7%, 85.7% and 70.0%, in ABX464 100 mg, 50 mg and placebo groups, respectively). When compared with placebo, a higher incidence of TEAEs was reported in the ABX464 groups, with a statistically significant difference for the 100 mg dose group (p=0.035) (online supplemental table 1). The onsets of headaches, nausea and vomiting were more rapid (mostly in the first 2 weeks) than the onset of diarrhoea (mostly in the two first months). Most dropouts in the 100 mg group took place rapidly in the first 2 weeks of treatment (online supplemental figure 1). One serious AE (SAE) was reported in each treatment group, including one atrial fibrillation in the 100 mg ABX464 group and a severe COVID-19 disease in the placebo group, of which both were treatment emergent. The patient hospitalised for a severe atrial fibrillation had experienced severe diarrhoea and the conclusion of hospitalisation was an atrial fibrillation reactive to hypokalemia secondary to diarrhoea in the context of study drug treatment. Another SAE was a severe RA worsening, as it required patient hospitalisation; it occurred before the first ABX464 50 mg dose. The incidence of severe AEs (grade 3 or above) was numerically higher in the ABX464 50 mg and 100 mg groups (14.3% and 15.8%, respectively) than in placebo group (5%).

TEAEs that occurred in >5% of patients in any ABX464 treatment group included headache (22 patients, 36.7%), followed by diarrhoea (12 patients, 20.0%), nausea (12 patients, 20.0%), abdominal pain upper (10 patients, 16.7%), vomiting (5 patients, 8.3%), dyspepsia (4 patients, 6.7%) and RA exacerbation (4 patients, 6.7%). Except for abdominal pain and RA exacerbation, the highest incidence of TEAEs occurred in the 100 mg ABX464 group (table 2). In the 100 mg ABX464 group, there were numerically higher incidences for neurological (dizziness, headache, taste disorder, tremor) and infectious AEs than for ABX464 50 mg and placebo groups. Drug discontinuation was mainly due to gastrointestinal disorders (online supplemental table 2) and was the main cause of patient withdrawals. Eleven patients were withdrawn for AEs (nine patients in the 100 mg ABX464 group and one in each of the other groups). Nine patients had temporary discontinuation (four patients in each of the ABX464 groups and one patient in the placebo group) with a mean duration of 4 days (ranging from 1 to 9 days). Vital signs and ECG parameters were similar across the ABX464 50 mg and 100 mg groups and the placebo group. No clinically

Table 1 Baseline patient demographic and clinical characteristics (Full Analysis set*)

		ABX464 100 mg (N=19)	ABX464 50 mg (N=21)	Placebo (N=20)	All (N=60)
Age (years)	Mean (SD)	54.4 (10.6)	57.9 (11.4)	58.6 (11.0)	57.0 (11.0)
Sex	Male	8 (42.1%)	6 (28.6%)	9 (45.0%)	23 (38.3%)
	Female	11 (57.9%)	15 (71.4%)	11 (55.0%)	37 (61.7%)
BMI (kg/m^2)	Mean (SD)	26.8 (5.0)	25.8 (4.4)	28.4 (5.9)	27.0 (5.2)
	Range	20.2–37.0	18.7–35.1	21.1–42.9	18.7–42.9
RA duration (years†)	Mean (SD)	6.0 (4.7)	7.3 (9.1)	5.8 (7.8)	6.4 (7.4)
	Range	0.9–18.8	0.6–29.7	0.4–32.7	0.4–32.7
MTX dose on day 0 (mg/week)	Mean (SD)	16.4 (3.0)	17.9 (3.4)	18.0 (3.4)	17.4 (3.3)
	Range	10–20	0–25	10–20	0–25
History of anti-TNFα therapy	N (%)	6 (31.6%)	6 (28.6%)	6 (30.0%)	18 (30.0%)
Anti-TNFα washout (months)	Mean (SD)	27.0 (35.2)	45.4 (89.2)	7.3 (2.3)	25.5 (51.2)
	Range	3–93.2	1.1–204.9	3.4–10.1	1.1–204.9
Patients with corticosteroids	N (%)	9 (47.4%)	10 (47.6%)	13 (65.0%)	32 (53.3%)
DAS28-CRP	Mean (SD)	5.5 (0.8)	5.5 (0.7)	5.3 (0.7)	5.4 (0.7)
	Range	3.7–6.5	4.2–6.8	3.7–6.4	3.7–6.8
DAS28-ESR	Mean (SD)	5.9 (1.1)	5.9 (0.8)	5.8 (0.8)	5.8 (0.9)
	Range	4.2–7.7	3.4–7.0	3.9–7.4	3.4–7.7
ESR	Mean (SD)	31.5 (18.4)	37.4 (24.8)	38.3 (24.2)	35.8 (22.6)
	Range	5–85	2–105	3–89	2–105
CRP (mg/L)	Mean (SD)	20.6 (28.6)	22.7 (24.6)	14.9 (13.5)	19.5 (22.9)
	Range‡	1.1–129.0	1.9–99.5	2.5–58.4	1.1–129.0
TJC	Mean (SD)	12.2 (5.8)	12.2 (4.6)	10.7 (4.5)	11.7 (4.9)
	Range	4–27	5–24	4–22	4–27
SJC	Mean (SD)	9.5 (3.8)	8.3 (3.5)	7.8 (3.4)	8.5 (3.6)
	Range	3–19	4–15	3–16	3–19
HAQ-DI	Mean (SD)	1.28 (0.48)	1.49 (0.63)	1.48 (0.66)	1.42 (0.60)
	Range	0.12–2.0	0–2.5	0–2.37	0–2.50
FACIT-Fatigue	Mean (SD)	28.3 (10.7)	26.7 (11.2)	24.4 (10.5)	26.5 (10.7)
	Range	7–49	3–45	12–45	3–49

The denominator for each percentage is the number of non-missing observations within the column.
*The Full Analysis dataset (FAS population) was defined as those patients included in the study, who had received at least one dose of the study treatment, and who had at least one baseline data.
†The disease onset was defined as the date of diagnosis.
‡A value of CRP ≥5 mg/L was requested at screening but eight patients had baseline CRP values ranging from 1.1 to 4.8 mg/L (two patients in the placebo; four patients in ABX464 50 mg group and 2 patients in ABX464 100 mg group).
BMI, body mass index; CDAI, clinical disease activity score; CRP, C reactive protein; DAS, disease activity score; ESR, erythrocyte sedimentation rate; FACIT, functional assessment of chronic illness therapy; HAQ-DI, Healthy Assessment Questionnaire—Disability Index; max, maximum; min, minimum; MTX, methotrexate; RA, rheumatoid arthritis; SDAI, simplified disease activity score; SJC, swollen joint count; TJC, tender/painful joint count.

meaningful changes were observed in laboratory data or other safety assessments (online supplemental table 3). No cases of OI were recorded, and the infestation rate was similar between placebo and ABX464 groups. No deaths or malignancies were reported. No AE persisted or had sequelae at the end of trial.

Efficacy

In the ITT population, the DAS28-CRP and DAS28-ESR decreased sequentially from baseline to week 12 after both ABX464 doses. Decreases in the DAS28-CRP and DAS28-ESR were statistically significant between the placebo group and the 50 mg ABX464 dose at week 12 (p=0.043 and p=0.035, respectively) (figure 2, table 3). In the PP population, significant decreases in the DAS28-CRP and DAS28-ESR were seen between placebo and the 50 mg or 100 mg ABX464 groups as early as week 8 (figure 2, online supplemental table 4).

Compared with placebo, mean changes in CDAI scores from baseline to week 12 were significantly different for the 50 mg ABX464 dose in the ITT population (p=0.020) (table 3) and for both ABX464 doses in the PP population (online supplemental table 4). No significant differences between groups were observed for SDAI scores change in ITT and PP populations.

Rates of categorical DAS28-CRP response at week 12 were significantly higher versus placebo at 50 mg and 100 mg ABX464 doses in the PP population (p=0.004 and p=0.038, respectively) (online supplemental table 5). These differences did not reach statistical significance in the ITT population (table 4).

In the ITT and PP populations, difference in LDA responses across groups did not reach statistical significance at week 12 (table 4, online supplemental table 5). There was a significant difference in CDAI remission rates at week 12 between the ABX464 50 mg group and the placebo group (ITT set: p=0.039; PP set: p=0.024). DAS28-ESR remission, SDAI remission and ACR/EULAR remission rates were not significantly different across groups (table 4, (online supplemental table 5).

In the ITT population, differences between treatment groups for the proportions of patients achieving ACR20, ACR50 and ACR50 did not reach statistical significance (table 4). When considering the very small-sized subsets of patients exposed or not to anti-TNFα, ACR20, ACR50 and ACR70 response rates at

Table 2 Most commonly occurring TEAEs by system organ class (>5% patients) and preferred term (Safety Set*)

	ABX464 100 mg (N=19)		ABX464 50 mg (N=21)		Placebo (N=20)	
	N	N (%)	N	N (%)	N	N (%)
Any TEAEs	93	18 (94.7%)	76	18 (85.7%)	34	14 (70.0%)
Gastrointestinal disorders	44	16 (84.2%)	24	11 (52.4%)	2	2 (10.0%)
Abdominal pain	1	1 (5.3%)	3	2 (9.5%)	0	0
Abdominal pain upper	10	4 (21.1%)	6	5 (23.8%)	1	1 (5.0%)
Diarrhoea	11	7 (36.8%)	7	4 (19.0%)	1	1 (5.0%)
Dyspepsia	3	3 (15.8%)	1	1 (4.8%)	0	0
Gastrointestinal pain	1	1 (5.3%)	0	0	0	0
Impaired gastric emptying	1	1 (5.3%)	0	0	0	0
Nausea	12	9 (47.4%)	4	3 (14.3%)	0	0
Splenic artery aneurysm†	1	1 (5.3%)	0	0	0	0
Vomiting	4	3 (15.8%)	2	2 (9.5%)	0	0
Nervous system disorders	19	10 (52.6%)	23	8 (38.1%)	10	5 (25.0%)
Dizziness	1	1 (5.3%)	0	0	1	1 (5.0%)
Headache	16	10 (52.6%)	19	8 (38.1%)	6	4 (20.0%)
Taste disorder	1	1 (5.3%)	0	0	0	0
Tremor	1	1 (5.3%)	0	0	0	0
Musculoskeletal and connective tissue disorders	9	6 (31.6%)	4	4 (19.0%)	8	4 (20.0%)
Arthralgia	3	2 (10.5%)	0	0	1	1 (5.0%)
Musculoskeletal pain	1	1 (5.3%)	0	0	0	0
Myalgia	2	1 (5.3%)	1	1 (4.8%)	0	0
Pain in extremity	2	1 (5.3%)	0	0	2	1 (5.0%)
Rheumatoid arthritis	1	1 (5.3%)	2	2 (9.5%)	1	1 (5.0%)
Infections and infestations	5	5 (26.3%)	4	3 (14.3%)	4	4 (20.0%)
COVID-19	0	0	0	0	2	2 (10.0%)
Oral herpes	1	1 (5.3%)	0	0	0	0
Peritonsillar abscess	1	1 (5.3%)	0	0	0	0
Rhinitis	1	1 (5.3%)	0	0	0	0
Sinusitis	1	1 (5.3%)	0	0	0	0
Urinary tract infection	1	1 (5.3%)	0	0	0	0

Number of events (n) and number and percentage of patients (N(%)).The denominator for each percentage is the number of patients within the column.
*the safety set corresponded to included patients who had received at least one dose of the study treatment.
†Asymptomatic incidental finding, unrelated.
AE, adverse event; TEAE, treatment emergent adverse event.

week 12 were not significantly different across groups (online supplemental table 6). In the PP population, the proportions of patients achieving ACR20 and ACR50 responses at week 12 were significantly higher (p=0.030 and p=0.037, respectively)

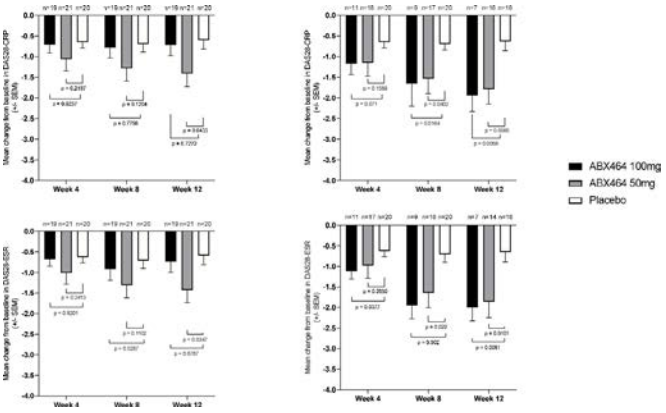

Figure 2 DAS28-CRP (A) and DAS28-ESR (B) mean (±SEM) changes from baseline at weeks 4, 8 and 12 in RA patients who received placebo or ABX464 (50 or 100 mg) once daily (ITT set). ITT, intent-to-treat; RA, rheumatoid arthritis.

in the ABX464 50 mg group than in placebo (online supplemental table 5). The ACR20 and ACR50 response rates were numerically higher in patients receiving ABX464 100 mg versus placebo, but statistical significance was not reached. ACR70 response rates and mean changes in HAQ-DI were not significantly different across groups at week 12. There was a significant difference in change in FACIT-Fatigue at week 12 between the ABX464 50 mg group and the placebo group in PP analysis (p=0.045, online supplemental table 4).

Two efficacy sets were initially considered: the 'raw' efficacy set 1 took into consideration only patients at time point (N=number of patients from whom efficacy variables were available at week 12). The efficacy set 2 used data imputation (last observation carried forward) from week 4 onwards. If there were no efficacy data available, then the dropout was considered as treatment failure. Main data obtained in the efficacy set 2 are provided in online supplemental tables 7 and 8.

The pharmacokinetics (PK) analysis was performed on the PK Analysis Set, which included all the patients who received at least one dose of ABX464 and for whom postdose concentrations were available without major protocol deviations or events implying bias for the PK evaluation. Summary of PK parameters

Table 3 Changes from baseline at week 12 in ESR, DAS28-CRP, DAS28-ESR, SDAI, CDAI, SJC, TJC, CRP, Pain-VAS, HAQ-DI and FACIT-Fatigue (ITT set)

		ABX464 100 **mg** (N=19)	ABX464 50 **mg** (N=21)	**Placebo (N=20)**
ESR	N	19 (100.0%)	21 (100.0%)	20 (100.0%)
	Mean (SD)	−0.3 (6.8)	−2.6 (19.3)	−2.7 (16.6)
	95% CI	−4 to 3	−11 to 6	−10 to 5
	Min – Max	−22 to 18	−37 to 49	−38 to 41
	p-value*	0.564	0.988	
DAS28-CRP	N	19 (100.0%)	21 (100.0%)	20 (100.0%)
	Mean (SD)	−0.72 (1.13)	−1.41 (1.45)	−0.60 (0.98)
	95% CI	−1.3 to −0.2	−2.1 to −0.7	−1.1 to −0.1
	Min – Max	−3.3 to 0.0	−4.8 to 0.1	−3.3 to 0.6
	p-value*	0.727	**0.043**	
DAS28-ESR	N	19 (100.0%)	21 (100.0%)	20 (100.0%)
	Mean (SD)	−0.7 (1.1)	−1.4 (1.3)	−0.5 (1.0)
	95% CI	−1.3 to −0.2	−2.1 to −0.8	−1.1 to −0.1
	Min – Max	−3.1 to 0.0	−4.5 to 0.0	−3.5 to 0.6
	p-value*	0.678	**0.034**	
SDAI	N	19 (100.0%)	21 (100.0%)	20 (100.0%)
	Mean (SD)	−9.3 (14.4)	−20.2 (33.2)	−7.5 (22.6)
	95% CI	−16.3 to −2.4	−35.4 to −5.1	−18.2 to 3.0
	Min – Max	−39.5 to 0.0	−85.2 to 62.7	−44.5 to 63.1
	p-value*	0.772	0.165	
CDAI	N	19 (100.0%)	21 (100.0%)	20 (100.0%)
	Mean (SD)	−9.9 (15.8)	−15.8 (13.2)	−6.9 (10.1)
	95% CI	−17.6 to −2.4	−21.9 to −9.8	−11.7 to −2.2
	Min – Max	−51.1 to 0.0	−48.0 to 0.3	−26.0 to 9.0
	p-value*	0.474	**0.020**	
SJC	N	19 (100.0%)	21 (100.0%)	20 (100.0%)
	Mean (SD)	−3.2 (5.3)	−4.4 (4.2)	−2.1 (4.2)
	95% CI	−6.0 to −1.0	−6.0 to −3.0	−4.0 to −0.0
	Min – Max	−18.0 to 0.0	−12.0 to 1.0	−12.0 to 5.0
	p-value*	0.492	0.084	
TJC	N	19 (100.0%)	21 (100.0%)	20 (100.0%)
	Mean (SD)	−4.1 (6.6)	−6.8 (6.4)	−2.9 (3.8)
	95% CI	−7.0 to −1.0	−10.0 to −4.0	−5.0 to −1.0
	Min – Max	−24.0 to 0.0	−24.0 to 0.0	−11.0 to 4.0
	p-value*	0.487	**0.022**	
CRP	N	19 (100.0%)	21 (100.0%)	20 (100.0%)
	Mean (SD)	0.6 (4.8)	−4.3 (28.8)	−0.6 (18.7)
	95% CI	−1.7 to −3.0	−17.4 to 8.8	−9.4 to 8.1
	Min – Max	−8.8 to 13.4	−54.9 to 87.5	−36.3 to 65.5
	p-value*	0.776	0.634	
Pain-VAS	N	19 (100.0%)	21 (100.0%)	20 (100.0%)
	Mean (SD)	−0.8 (1.9)	−2.6 (2.4)	−0.7 (2.3)
	95% CI	−1.8 to −0.1	−3.7 to −1.5	−1.9 to 0.3
	Min – Max	−8.2 to 0.0	−7.3 to 0.0	−7.0 to 3.6
	p-value*	0.876	**0.018**	
HAQ-DI	N	19 (100.0%)	21 (100.0%)	20 (100.0%)
	Mean (SD)	−0.10 (0.34)	−0.43 (0.61)	−0.18 (0.48)
	95% CI	−0.27 to 0.06	−0.71 to −0.15	−0.40 to 0.04
	Min – Max	−1.37 to 0.37	−1.75 to 0.62	−1.75 to 0.87
	p-value*	0.576	0.153	
FACIT-Fatigue	N	19 (100.0%)	21 (100.0%)	20 (100.0%)
	Mean (SD)	2.9 (7.4)	6.2 (6.0)	3.1 (6.3)
	95% CI	−1 to 6	3 to 9	0 to 6
	Min – Max	−4 to 26	0 to 20	−11 to 15
	p-value*	0.944	0.105	

*Analysis of covariance, ABX-464 versus placebo; mixed model analysis of covariance is conducted for the changes from baseline for each parameter.
CDAI, clinical disease activity score; CRP, C reactive protein; DAS, disease activity score; ESR, erythrocyte sedimentation rate; FACIT, functional assessment of chronic illness therapy; HAQ-DI, Healthy Assessment Questionnaire - Disability Index; max, maximum; min, minimum; Pain-VAS, Patient assessment of joint pain; SDAI, simplified disease activity score.

Table 4 Patients' responses and remissions at week 12 (ITT set)

		ABX464 100 mg (N=19)	ABX464 50 mg (N=21)	Placebo (N=20)
ACR20 response	N	19	21	20
	Yes	3 (15.8%)	9 (42.9%)	4 (20.0%)
	95% CI	3.4 to 39.6	21.8 to 66.0	5.7 to 43.7
	p-value*	0.731	0.112	
ACR50 response	N	19	21	20
	Yes	2 (10.5%)	5 (23.8%)	1 (5.0%)
	95% CI	1.3 to 33.1	8.2 to 47.2	0.1 to 24.9
	p-value*	0.514	0.076	
ACR70 response	N	19	21	20
	Yes	1 (5.3%)	4 (19.0%)	1 (5.0%)
	95% CI	0.1 to 26.0	5.4 to 41.9	0.1 to 24.9
	p-value*	0.970	0.155	
Categorical DAS28-CRP response	N	19	21	20
	Yes	6 (31.6%)	14 (66.7%)	8 (40.0%)
	95% CI	12.6 to 56.6	43.0 to 85.4	19.1 to 63.9
	p-value*	0.583	0.085	
DAS28-ESR remission	N	19	21	20
	Yes	0	2 (9.5%)	0
	95% CI	82.4 to 100.0	1.2 to 30.4	83.2 to 100.0
	p-value*	NC	0.095	
Low disease activity	N	19	21	20
	Yes	2 (10.5%)	4 (19.0%)	2 (10.0%)
	95% CI	1.3 to 33.1	5.4 to 41.9	1.2 to 31.7
	p-value*	0.956	0.408	
SDAI remission	N	19	21	20
	Yes	0	1 (4.8%)	0
	95% CI	82.4 to 100.0	0.1 to 23.8	83.2 to 100.0
	p-value*	NC	0.243	
CDAI remission	N	19	21	20
	Yes	0	3 (14.3%)	0
	95% CI	82.4 to 100.0	3.0 to 36.3	83.2 to 100.0
	p-value*	NC	**0.039**	
ACR/EULAR remission	N	19	21	20
	Yes	0	1 (4.8%)	0
	95% CI	82.4 to 100.0	0.1 to 23.8	83.2 to 100.0
	p-value*	NC	0.243	

*χ^2 test ABX-464 versus placebo. NC—no statistics from χ^2 test produced because response has fewer than two non missing levels.
ACR, American College of Rheumatology; CDAI, clinical disease activity score; DAS, disease activity score; ESR, erythrocyte sedimentation rate; EULAR, European League Against Rheumatism; SDAI, simplified disease activity score.

following administration of ABX464 50 mg and 100 mg is provided in online supplemental table 9.

miR-124

The expression of miR-124 was measured in 27 blood samples from patients with RA and a miR-124 induction was observed at week 8 compared with baseline in patients receiving ABX464. The fold changes of miR-124 were statistically significant between placebo and ABX464 50 and 100 mg doses ($p<0.001$ and $p<0.01$, respectively) with medians equal to 0.2 for placebo, 174.5 and 119.2 for ABX464 50 and 100 mg, respectively (online supplemental figure 2).

DISCUSSION

This is the first multicentre, randomised, double-blind, placebo-controlled study to evaluate the safety and efficacy of two daily doses (50 mg and 100 mg) of ABX464 (obefazimod) for 12 weeks in patients with moderate to severe active RA who had an inadequate response to MTX and/or to an anti-TNFα therapy.

A daily dose of 50 mg of ABX464 appeared safe in those patients and no new safety signals were identified. ABX464 AEs were mild, dose-dependent, and the nature of these AEs was consistent with what has been observed in more than 850 subjects who have so far been treated in other clinical trials with ABX464 across different indications (HIV/AIDS, COVID-19 and UC). Compared with placebo group, a larger proportion of patients in ABX464 groups experienced TEAEs. An increased incidence of largely mild-to-moderate gastrointestinal AEs in the 100 mg treatment group led early to a high drop-out rate of patients, and, therefore, may not be a relevant dose to use. Rates of AEs—in particular, abdominal pain upper, diarrhoea, vomiting and headache—were more elevated than rates of AEs observed throughout the development programme of ABX464 with the dose 50 mg and 100 mg[16]; these higher rates were likely

driven by an additive effect due the concomitant administration of MTX since gastrointestinal AEs are the most common side effect with this treatment.[17] The ABX464 100 mg group seems to have higher incidences not only for gastrointestinal but also for neurological (dizziness, headache, taste disorder, tremor) and infectious AEs; the ABX464 100 mg/day dose is probably too high and the future development programme will explore doses lower than 50 mg. In the present study, no malignancies were reported, and no OI was observed during the study, with an infestation rate similar between placebo and ABX464 all doses.

Although the sample size of this proof-of-concept study was limited, multiple early efficacy endpoints showed signs of promise with the ABX464 50 mg daily dose, whereas no clear efficacy was demonstrated with the highest dose, likely due to the high drop-out rate of patients. In the ITT population, DAS28-CRP and DAS28-ESR of ABX464 50 mg-treated patients decreased sequentially to reach a statistically significant difference versus placebo after 12 weeks. In addition, compared with placebo, changes in CDAI scores from baseline to week 12 in the ITT population were significantly in favour of ABX464 50 mg, as were differences in CDAI remission rates. Decrease in CRP levels did not reach significance, presumably due to a very large SD. In general, production of CRP correlates with IL-6 levels, which were significantly decreased in the serum of patients with RA (data not shown). Significant changes were likewise observed in the PP population, with additional endpoints in favour of ABX464, such as categorical DAS28-CRP response rates and ACR20 and ACR50. In the PP set, more than half of ABX464 patients dosed with 50 mg reached the ACR20 endpoint at week 12.

From a mechanism of action standpoint, a striking increase in miR-124 expression was seen at week 8 in blood samples of patients with RA receiving ABX464, which indicates that the treatment promotes the production of a key agent that has the potential to reduce several inflammatory-activated pathways. Indeed, miR-124 target genes that control the production of a number of inflammatory mediators (eg, TNF-α, IL-6, IL-17) implicated in the intense inflammatory reaction that drives RA.[5–9] An upregulated biogenesis of miR-124 has been consistently reported with ABX464, across non-clinical and clinical studies in different indications,[15] still a definitive correlation with clinical response remains to be established. Interestingly though, no significant difference in miR-124 upregulation was seen between the two ABX464 doses, indicating the absence of a dose-dependent effect, which reminds the absence of dose-related efficacy of the drug reported in clinics in patients with UC.[14]

In conclusion, this proof-of-concept study provides the first clinical evidence that ABX464 50 mg/day for 12 weeks appeared to be safe and well tolerated by patients with active RA, whereas 100 mg may not be a relevant dose to use in patients with RA. Despite a limited number of patients *per* group, multiple efficacy endpoints showed promising results with the ABX464 50 mg/day. A total of 40 patients from the present cohort have been enrolled in an ongoing open-label 52-week maintenance study with ABX464 50 mg per day to collect further safety and efficacy data. Though preliminary, these encouraging findings warrant further exploration of the efficacy and safety of ABX464 at 50 mg per day or less as an RA treatment.

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Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by Federal Agency for Medicines and Health ProductsEurostation II—Place Victor Horta 40/401060 BrusselsState Institute For Drug Control (SUKL) Srobarova 48 10041 Praha 10, CZANSM—143/147, bd Anatole France—93285 Saint-Denis Cedex, FR OGYÉI/33889-6/2019 1051 Budapest, Zrínyi utca 3.Levélcím: 1372 Postafiók 450, HNClinmark ul. Wiktorska 63 02-587 Warszawa, PL. Participants gave informed consent to participate in the study before taking part.

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TRANSLATIONAL SCIENCE

Multiomics analysis of rheumatoid arthritis yields sequence variants that have large effects on risk of the seropositive subset

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ABSTRACT

Objectives To find causal genes for rheumatoid arthritis (RA) and its seropositive (RF and/or ACPA positive) and seronegative subsets.

Methods We performed a genome-wide association study (GWAS) of 31 313 RA cases (68% seropositive) and ~1 million controls from Northwestern Europe. We searched for causal genes outside the HLA-locus through effect on coding, mRNA expression in several tissues and/or levels of plasma proteins (SomaScan) and did network analysis (Qiagen).

Results We found 25 sequence variants for RA overall, 33 for seropositive and 2 for seronegative RA, altogether 37 sequence variants at 34 non-HLA loci, of which 15 are novel. Genomic, transcriptomic and proteomic analysis of these yielded 25 causal genes in seropositive RA and additional two overall. Most encode proteins in the network of interferon-alpha/beta and IL-12/23 that signal through the JAK/STAT-pathway. Highlighting those with largest effect on seropositive RA, a rare missense variant in *STAT4* (rs140675301-A) that is independent of reported non-coding *STAT4*-variants, increases the risk of seropositive RA 2.27-fold ($p=2.1\times10^{-9}$), more than the rs2476601-A missense variant in *PTPN22* (OR=1.59, $p=1.3\times10^{-160}$). *STAT4* rs140675301-A replaces hydrophilic glutamic acid with hydrophobic valine (Glu128Val) in a conserved, surface-exposed loop. A stop-mutation (rs76428106-C) in *FLT3* increases seropositive RA risk (OR=1.35, $p=6.6\times10^{-11}$). Independent missense variants in *TYK2* (rs34536443-C,

Key messages

What is already known about this subject?

⇒ Although many genetic risk loci have been identified in rheumatoid arthritis (RA) overall, there are limited data available on the seropositive and seronegative subsets. Furthermore, most reported RA associations outside the HLA-locus are with common non-coding variants with low risk,which lack a compelling candidate gene mediating the effect on RA.

Key messages

What does this study add?

⇒ In this largest genome-wide association study on RA to date, we studied both RA overall and the seropositive and seronegative RA subsets and found several unreported sequence variants with large effect on the risk of seropositive RA, while associations with seronegative RA were scarce. Through a genomic, transcriptomic and proteomic analysis, we identified candidate causal genes for most signals and show that the majority of those associated with seropositive RA are in the interferon alpha/beta and IL-12/23 signalling networks. Furthermore, most sequence variants that confer the largest risk of seropositive RA point to causal genes encoding proteins in the JAK/STAT-pathway and have not been reported in RA before. This includes a missense variant in the *STAT4* gene that confers 2.27-fold risk, larger than the lead signals at the well-known *HLA-DRB1* and *PTPN22* loci, and two unreported missense variants in the *TYK2* gene, affecting levels of the interferon-alpha/beta receptor 1 (IFNAR1).

How might this impact on clinical practice or future developments?

⇒ These findings highlight how a multiomics approach can reveal causal genes. Our findings support treatment of seropositive RA with the already registered JAK and IL-6R inhibitors as well as CTLA4-Ig but also open for repurposing of other drugs that target proteins in the JAK/STAT-pathway, including inhibitors of FLT3, TYK2 and IFNAR1.

rs12720356-C, rs35018800-A, latter two novel) associate with decreased risk of seropositive RA (ORs=0.63–0.87, p=10^{-9}–10^{-27}) and decreased plasma levels of interferon-alpha/beta receptor 1 that signals through TYK2/JAK1/STAT4.

Conclusion Sequence variants pointing to causal genes in the JAK/STAT pathway have largest effect on seropositive RA, while associations with seronegative RA remain scarce.

INTRODUCTION

Rheumatoid arthritis (RA) is a heterogeneous clinical syndrome that affects around 0.5%–1% of the general population. It is characterised by inflammatory polyarthritis and progressive joint damage if insufficiently treated.[1] RA is divided into seropositive and seronegative RA, where around two-thirds of RA patients are in the seropositive subset, based on autoantibodies (rheumatoid factor (RF) and/or antibodies against citrullinated peptide antigens (ACPA)).[1 2] Although many risk loci have been identified in previous genome-wide association studies (GWAS), most reported RA associations are with common non-coding variants that confer low risk and lack a compelling candidate gene mediating the effect on RA.[1 3–6] The main exceptions are the shared epitope encoded by certain alleles of *HLA-DRB1* and two missense variants in the *PTPN22* (rs2476601-A) and *TYK2* (rs34536443-C) genes.[1 3]

Previous GWAS have focused on RA overall,[3–6] except for one study on ACPA-positive (n=1147) and ACPA-negative (n=774) RA that confirmed the strong association of HLA-DRB1 alleles with ACPA-positive RA but did not identify any genome-wide significant signals outside the HLA-locus[7] and another report on ACPA-negative RA only (n=1922) that identified two genome-wide significant signals.[8]

Here, we searched for sequence variants outside the HLA-locus affecting the risk of RA overall, the seropositive and/or seronegative subsets of RA, using the largest GWAS study population to date in RA (31 313 cases and ~1 million controls) from six countries in Northwestern Europe and searched for candidate causal genes through a genomic, transcriptomic and proteomic analysis.

METHODS

Study populations

Cases with RA were diagnosed by rheumatologists and/or captured through the nationwide Scandinavian rheumatology quality registries and/or the 10th revision of the International Statistical Classification of Diseases (ICD-10) code-based registration of all inpatient and outpatient healthcare visits (see four-digit based ICD-10 codes in table 1). If available, RF and anti-CCP measurement were used to define the seropositive/seronegative RA subsets, according to classification criteria.[2 9]

An overview of the study populations is provided in table 1. In the study populations from *Iceland* (3613 cases and 341 788 controls), *UK Biobank* (5798 cases and 402 767 controls of self-reported white British ancestry, confirmed by genetic analysis)[10] and *FinnGen* (https://www.finngen.fi/en/access_results version R4: 4701 cases and 125 923 controls), RA cases were compared with the remaining non-RA individuals, with the Icelandic study covering a large part of the Icelandic population and the latter two being nationwide genetic cohort studies. From *Sweden*, we included: (1) the population-based EIRA case–control study (www.eirasweden.se) with 3436 newly diagnosed cases and 3058 controls matched for age, sex and geographical area from mid and Southern parts of Sweden. In addition, we included 7488 controls from the parallel Swedish EIMS study (ki.se/imm/eims-epidemiologisk-undersokning-av-riskfaktorer-for-multipel-skleros); (2) the RA cohort from Umea (n=1935) and 1156 controls from Umea biobank, matched for age and sex (www.umu.se/en/biobank-research-unit); and (3) the Swedish Rheumatology Quality Register Biobank (n=3287, www.srq.nu).

From *Denmark*, RA cases were identified in four study populations: (1) Danish Biomarker Protocol[11] (n=2544 with samples in the Danish Rheumatological Biobank and clinical data in the Danish Rheumatology Quality Register, DANBIO)[12] (2) the Copenhagen Hospital Biobank (n=3282), (3) the TARCID cohort (n=1826) and (4) the nationwide Danish Blood Donor Study (DBDS; 10 RA cases).[13] Controls for these 7662 cases were age-matched and sex-matched non-RA individuals from DBDS (n=86 964).

From *Norway*, 881 RA cases from the Oslo RA cohort and 28 517 population-based controls from the Norwegian Mother, Father and Child Cohort Study were included.[14 15]

Patients were involved in the design and conduct of several of the studies that are included in this report.

Genotyping and multiomics analyses

For a detailed methodological description, see online supplemental information 2. In short, genotyping of all cohorts except UK Biobank and FinnGen was performed at deCODE genetics using the Illumina technology, and the sequence variants for imputation were identified through whole-genome sequencing of 67 645 individuals.

We used logistic regression to test the association of ~64 million sequence variants with RA overall, the seropositive and

Table 1 RA study populations from six Northwestern European countries included in the present study*

	Total cases	Total controls	Sweden		Denmark		Iceland		Norway		UK biobank		FinnGen	
			Ca	Co	Ca	Co	Ca	Co	Ca	Co	Ca	Co	Ca	Co
RA overall	31 313	995 377	8658	9418	7662	86 964	3613	341 788	881	28 517	5798	402 767	4701	125 923
Seropositive RA	18 019	991 604	6455	9423	4850	86 964	1746	313 704	587	28 517	913	407 652	3468	145 344
Seronegative RA	8515	1 015 471	1852	9436	2652	86 966	1069	322 808	455	28 517	1051	407 514	1436	143 312
Serology lacking	4779	–	351	–	160	–	798	–	0	–	3834	–	0	–

*The following ICD-10 codes were used, in addition to clinical diagnoses validated by physicians, from case–control studies on RA or Scandinavian rheumatology quality and patient registers: RA overall (M05.8, M05.9, M06.0, M06.8, M06.9), seropositive RA (M05.8, M05.9 and/or positive rheumatoid factor (RF) and/or anti-CCP antibody measurement), seronegative RA (M06.0, M06.8 or M06.9 with negative RF measurement (and negative anti-CCP measurement if available). See Methods for further details.
Ca, number of cases; Co, number of controls; RA, rheumatoid arthritis.

the seronegative subset.[16] Sequence variants were split into five classes based on their genome annotation, and the significance threshold for each class was based on the number of variants in that class,[17] thereby adjusting for all ~64 million variants tested, maintaining an unadjusted significance threshold of 8×10^{-10}. The primary signal at each genomic locus has the lowest Bonferroni-adjusted p value. Conditional analysis was used to search for possible secondary signals (<500 kB from the primary signal, excluding HLA-locus). We tested whether primary and secondary signals were in strong linkage disequilibrium (R^2 >0.8) with top cis-eQTL variants for genes expressed in various tissues (online supplemental tables 5 and 6), and/or with levels of 4789 proteins in plasma (pQTL, SomaScan, Somalogic) in 35 559 Icelanders (online supplemental table 7).[18–21]

We used the Ingenuity Pathway Analysis software (QIAGEN Inc) to evaluate whether there is experimental evidence for direct or indirect interaction between the proteins coded by candidate causal genes, supporting biological connection.

RESULTS

Genome-wide association study

Of the 31 313 RA cases, 26 534 (84.7%) had information on serological status. Of these, 18 019 (67.9%) were seropositive and 8515 (32.1%) seronegative (table 1).

In separate meta-analyses of RA overall and the seropositive and seronegative RA subsets, we found in total 37 sequence variants at 34 non-HLA loci (online supplemental figure 1a–c), as summarised in table 2. Thus, we identified 25 lead signals for RA overall (online supplemental table 2), 33 for seropositive and 2 for seronegative RA (online supplemental table 3). When we searched for novel sequence variants, we adjusted for 82 independent sequence variants previously reported to associate with RA ($p<5\times10^{-8}$ in the largest meta-analysis to date),[4 6] and 15 of the 37 sequence variants are previously unreported. The 15 novel associations are at 12 loci and six of those loci are previously unreported. Little heterogeneity was observed between the study populations (see online supplemental tables 2 and 3 (P_{het}) and online supplemental figure 4 (average effect)).

Replication of previously reported signals

We replicated 53 of the 82 previously reported variants (online supplemental table 1, correcting for multiple testing, p value threshold=0.05/82 variants /3 phenotypes=2.03×10^{-4}). However, only 36 of the 82 variants were previously reported to be genome-wide significant in Europeans,[4 6] and we replicated 34 of these 36 variants (94%).

Comparison of RA subsets

The heritability estimates (total observed scale h2) were higher for seropositive RA (0.19 (0.022)) than for seronegative RA (0.099 (0.019)). For a substantial proportion of the RA-associated sequence variants, their effect was greater on seropositive RA than seronegative RA risk (table 2, figure 1). However, the genetic correlation between seropositive and seronegative RA was high (rg 0.87, SE 0.13, $p=4.5\times10^{-12}$ (online supplemental table 9).

Genomic, transcriptomic and proteomic analysis of lead signals

We searched for candidate causal genes with an omics approach (figure 2A) and evaluated the effect of lead signals (or correlated variants, R^2 >0.8) on amino acid sequence (online supplemental tables 2–4), mRNA expression (cis-eQTL (online supplemental tables 5 and 6) and/or plasma levels of proteins (pQTL (online supplemental table 7). This yielded a total of 27 candidate causal genes in RA overall and/or its subsets.

Seropositive RA

Twenty-four of the 33 lead signals in seropositive RA pointed to 25 candidate causal genes, as shown in figure 2B ranked by effect. The one with the largest effect is a rare (MAF=0.14%) missense variant in the *STAT4* gene (rs140675301-A, Glu128Val) that associates with 2.27-fold increased risk ($p=2.1\times10^{-9}$, table 2 and figure 2B). Rs140675301-A is the first coding variant identified at the *STAT4* locus that associates with RA and has not been reported in any disease before. This signal is independent (online supplemental table 8) of the common lead *STAT4* intronic variant (rs4853458-A), which is strongly correlated ($R^2=1$) with other intronic variants in *STAT4*, previously reported to associate with RA[22 23] (figure 3A and online supplemental table 1). STAT4 contains six domains that have different functions, and the rare missense rs140675301-A variant leads to an amino acid change from negatively charged, hydrophilic, glutamic acid to non-polar hydrophobic valine at position 128 (Glu128Val) in a loop on the surface of the protein (figure 3B), between the N-terminal domain and the helical coiled coil domain. The coiled coil domain provides a carbonised hydrophilic surface that binds to regulatory factors.[24] The amino acid sequence and secondary structure of the loop is highly conserved between species (figure 3C) and within the family of STAT proteins,[24 25] indicating its importance for the function of STAT4. Tetramer formation of STAT at DNA binding sites is necessary for full transcriptional activation of many of its target genes,[26] and STAT without the N-terminal domain cannot form tetramers.[27]

The second largest effect on the risk of seropositive RA had the well-known missense variant rs2476601-A in the *PTPN22* gene, followed by a novel missense variant in the *TYK2* gene (rs35018800-A, Ala928Val), encoding tyrosine kinase 2, which is a member of the JAK/STAT-pathway like STAT4. This rare (MAF=0.60%) missense variant in TYK2 conferred reduced risk

Table 2 Sequence variants outside the HLA locus that associate with RA overall, seropositive (rheumatoid factor and/or anti-CCP antibody positive) and/or seronegative RA in GWAS meta-analysis within six Northwestern-European countries (table 1). Association results are shown for the lead signals for all three RA groups, and the heterogeneity between the seropositive and seronegative subsets.† Effect alleles with novel associations are marked with.*

					Seropositive RA		Seronegative RA		RA overall		
Chr	Position	Effect allele*	*Close gene*	Annotation	OR	*P value*	OR	*P value*	OR	*P value*	P_{het}
chr1	2 800 059	rs897628-T*	***TTC34***	Missense	0.90	*3.3E-16*	0.98	*0.18*	0.94	*1.9E-10*	*1.6E-05*
chr1	113 834 946	rs2476601-A	***PTPN22***	Missense	1.59	*1.3E-160*	1.29	*2.9E-27*	1.41	*3.9E-144*	*7E-13*
chr1	161 506 414	rs9427397-T*	***FCGR2A***	Missense	1.11	*2.2E-08*	1.02	*0.55*	1.07	*3.3E-06*	*0.026*
chr2	60 881 694	rs67574266-A	***REL,PUS10***	5-prime UTR	1.08	*6.2E-10*	1.01	*0.57*	1.05	*3.6E-07*	*2.0E-03*
chr2	111 119 036	rs72836346-C*	***BCL2L11***	Upstream gene	1.14	*2.5E-10*	1.01	*0.75*	1.10	*7.5E-09*	*1.4E-03*
chr2	191 073 180	rs140675301-A*	***STAT4***	Missense	2.27	*2.1E-09*	1.23	*3.4E-01*	1.63	*3.9E-06*	*0.017*
chr2	191 094 763	rs4853458-A	***STAT4,GLS***	Intron	1.11	*5.2E-14*	1.10	*1.1E-06*	1.10	*2.7E-19*	*0.71*
chr2	203 880 280	rs11571297-C	***CTLA4***	Regulatory	0.89	*2.9E-20*	0.95	*2.2E-03*	0.92	*4.4E-19*	*7.5E-04*
chr3	58 197 909	rs35677470-A	***DNASE1L3***	Missense	1.13	*2.0E-07*	1.16	*7.4E-07*	1.10	*1.8E-08*	*0.43*
chr4	26 083 889	rs10517086-A	*LINC02357*	Intergenic	1.11	*6.2E-16*	1.06	*1.8E-03*	1.09	*7.1E-18*	*0.025*
chr5	56 148 856	rs7731626-A	***ANKRD55***	Intron	0.87	*1.2E-26*	0.87	*8.4E-17*	0.88	*1.1E-39*	*0.83*
chr6	137 678 425	rs35926684-G	*TNFAIP3*	Regulatory	1.12	*4.3E-16*	1.02	*0.24*	1.09	*1.5E-14*	*1.3E-04*
chr6	159 085 568	rs2451258-C	.	Regulatory	0.91	*1.6E-12*	0.99	*0.75*	0.96	*1.2E-05*	*4.2E-05*
chr6	167 127 770	rs3093017-C	***CCR6***	Intron	1.11	*1.8E-18*	1.04	*0.03*	1.07	*7.0E-15*	*6.1E-04*
chr7	50 313 596	rs10261758-G*	*IKZF1*	Intron	1.07	*6.9E-07*	1.04	*0.04*	1.07	*3.6E-12*	*0.17*
chr7	128 938 247	rs2004640-G*	***IRF5***	Splice donor	0.92	*1.4E-11*	0.94	*1.9E-04*	0.94	*5.1E-13*	*0.25*
chr8	11 480 078	rs2409780-C	***BLK,FAM167A***	Regulatory	1.09	*1.1E-09*	1.05	*9.1E-03*	1.08	*1.3E-12*	*0.1*
chr8	100 105 506	rs1471293-A*	*RGS22*	5-prime UTR	1.08	*7.4E-10*	1.04	*3.4E-02*	1.05	*9.1E-08*	*0.039*
chr9	120 933 192	rs35942002-A	***TRAF1***	Upstream gene	1.09	*6.3E-13*	1.05	*9.1E-04*	1.06	*2.8E-09*	*0.1*
chr10	6 056 986	rs706778-T	***IL2RA***	Intron	1.09	*1.2E-11*	1.07	*3.7E-05*	1.07	*2.4E-12*	*0.36*
chr10	31 122 426	rs1538981-C	***ZEB1***	Regulatory	0.91	*8.1E-14*	0.99	*0.40*	0.94	*9.4E-12*	*9.4E-05*
chr11	64 340 005	rs479777-C*	*CCDC88B*	Upstream gene	0.93	*2.7E-09*	0.92	*7.4E-07*	0.94	*1.4E-10*	*0.68*
chr11	118 870 448	rs7117261-T	.	Regulatory	0.90	*2.0E-12*	0.94	*1.3E-03*	0.92	*7.6E-13*	*0.13*
chr11	128 627 057	rs73013527-C	*LOC105369568*	Intergenic	1.08	*2.7E-10*	1.04	*0.03*	1.06	*7.7E-10*	*0.045*
chr12	111 446 804	rs3184504-T	***SH2B3***	Missense	1.10	*7.6E-16*	1.08	*1.6E-06*	1.08	*1.1E-17*	*0.38*
chr13	28 029 870	rs76428106-C*	***FLT3***	Intron	1.35	*6.6E-11*	1.15	*0.03*	1.23	*1.7E-08*	*0.041*
chr13	39 788 092	rs8002731-C	***COG6***	Intron	0.92	*3.5E-10*	0.94	*2.1E-04*	0.93	*1.7E-14*	*0.35*
chr14	92 651 884	rs117068593-T*	***RIN3***	Missense	0.93	*3.2E-05*	0.94	*9.8E-03*	0.93	*1.9E-09*	*0.59*
chr15	69 751 888	rs11636401-G*	.	TF binding site	0.91	*2.0E-16*	0.95	*7.1E-04*	0.93	*4.3E-15*	*0.045*
chr16	85 982 485	rs9939427-A	***IRF8***	Intergenic	1.10	*5.2E-11*	1.06	*4.6E-03*	1.07	*1.7E-10*	*0.14*
chr16	88 981 246	rs62045818-C*	*CBFA2T3*	Upstream gene	0.93	*8.9E-10*	1.00	*9.3E-01*	0.96	*3.1E-05*	*5.7E-04*
chr17	39 908 216	rs11078928-C	***GSDMB***	Splice acceptor	1.07	*1.3E-07*	1.05	*1.3E-03*	1.04	*1.9E-05*	*0.34*
chr19	10 352 442	rs34536443-C	***TYK2***	Missense	0.69	*2.7E-27*	0.81	*1.6E-06*	0.75	*2.5E-29*	*4.0E-03*
chr19	10 359 299	rs12720356-C*	***TYK2***	Missense	0.87	*2.3E-09*	0.90	*7.5E-04*	0.90	*4.3E-10*	*0.38*
chr19	10 354 167	rs35018800-A*	***TYK2***	Missense	0.63	*1.4E-11*	0.86	*0.07*	0.77	*1.4E-07*	*3.7E-03*
chr21	35 340 290	rs8129030-T	.	Regulatory	0.92	*1.1E-11*	0.96	*0.01*	0.95	*2.3E-08*	*0.038*
chr21	44 236 891	rs11558819-T*	***ICOSLG***	Missense	0.91	*1.6E-09*	0.98	*0.26*	0.95	*1.2E-05*	*1.9E-03*

*Sequence variants that remain significant after adjustment for previously reported sequence variants (online supplemental table 1). Bold indicates candidate causal genes (summarised in figure 2).
†We performed a meta-analysis using logistic regression analysis assuming a multiplicative model, reporting OR and two-sided p values adjusted for year of birth, sex and origin (Iceland) or the first 20 principal components (other countries). Variants were split into five classes based on their genome annotation and significance threshold based on the number of variants in each class. The adjusted significance thresholds are 1.3×10^{-7} for variants with high impact (splice donor, splice acceptor, stop gained, frameshift, stop lost, initiator codon), 2.6×10^{-6} for variants with moderate impact (missense, splice region, stop retained, inframe indels), 2.4×10^{-5} for low-impact variants (synonymous, 5′ UTR, 3′ UTR, upstream and downstream), 1.2×10^{-6} for other low-impact variants in DNase I hypersensitivity sites (intronic, intergenic, regulatory-region) and 5.92×10^{-10} for all other variants not in DNase I hypersensitivity sites. Primary signal at each locus (1 Mb) was selected based on conditional association analysis of all variants at each locus, using Bonferroni corrected p values (0.05×P/class-specific p value threshold). We report the coding signal when two markers are equivalent after conditional analysis. Secondary signals are sequence variants that remained GWAS significant after adjustment for the lead signal and other independent (secondary) signals at the locus. When different but correlated variants are lead in RA overall and seropositive RA, the seropositive RA signal is presented here. See further in online supplemental tables 2 and 3.
GWAS, genome-wide association study; Phet, a p value for test of heterogeneity between the effects in seropositive and seronegative RA subsets; RA, rheumatoid arthritis.

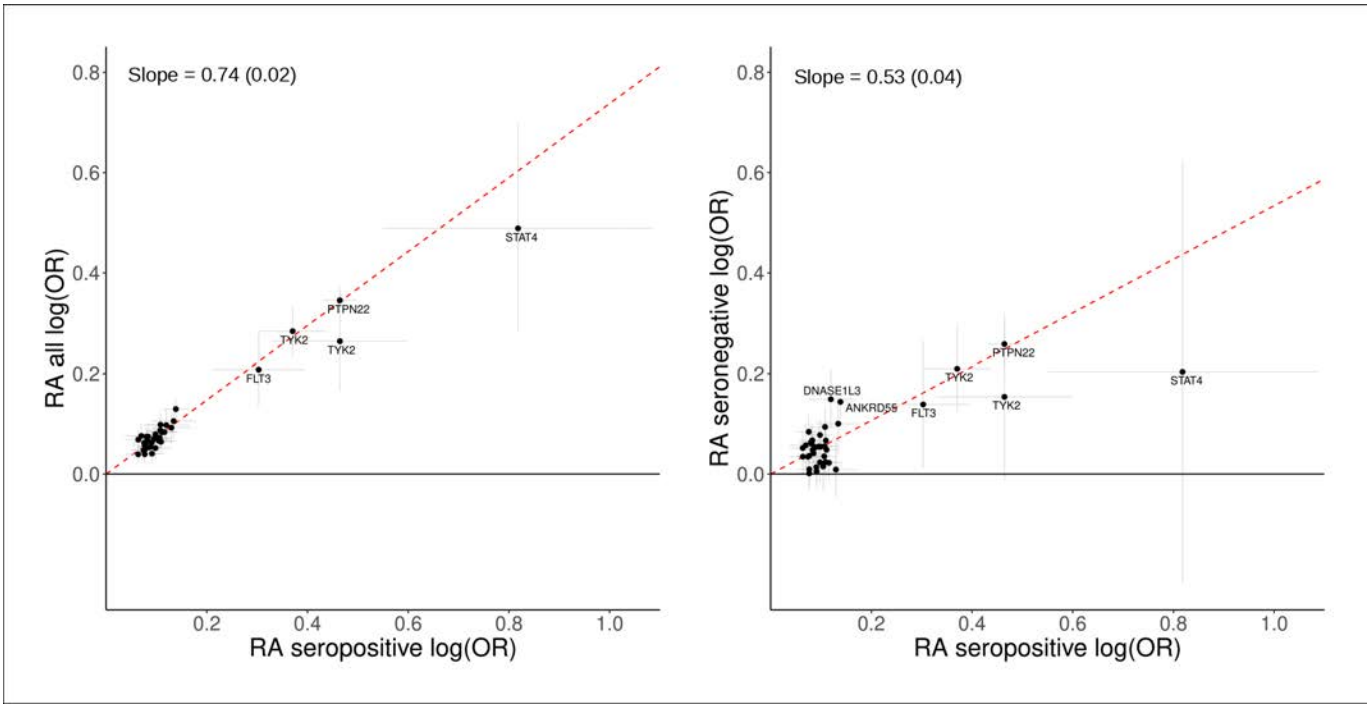


Figure 1 Effects of the lead sequence variants associated with seropositive RA (18 019 cases) compared with RA overall (31 313 cases, left graph) and seronegative RA (8515 cases, right graph). The x-axis and the y-axis show the logarithmic estimated ORs for the associations with the three phenotypes. All effects are shown for the RA risk increasing allele based on current meta-analysis of study population from six countries in Northwestern Europe (table 1). Error bars represent 95% CIs. The red line represents slope (SD) based on a simple linear regression through the origin using MAF (1-MAF) as weights. See further results in table 2 and online supplemental tables 2; 3.

of seropositive RA (OR=0.63, $p=1.4\times10^{-11}$), independently of a known missense variant in *TYK2* (rs34536443-C, Pro1104Ala, MAF 4.3%), which we also found to decrease the risk of RA overall (OR=0.75, $p=2.5\times10^{-29}$), and here, we extend this association to the seropositive RA subset (OR=0.69, $p=2.7\times10^{-27}$; table 2, online supplemental table 3 and online supplemental figure 2). In addition, we identified a common missense variant in *TYK2* that independently associated with reduced risk of seropositive RA (rs12720356-C, Ile684Ser, MAF=8.82%, OR=0.87, $p=2.3\times10^{-9}$). Analysis of the plasma proteome (online supplemental table 7) showed that the minor alleles of the variants encoding both Ile684Ser and Pro1104Ala in TYK2 are the only sequence variants that associate in trans with plasma levels of interferon alpha/beta receptor 1 (IFNAR1, Ile684Ser: effect=−0.19 SD, $p=7\times10^{-25}$; Pro1104Ala, effect=−0.13 SD, $p=6\times10^{-10}$). These variants did not associate with levels of any other plasma protein measured. Notably, both the missense variants in *TYK2* and *STAT4* are predicted to damage the function of the encoded protein (online supplemental table 4).

An intronic variant (rs76428106-C) in the *FLT3* gene, encoding another tyrosine kinase receptor that signals through the JAK/STAT-pathway, conferred 35% increase in risk of seropositive RA ($p=6.6\times10^{-11}$). This is in accordance with our previous report, where we discovered this variant in a GWAS on autoimmune thyroid disease and found that it also associated nominally with the risk of seropositive RA (OR=1.41, $p=4.3\times10^{-4}$) and with increased levels of 22 proteins in plasma (trans-pQTL), including the FLT3 ligand[18] (online supplemental table 7). rs76428106-C associated with increased mRNA expression of FLT3 in lung tissue (beta=0.82 SD, $p=1.3\times10^{-10}$, online supplemental table 6).

We performed a network analysis of the 25 seropositive RA candidate causal genes and found that 18 of them encode proteins that are linked in the same network (online supplemental figure 3), either through direct protein–protein interaction (eg, STAT4-TYK2, PTPN22-IRF5 and FLT3-SH2B3) or indirectly (eg, one affecting the level of another). Other molecules that are central in this network, and directly interact with proteins encoded by the candidate genes, are interferon alpha/beta and IL12/IL-23.

Among the other candidate causal genes, we also identified novel loss-of-function variants in genes encoding molecules in this network, although with more modest effect on seropositive RA risk (table 2 and figure 2B). This includes a splice-donor variant in the *IRF5* gene (rs2004640-G, OR=0.92, $p=1.44\times10^{-11}$) that encodes interferon regulatory factor 5. *IRF5* rs2004640-G association with decreased risk of seropositive RA was independent from previously reported non-coding variants at the *IRF5* locus (online supplemental table 1) and rs2004640-G is also associated with decreased mRNA expression of *IRF5* in several tissues (online supplemental table 6). Other novel coding variants pointing to putative causal genes were missense variants in *ICOSLG* (rs11558819-T, OR=0.91, $p=1.56\times10^{-9}$) encoding ICOS ligand and *TTC34* (rs897628-T, OR=0.90, $p=3.28\times10^{-16}$). *TTC34* encodes tetratricopeptide repeat protein 34 that has an unknown role in the pathogenesis of RA and belongs to another network that includes the remaining seven candidate causal genes for seropositive RA (online supplemental figure 3).

Seronegative RA

Both signals in seronegative RA were also found in seropositive RA and pointed to causal genes: a missense variant rs2476601-A in *PTPN22* and intronic variant rs7731626-A in *ANKRD55* (table 2 and online supplemental tables 2; 3).

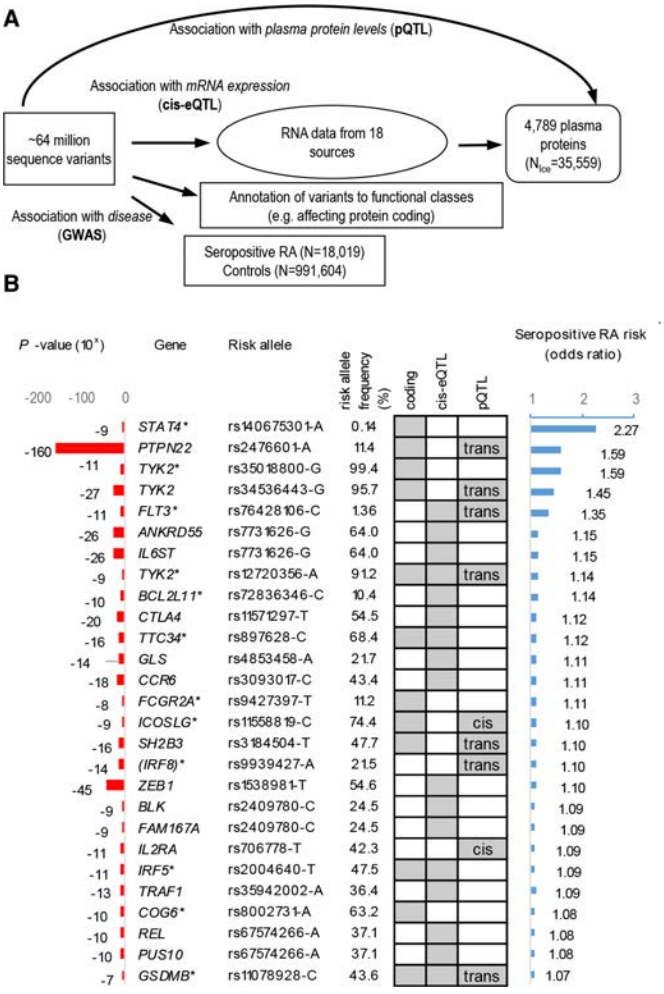


Figure 2 Identification of sequence variants that associate with seropositive RA and the multiomics approaches used to recognise candidate causal genes. (A) schematic overview of the experimental approach used to identify sequence variants that associate with seropositive RA and their systematic annotation, applying multiomics approach to identify candidate causal genes, that is, based on whether lead variants or correlated variants (R^2 >0.8) affect protein *coding* (online supplemental tables 2–4), *mRNA expression* (cis-eQTL (online supplemental tables 5 and 6)) or *levels of proteins* in plasma (pQTL (online supplemental table 7)). (B) Out of 33 lead variant associations outside the HLA-locus (online supplemental table 3), 25 candidate causal genes were identified as listed, ranked by effect (OR). All effects are shown for the risk increasing allele based on GWAS in RA study populations from Northwestern Europe (table 1). Associations that are previously unreported in RA are marked with *. Grey boxes highlight where data point to a candidate causal gene. GWAS, genome-wide association study; RA, rheumatoid arthritis.

PTPN22 rs2476601-A associated with plasma levels of several proteins (trans-pQTL), and it was the only variant in the genome to affect the levels of these proteins (online supplemental table 7). *ANKRD55* rs7731626-A associated with a decreased risk of RA and its subsets and a decreased mRNA expression in whole blood of two neighbouring genes at the locus: *ANKRD55* and *IL6ST*.

RA overall

The lead signals pointing to causal genes in RA overall were also identified in the seropositive subset (table 2), with two

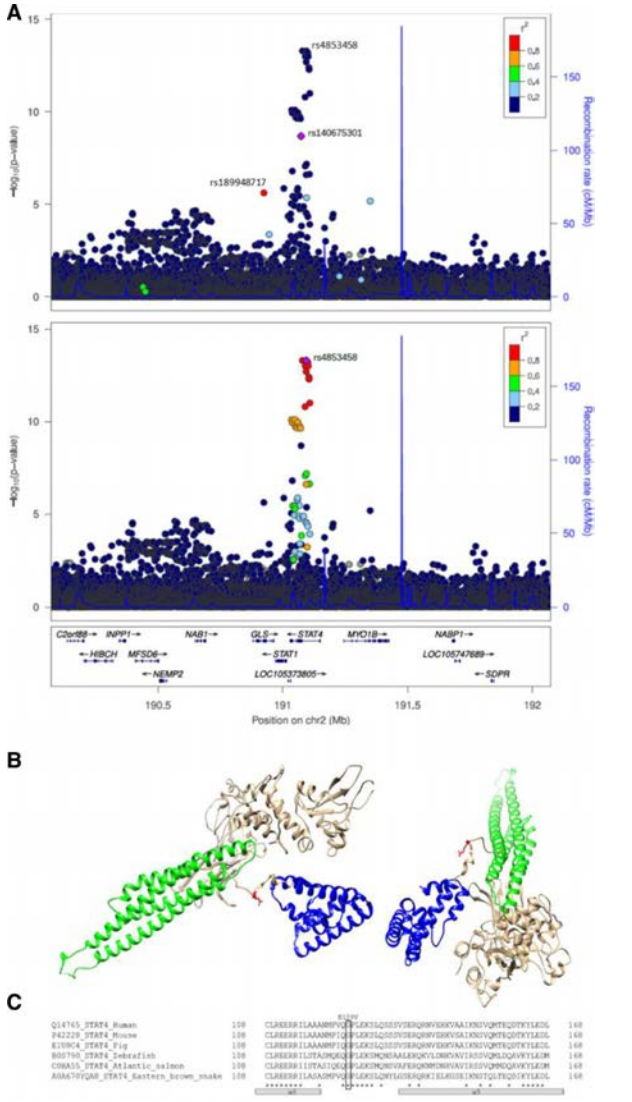


Figure 3 *STAT4* missense variant rs140675301 is associated with seropositive RA (18 019 cases), is not correlated with previously reported variants at the locus and leads to an amino acid change in a highly conserved area of the protein. (A) Locus plot for the association of variants at the *STAT4* locus with seropositive RA. The upper graph illustrates that the intronic variant rs4853458, that is the lead variant at the locus, is not correlated (r^2 <0.2) with the missense variant rs140675301, that is coloured in purple. The missense variant rs140675301 is only highly correlated (r^2 >0.8) with one variant, the intronic variant rs189948717 (coloured in red), that has less effect (seropositive RA: OR=1.81, p=3.69×10^{-6}). Neither of these variants have previously been reported in any disease. The lower graph highlights that the lead variant at the locus (rs4853458, coloured in purple) has many correlated variants, coloured by degree of correlation (r^2) with rs4853458. (B) Secondary structure of STAT4 (viewed from two angles) based on a structural model with STAT1 crystal structure (PDB code: 1yvl.1.A (Mao *et al*, *Molecular Cell* 2005;17:761–71) as template. Glu128Val (red) is located in a loop connecting the N-terminal domain (blue), important for tetramer formation of STATs and nuclear translocation, and the coiled coil domain (green), which provides a carbonised hydrophilic surface that binds to regulatory factors.[24] α-Helices are drawn as cylinders. Invariant residues are marked with asterix. (C) multiple sequence alignment of the conserved STAT4 loop between the N-terminal domain (α8) and the coiled coil (α9) domain, performed with Clustal omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). RA, rheumatoid arthritis.

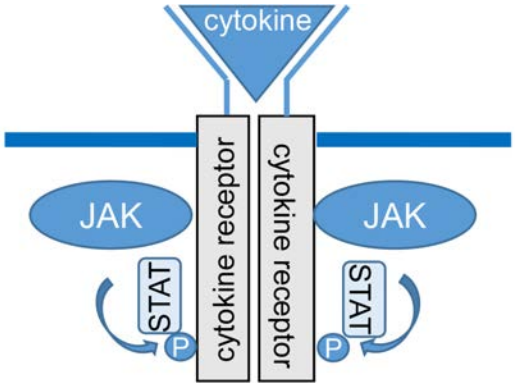
exceptions: missense variants in *DNASE1L3* (rs35677470-A) and *RIN3* (rs117068593-T) (online supplemental table 2). Both these missense variants are predicted to damage the function of the encoded protein (online supplemental table 4). *DNASE1L3* rs35677470-A is a known signal in RA, but the *RIN3* locus has to our knowledge not been reported to associate with any disease before. It encodes Ras and Rab interactor 3 that functions as a guanine nucleotide exchange factor of unknown relevance in RA.

DISCUSSION

In this largest GWAS study on RA to date, we studied both RA overall and the seropositive and seronegative RA subsets and found 37 sequence variants of which 15 were previously unreported. Several of these have large effect on seropositive RA risk, while only two signals were identified in the seronegative subset, both previously reported in RA overall. Through a multiomics approach, we identified candidate causal genes for most signals and show that the majority of those associated with seropositive RA are in the interferon alpha/beta and IL-12/23 signalling networks, with largest risk associated with sequence variants in genes encoding proteins in the JAK/STAT pathway.

Novel missense variant in the *STAT4* gene (rs140675301-A) confers 2.27-fold increased risk that is higher risk than any previously reported RA association, including the well-known *HLA-DRB1* shared epitope and the lead missense variant at the *PTPN22* locus. Although the STAT4 locus has been reported in genome-wide studies, this is the first *STAT4* coding variant found to associate with RA. This coding variant points directly to STAT4 as the causal gene at the locus. It has not been reported for any other disease before, and we found that it leads to an amino acid change in a surface loop of the protein that is highly conserved, thereby underscoring its importance for STAT4 function. *STAT4* encodes STAT4, a cytoplasmic transcription factor that regulates gene expression through the JAK/STAT-pathway.[28] It is phosphorylated in response to various cytokines and displacement of the N-terminal and coiled coil domains within the protein structure could interfere with DNA binding, transcriptional activation and/or target selectivity. As highlighted in the network analysis and illustrated in figure 4, both interferon alpha, IL-12 and IL-23, signal through STAT4 via TYK2/JAK1 and TYK2/JAK2.[29] Another RA-associated variant in STAT4 (rs7574865-T, R^2=0.99 to lead intron variant rs4853458-A)[23] increases IL-12-induced IFN-γ production in T cells.[30] STAT4 is expressed at inflammatory sites in activated peripheral blood monocytes, fibroblasts, dendritic cells and macrophages and also in synovial macrophages and dendritic cells from patients with seropositive RA.[28 31–34] Furthermore, reduced expression of STAT4 has been observed in RA patients that have responded well to disease-modifying treatment.[32] Thus, STAT4 may have a central role in the inflammatory cascade in joints of RA patients.

Tyrosine kinase 2, encoded by the *TYK2* gene, is another key molecule in the JAK/STAT pathway that regulates signal transduction pathways downstream of the receptors for several cytokines, including interferon alpha/beta and IL-23/IL12 as described previously. We found that three independent coding variants in *TYK2* associated with 25%–37% reduced risk of seropositive RA, and they associated with lower plasma levels of the IFNAR1 receptor for interferon-alpha/beta. Accordingly, one of the missense variants (Pro1104Ala) is located in the catalytic kinase domain of TYK2 and has previously been shown to reduce signalling through IFNAR1.[35]



cytokine	receptor	JAK	STAT
IFN-alpha	**IFNAR1****	**TYK2**/JAK1***	**STAT4**
IL-12	p35-p40	**TYK2**/JAK2***	**STAT4**
IL-23	p19-p40	**TYK2**/JAK1***	**STAT3/4**
FLT3-ligand	**FLT3****	JAK*	STAT5
IL-6	**IL-6R***	**TYK2**/JAK1/2*	STAT3

Figure 4 The JAK-STAT pathway. The figure and table shows which receptors, JAK and STAT subtypes certain cytokines bind to, highlighting proteins encoded by and/or affected by causal genes in seropositive RA, based on the multiomics analysis of sequence variants associated with risk of seropositive RA (shown in bold). Binding of a cytokine to its receptor activates the associated Janus kinases (JAK). The JAK in turn phosphorylates (P) the receptor, which provides a docking for signal transducers and activators of transcription (STATs) and other signalling molecules to bind to the receptor. STATs also become phosphorylated and translocate to the nucleus, where they regulate gene expression. *Protein targeted by drugs that are registered for RA. **Proteins targeted by drugs registered or in pipeline for other diseases. RA, rheumatoid arthritis.

TYK2 also mediates the signalling of IL-6, IL-10 and IL-4/IL-13.[36] IL-6 signals through the IL-6 receptor (IL-6R), thereby inducing IL6ST homodimerisation and activation of TYK2/JAK1/2 and STAT3 signalling pathway (figure 4), known to play a role in RA.[37] The intronic variant rs7731626-A in *ANKRD55* associated with a reduced risk of both seropositive and seronegative RA and also reduced expression of *ANKRD55* and *IL6ST*. The effect on *IL6ST* expression and its biological function points to *IL6ST* as a candidate causal gene at that locus. Accordingly, drugs inhibiting IL-6R are effective in RA.[38]

The FLT3 receptor is another activator of the JAK/STAT pathway that signals through STAT5[39] (figure 4), and an intronic variant in the *FLT3* gene (rs76428106-C) conferred 35% increase in risk of seropositive RA. This confirms a non-genome-wide significant signal in our previous report, in which we identified this variant as a strong risk factor for autoimmune thyroid disease and found that it generates a cryptic splice site, introducing a stop codon in 30% of transcripts that are predicted to encode a truncated protein, lacking its tyrosine kinase domains.[18] *FLT3* encodes fms-related tyrosine kinase 3 receptor, a key regulator in the development of monocytes and dendritic cells. The cell-surface receptor is expressed on common dendritic cells and lymphoid/myeloid progenitors that give rise to both classical and plasmacytoid dendritic cells, which produce large amount

of interferons when activated.[40] As previously reported, *FLT3* rs76428106-C increases plasma levels of the FTL3 ligand,[18] and RA patients have increased levels of FLT3 ligand both in serum and synovial fluid of inflamed joints.[41 42] FLT3 ligand deficient mice are protected against collagen-induced arthritis,[42] and in a mouse model of collagen-induced arthritis, an oral inhibitor of FLT3/JAK2/c-Fms was found to block signalling through TYK2 and STAT4 and decrease both inflammation and bone resorption.[43]

Yet another variant affecting interferon signalling is a splice-donor variant in the *IRF5* (rs2004640-G) gene that encodes interferon regulatory factor 5 and reduced both RA risk and IRF5 expression. *IRF5*-rs2004640-G has not been reported in GWAS on RA before, although the locus is known, and a tentative association was reported in a meta-analysis of candidate gene studies (4818 cases, p=0.003).[44]

The size and homogeneous background of the study populations, with ~64 million sequence variants derived from over 67 thousand whole-genome sequenced individuals, increases the likelihood to detect rare and low-frequency sequence variants that associate with disease. Furthermore, we were able to test their functional relevance through analysis of RNA sequence and plasma proteome. However, it remains to be seen whether the sequence variants associate with RA in populations of another ancestries.

The SNP-based heritability estimate for seropositive RA was the same as in a previous study (0.19),[45] while lower for seronegative RA (0.099) where previous findings are scarce.[46]

In addition to the causal genes highlighted previously, the network analysis illustrated how majority of all candidate causal genes encode proteins in the interferon alpha/beta and IL-12/IL-23 signalling network. Furthermore, we observed a consistent direction of the effect on seropositive RA risk, gene expression and protein levels in plasma, indicating that increased signalling through the JAK/STAT-pathway is central in the inflammatory cascade in seropositive RA. Our findings are in line with the documented effectiveness of IL-6 receptor and JAK inhibitors (baricitinib, tofacitinib, filgotinib and upadacitinib) as well as CTLA4-Ig in RA.[1 36 38 47] Furthermore, there are inhibitors of other proteins in this pathway that are in development or already marketed for other diseases but have to our knowledge not been tested for treatment of RA, including FLT3 inhibitors used to treat acute myeloid leukaemia and other cancer forms,[48] TYK2 inhibitors that show promising results in clinical trials for psoriatic arthritis[49] and IFNAR1 inhibitors in systemic lupus erythematosus.[50]

In summary, through a large genome, transcriptome and proteome analysis of RA and its subsets, we identified new RA risk loci and highlight candidate causal genes at the majority of RA-associated loci. Most sequence variants have larger effect on the risk of seropositive than seronegative RA. Majority of those with largest effect on RA risk have not been reported before and point to candidate causal genes encoding proteins in the network of interferon alpha/beta and IL-12/IL-23 that signal through the JAK/STAT pathway. Together, these data thus shed light on the molecular mechanism affected by most non-HLA sequence variants that predispose to seropositive RA. In contrast, the genetic background of seronegative RA remains largely unexplained.

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Data availability statement Data are available in a public, open access repository. All data relevant to the study are included in the article or uploaded as supplementary information. The GWAS summary statistics are available at https://www.decode.com/summarydata/. Sequence variants passing GATK filters will be deposited in the European Variation Archive (https://www.ebi.ac.uk/ena/data/view/). We used publicly available software (URLs listed further) in conjunction with the algorithms in the sequencing processing pipeline (whole-genome sequencing, association testing, RNA-sequence mapping and analysis, see methods description in Supplementary Information 2): BWA 0.7.10 mem (https://github.com/lh3/bwa);

GenomeAnalysisTKLite 2.3.9 (https://github.com/broadgsa/gatk/); Picard tools 1.117 (https://broadinstitute.github.io/picard/); SAMtools 1.3 (http://samtools.github.io/); Bedtools v2.25.0-76-g5e7c696z (https://github.com/arq5x/bedtools2/); Variant Effect Predictor (https://github.com/Ensembl/ensembl-vep); Read_haps (http://github.com/DecodeGenetics/read_haps); In-silico prediction of missense variants (https://sites.google.com/site/ jpopgen/dbNSFP).

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TRANSLATIONAL SCIENCE

Isolation of HLA-DR-naturally presented peptides identifies T-cell epitopes for rheumatoid arthritis

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ABSTRACT

Objective Rheumatoid arthritis (RA) immunopathogenesis revolves around the presentation of poorly characterised self-peptides by human leucocyte antigen (HLA)-class II molecules on the surface of antigen-presenting cells to autoreactive CD4 +T cells. Here, we analysed the HLA-DR-associated peptidome of synovial tissue (ST) and of dendritic cells (DCs) pulsed with synovial fluid (SF) or ST, to identify potential T-cell epitopes for RA.

Methods HLA-DR/peptide complexes were isolated from RA ST samples (n=3) and monocyte-derived DCs, generated from healthy donors carrying RA-associated shared epitope positive HLA-DR molecules and pulsed with RA SF (n=7) or ST (n=2). Peptide sequencing was performed by high-resolution mass spectrometry. The immunostimulatory capacity of selected peptides was evaluated on peripheral blood mononuclear cells from patients with RA (n=29) and healthy subjects (n=12) by flow cytometry.

Results We identified between 103 and 888 HLA-DR-naturally presented peptides per sample. We selected 37 native and six citrullinated (cit)-peptides for stimulation assays. Six of these peptides increased the expression of CD40L on CD4 +T cells patients with RA, and specifically triggered IFN-γ expression on RA CD4 +T cells compared with healthy subjects. Finally, the frequency of IFN-γ-producing CD4 +T cells specific for a myeloperoxidase-derived peptide showed a positive correlation with disease activity.

Conclusions We significantly expanded the peptide repertoire presented by HLA-DR molecules in a physiologically relevant context, identifying six new epitopes recognised by CD4 +T cells from patients with RA. This information is important for a better understanding of the disease immunopathology, as well as for designing tolerising antigen-specific immunotherapies.

Key messages

What is already known about this subject?

⇒ Rheumatoid arthritis (RA) is triggered and sustained by CD4 +T cells recognising poorly characterised self-peptides on human leucocyte antigen (HLA)-class II molecules, displayed on the surface of antigen-presenting cells.

What does this study add?

⇒ Our study confers key insights into the repertoire of naturally presented self-peptides in HLA-DR molecules in the context of RA.

⇒ We identified six new epitopes, four native and two citrullinated, recognised by CD4 +T cells from patients with RA.

How might this impact on clinical practice or future developments?

⇒ These results contribute to the understanding of the immunopathogenesis of the disease and the design of tolerising antigen-specific therapeutic strategies.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterised by synovial infiltration of adaptive immune cells, including T and B cells, as well as specialised antigen-presenting cells (APCs) such as dendritic cells (DCs). It is believed that RA is initiated and perpetuated by autoreactive CD4 +T cells that recognise self-peptides presented by human leucocyte antigen (HLA)-class II molecules displayed by APCs.[1] A group of HLA-class II molecules encoded by *HLA-DRB1* alleles such as *HLA-DRB1*0101*, **0401*, **0404*, **0405*, **1001* and **1402*, known as 'shared epitope' (SE) alleles, has been shown to confer increased susceptibility and severity for RA.[2 3]

SE alleles have also been associated with anti-citrullinated protein/peptide antibody (ACPA) seropositivity in RA.[4] Citrullination is a post-translational modification (PTM) that involves the enzymatic conversion of positively charged arginine to neutral citrulline within a peptide. Since SE molecules share a positively charged peptide-binding pocket 4 (P4), it has been proposed that peptides containing a citrulline in P4 present a higher binding affinity for SE molecules compared with their arginine-bearing counterparts.[5 6] Alternatively, it has been shown that citrulline on certain citrullinated (cit)-peptides can be beneficial in interacting with other SE anchor pockets, or could directly contact the T-cell receptor (TCR), selecting citrulline-specific T-cell clones.[7–9] Cit-peptides from several proteins have been reported to activate CD4 +T cells in patients with RA, which are thought to support the differentiation of ACPA-secreting plasma cells, although definitive evidence on this point is still needed.[10–12]

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Numerous autoantigens have been proposed for RA, however, information regarding the epitopes responsible for T-cell activation remains limited. Some CD4 +T cell epitopes derived from proteins that can be found in the synovium, such as type-II collagen, fibrinogen and proteoglycan-aggrecan, have been identified by analysing T-cell responses to peptides encompassing whole protein sequences,[13 14] or in silico predicted HLA-class II-binding peptides.[15 16] Nevertheless, those approaches have mainly focused on proteins previously described as autoantibody targets and have not taken into consideration the restrictions imposed by antigen-processing and peptide-loading onto HLA molecules. These limitations can be circumvented by the isolation of peptide/HLA complexes (pHLA) and further sequencing of naturally presented peptides (NPPs) by liquid chromatography coupled to mass spectrometry (LC-MS/MS), a technology that has already identified several autoimmunity-associated T-cell epitopes,[17–19] as well as post-translationally modified NPPs.[20 21]

So far, only a few reports have described NPPs potentially involved in RA. These studies used synovial tissue (ST),[20 22] and peripheral blood (PB) or synovial fluid (SF) mononuclear cells (PBMCs and SFMCs, respectively)[22] as pHLA sources, and succeeded in identifying two RA T-cell epitopes[23]; however, no cit-peptides were reported. Here, we extend these results by describing six novel T-cell epitopes for RA, including two cit-peptides, out of a repertoire of HLA-DR-bound peptides isolated from ST-resident APCs, as well as SF and ST-pulsed monocyte-derived (Mo)DCs.

METHODS

Study participants

We recruited healthy controls (HC), who donated buffy coats (n=10) and PB (n=12), as well as patients with RA donating SF (n=15), ST (n=3) and PB (n=29), from Hospital Clínico Universidad de Chile, Hospital del Salvador and Biobanco del Centro de Hemoterapia y Hemodonación de Castilla y León (online supplemental tables 1 and 2).

Generation of MoDCs

MoDCs were generated from HC PB monocytes as previously described.[24 25] MoDCs were first pulsed with a pool of albumin and IgG-depleted RA SF (SF-DCs) (online supplemental figure 1), or RA ST lysate obtained from a single donor (ST-DCs), and then maturated with lipopolysaccharide. Unpulsed DCs (UP-DCs) were used as controls. After harvesting, MoDCs were characterised by flow cytometry and dry cell pellets were prepared.

Isolation of pHLA-DR complexes

Solubilised membranes from dry cell pellets and ST lysates were subjected to immunoprecipitation with an anti-HLA-DR antibody (clone B8.11.2). HLA-DR-bound peptides were disassembled by acidic elution.

Mass spectrometry analysis

Eluted peptides were fractionated by strong cation-exchange chromatography. Fractions were analysed by LC-MS/MS on an LTQ-Orbitrap-XL mass spectrometer. Additionally, MoDCs, SF and ST lysates were trypsinised and analysed by LC-MS/MS. Fragmentation spectra were searched on the software Proteome Discoverer v1.4.

T-cell stimulation

RA PBMCs were stimulated with pools of 5–6 peptides or single peptides for 12–16 hours, to evaluate CD40L expression on CD4 +T cells by flow cytometry. The peptides with the highest CD40L induction capacity were used to stimulate PBMCs from patients with RA and HC for 5 days, to assess CD4 +T cells IFN-γ production by flow cytometry.

Flow cytometry analysis

Cells were stained with fluorochrome-conjugated antibodies and analysed on a LSR Fortessa X-20 or a FACS Canto flow cytometer. Data analysis was done on FlowJo V.10.4.

Statistical analysis

Statistical analyses were performed on IBM-SPSS Statistics V.27.0. A $p<0.05$ was considered significant.

Detailed experimental methods are available as online supplemental material.

RESULTS

Characterisation of the HLA-DR immunopeptidome generated by a combined approach

HLA-DR-bound peptides were isolated from RA ST, SF-DCs and ST-DCs carrying SE-positive molecules. For the last two approaches, UP-DCs were used as controls. Both, UP-DCs and SF/ST-DCs showed high expression of CD83, CD86 and HLA-DR (online supplemental figure 2B,C), indicating that the exogenous load did not affect DCs maturation state. HLA-DR expression was also detected in ST samples (online supplemental figure 2D), attesting the presence of resident or infiltrating APCs.

The identity of peptides isolated from HLA-DR molecules was defined based on the fragmentation spectra obtained from LC-MS/MS sequencing. Sequences derived from skin-specific proteins were considered contaminants and discarded from further analyses. Between 103 and 888 peptides were obtained per sample (table 1). The number of peptides identified from MoDC samples positively correlated with starting MoDC counts (figure 1A). Over 90% of identified sequences were 9–26

Table 1 Number of HLA-DR-bound peptides isolated from each sample

Sample*	*HLA-DRB1* alleles†	Initial cell no ($x10^6$)	No of peptides‡
UP-DC1	***0101**/*1104	5.0	204
UP-DC2	***0404**/*0407	8.0	493
UP-DC3	***1402**/*1301	5.7	327
UP-DC4	***0101**/*0301	11.3	595
UP-DC5	***0401**/*0301	5.4	509
SF-DC1	***0101**/*1104	10.0	329
SF-DC2	***0404**/*0407	3.9	186
SF-DC3	***1402**/ *1301	2.1	156
SF-DC4	***0101**/*0301	8.5	888
SF-DC6	***0405**/*0701	7.3	724
SF-DC7	***0401**/*1101	1.0	319
SF-DC8	***0401**/*0407	5.0	341
ST-DC9	***0101**/*0301	5.9	585
ST-DC10	***0101/*0401**	4.5	506
ST1	***0401**/*1101–04	N/A	197
ST2	***0405**/*0701	N/A	103
ST3	***0401**/*1602	N/A	305

*DC samples with the same code number derive from the same donor.
†*HLA-DRB1* alleles expressing the SE are highlighted in bold.
‡Number of peptides obtained after discarding sequences derived from skin-specific proteins.
DC, dendritic cells; HLA, human leucocyte antigen; N/A, not assessed; SF-DC, synovial fluid-pulsed DCs; ST, synovial tissue; UP-DC, unpulsed DCs.

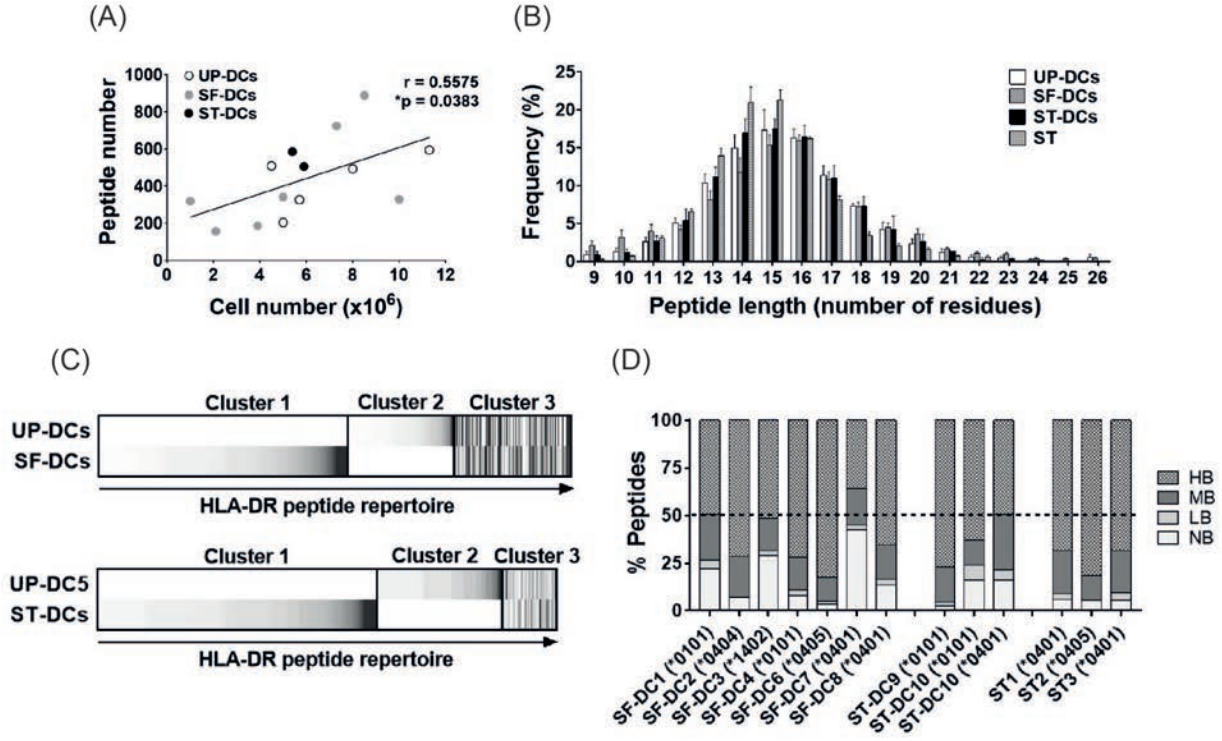


Figure 1 Characterisation of peptides isolated from HLA-DR molecules. HLA-DR/peptide complexes from dendritic cells (DCs) and synovial tissue (ST) samples were immunoprecipitated and peptides were sequenced by liquid chromatography coupled to mass spectrometry. (A) Correlation between the number of isolated peptides and the initial number of DCs (n=14). Data from unpulsed DCs (UP-DCs), synovial fluid-pulsed DCs (SF-DCs) and ST-pulsed DCs (ST-DCs) are shown. (B) Peptide length distribution in UP-DCs, SF-DCs, ST-DCs and ST samples. Bars represent the average peptide length frequency (%)±SD for all donors. (C) Relative abundance of peptides obtained from UP-DCs, SF-DCs and ST-DCs. Grouped data from UP-DC1 to UP-DC4 was compared with grouped data from SF-DC1 to SF-DC8 (5 day DCs), while data from UP-DC5 was compared with grouped data from ST-DC9 and ST-DC10 (7 day DCs). (D) Theoretical binding affinities of selected peptides to the SE-positive HLA-DR molecule present in the respective source sample. Bars show the cumulative percentage of high (HB), medium (MB), low (LB) and non-binder (NB) peptides isolated from each sample.

residues long, with a mode of 15 residues (figure 1B), which is consistent with reported data for HLA-class II peptides.[26]

When comparing NPPs obtained from UP-DCs and SF/ST-DCs, a significant number of differentially isolated peptides was found. This can be partially explained by the low number of peptides retrieved and the omission of less abundant sequences. Nevertheless, with the aim to work with peptides more likely to be derived from synovial proteins, non-redundant sequences obtained exclusively from SF/ST-DCs (cluster 1 in figure 1C) were considered for subsequent analyses.

After discarding peptides derived from HLA chains and those shorter than nine residues (HLA-class II minimum core), the universe of non-redundant sequences was reduced to 731, 348 and 210, for SF-DCs, ST-DCs and ST, respectively. The frequency of peptides belonging to nested sets, families of peptides with a common 9-mer binding core but different C- and N-termini lengths, which are a common attribute of HLA-class II peptides,[26] varied between 21% and 80% (table 2).

Numerous non-redundant sequences and parental proteins were found in more than one sample of the same type (table 2), and across different sample sources (online supplemental figure 3A,B). Furthermore, five non-redundant sequences and 35 parental proteins were present in all three sources (online supplemental figure 3C), suggesting that ST-resident APCs can uptake, process and present local antigens alike ex vivo-pulsed MoDCs. Since cit-proteins are prominent autoantigens in RA, citrullination was included as a variable PTM in the MS/MS database search. As a result, 13 non-redundant cit-sequences were detected and further confirmed by manual inspection (table 2).

According to a theoretical analysis for HLA-DR binding affinity, for most samples, over 50% of peptides were assigned as high binders (HB) and around 25% were medium binders for the SE-positive molecule expressed by the sample donor (figure 1D). Only one donor was SE double-positive (ST-DC10), for whom numerous peptides were considered HB for both alleles (figure 1D). In contrast, lower percentages of HB were assigned for SE-negative molecules (online supplemental figure 4A). Nevertheless, most peptides were predicted to bind to several different HLA-DR molecules (online supplemental figure 4B).

A total of 502, 225 and 177 parental proteins were identified from SF-DCs, ST-DCs and ST samples, respectively (table 2), which were associated with a variety of cellular processes (figure 2A). As expected for HLA-class II molecules, most parental proteins were assigned to subcellular compartments associated with the endocytic processing pathway[27] (figure 2B). Regarding tissue distribution, most proteins were considered ubiquitous or widely expressed, while a minor percentage was regarded as tissue specific or secreted in plasma (figure 2C). Interestingly, over 20% of parental proteins have been reported to be increased in SF, ST, fibroblast-like synoviocytes (FLS) or serum from patients with RA. Also, over 10% have been described as targets for RA autoantibodies, either in their native or PTM

Table 2 Characteristics of peptides isolated from HLA-DR molecules

	SF-DC1	SF-DC2	SF-DC3	SF-DC4	SF-DC6	SF-DC7	SF-DC8	ST-DC9	ST-DC10	ST1	ST2	ST3
Peptides*, n	132	56	42	231	525	155	102	511	336	186	93	280
Non-redundant peptides†, n	107	47	38	174	254	109	79	197	163	88	53	138
Parental proteins, n	98	47	38	149	210	71	68	130	125	74	49	126
Unique peptides‡, (%)	60	68	79	54	27	45	49	20	29	22	35	28
Peptides in nested sets, (%)	40	32	21	46	73	55	51	80	71	78	65	72
Cit-peptides, n	0	0	1	4	0	10	1	0	0	1	1	1
Non-redundant cit-peptides, n	0	0	1	2	0	6	1	0	0	1	1	1
Total non-redundant peptides§, n				**731**				**348**			**210**	
Non-redundant peptides in one sample, n (%)	661 (90.4)							335 (96.3)		147 (70)		
Non-redundant peptides in ≥2 samples, n (%)	70 (9.6)							13 (3.7)		63 (30)		
Total parental proteins§, n				**502**				**225**			**177**	
Parental proteins in one sample, n (%)	383 (76.3)							195 (86.7)		116 (65.5)		
Parental proteins in ≥2 samples, n (%)	119 (23.7)							30 (13.3)		61 (34.5)		
Total non-redundant cit-peptides, n				**10**				**0**			3	
Total parental cit-proteins, n				**8**				**0**			3	

*Number of peptides obtained after discarding sequences present in UP-DCs, derived from HLA chains, or shorter than nine amino acids.
†Non-redundant peptides include unique peptides and peptides belonging to nested sets but as the latter share the same core, the whole family is considered as one in the counting.
‡Unique peptides are sequences not belonging to a nested set.
§The total number indicated is not equal to the sum of peptides/proteins obtained per sample, as some of them were found in more than one sample.
DC, dendritic cells; HLA, human leucocyte antigen; SF-DC, synovial fluid-pulsed DCs; ST, synovial tissue; UP, unpulsed.

forms, and a lower percentage have been recorded as eliciting T-cell responses in patients with RA. No reported association to RA was found for over 50% of parental proteins (figure 2D). To collate the origin of parental proteins, the proteome of MoDC, SF and ST lysates was compared with parental proteins from SF-DCs, ST-DCs and ST NPPs. These analyses showed that parental proteins from SF-DCs and ST-DCs can be found in both, MoDC lysates and the material used to pulse MoDCs (SF and ST lysates, respectively) (figure 2E,F), suggesting that MoDCs were able to uptake and process exogenous synovial proteins. Similar results were obtained when analysing parental proteins from ST NPPs (figure 2G).

Selection of NPPs for T-cell stimulation assays

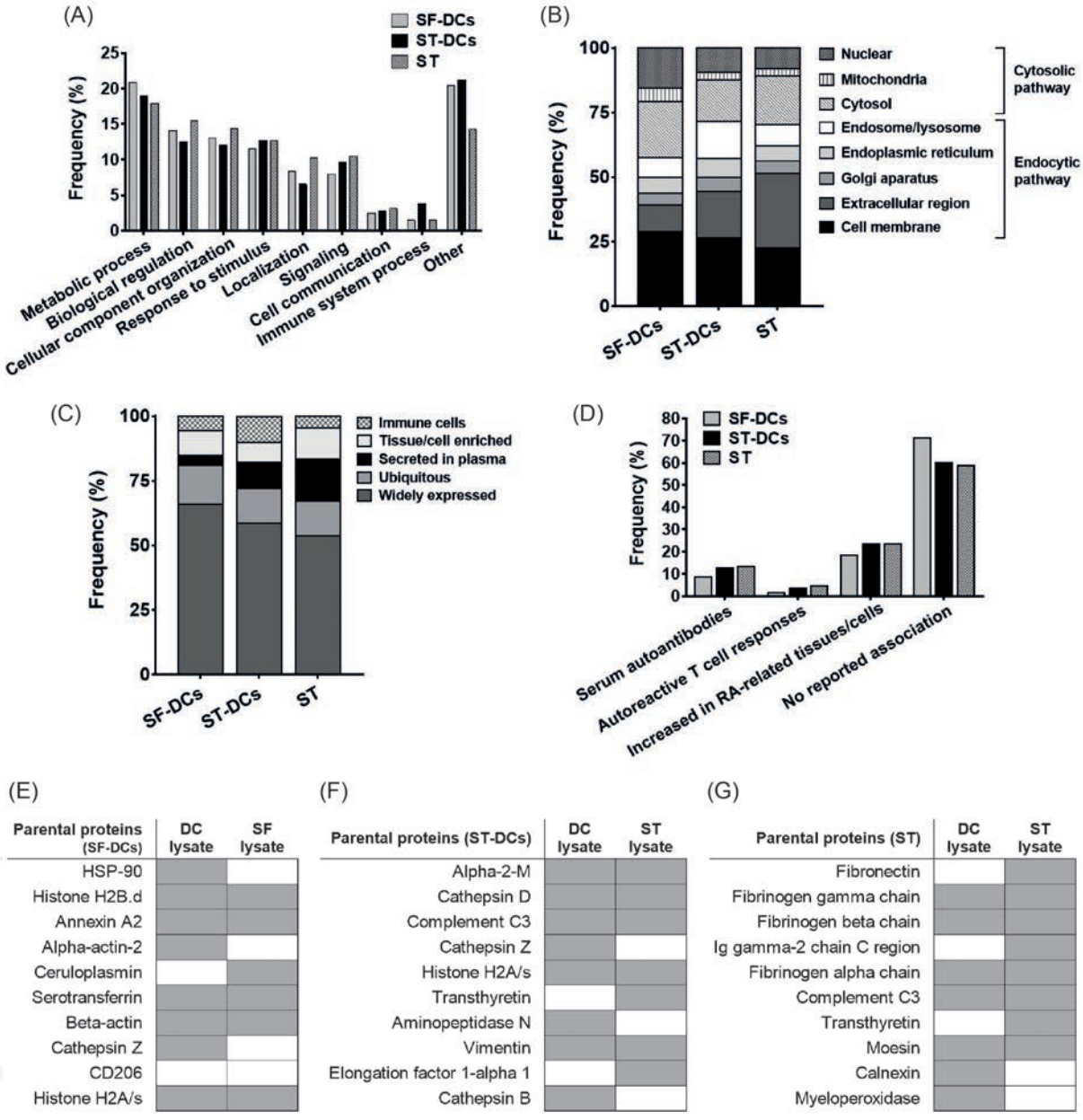
In order to screen for potential T-cell epitopes, a group of 43 NPPs was selected among those peptides that were most frequently detected, showed a higher theoretical affinity for SE-positive molecules and promiscuity for other HLA-DR molecules, and whose parental proteins had been described as autoantigens or highly expressed in patients with RA (table 3). These candidates also exhibited common features of HLA-class II peptides, such as the nature of core-flanking residues and intra-protein localisation[28 29] (online supplemental figure 5A-C). Among these NPPs, six cit-peptides and their native counterparts were included. The presence of citrulline was confirmed by a manual compared analysis of MS/MS spectra from cit-peptides and their eluted native counterparts when available (online supplemental figure 6), as well as from their synthetic versions (online supplemental figure 7).

A predictive analysis assessing the contribution of citrulline on the binding of cit-peptides to the SE-positive alleles displayed by the donors from which these cit-peptides were retrieved (DR*0401 and *0101), suggested that three cit-peptides, deriving from cit-alpha-actin-2, cit-histone H2B and cit-cathepsin Z, could accommodate citrulline in the P4 pocket, which should increase their binding affinity compared with their unmodified versions, according to previous reports[5 6] (online supplemental figure 8A). Instead, for cit-proteoglycan-4 (PG4), citrulline was predicted to match the P9 pocket, whereas for the remaining cit-peptides (cit-fibronectin and cit-gelsolin), citrulline was predicted to be located outside the binding core. Since HLA-DR binding algorithms for cit-peptides have not been widely validated, these predictions should be regarded with caution. To extend these analyses, binding competition assays were performed using soluble HLA-DR molecules. These assays demonstrated that four cit-peptides were able to bind to SE-positive molecules (figure 3). Of note, an increase in experimental affinity of a cit-peptide versus its native counterpart was only observed for cit-PG4, although the difference was very modest (figure 3). In addition, we assessed the binding capacity of three cit-peptides to their corresponding SE-negative allele (DR*1101). While cit-fibronectin was able to bind to DR*0401 but not to DR*1101; cit-histone H2B bound to DR*1101 but not to DR*0401. On the other hand, cit-alpha-actin did not bind to either allele (figure 3).

NPPs activate CD4+ T cells from patients with RA

For an initial set of T-cell stimulation assays, 8 pools consisting of 5–6 peptides each, were designed (table 3). Only pools 5 and 6 increased the expression of CD40L by RA CD4 +T cells relative to unstimulated cells, and above the median increase achieved by an autoantigenic cit-aggrecan peptide[11] (figure 4A and online supplemental figure 9B).

When peptides from pools 5 and 6 were assessed individually, a positive CD40L response was detected against peptides derived from gelsolin, histone H2B, cit-histone H2B, cit-PG4, histone H4 and myeloperoxidase (MPO) (figure 4B, online supplemental figures 9C and 10C). These six peptides were further evaluated in their capacity to trigger T-cell responses in patients with RA versus HC, including both SE-positive and SE-negative donors (online supplemental table 2). All peptides elicited stronger IFN-γ CD4 +T cell responses among patients with RA (figure 4C and online supplemental figure 11), being cit-PG4, gelsolin and MPO the most RA-specific (online supplemental table 3). Although no significant differences on T-cell responses were found between SE-positive and SE-negative patients with RA, among HC the only appreciable responses to any peptide were detected in SE-positive individuals (online supplemental



(E)

Parental proteins (SF-DCs)	DC lysate	SF lysate
HSP-90	■	
Histone H2B.d	■	■
Annexin A2	■	■
Alpha-actin-2	■	
Ceruloplasmin		■
Serotransferrin	■	■
Beta-actin	■	■
Cathepsin Z	■	
CD206		
Histone H2A/s	■	■

(F)

Parental proteins (ST-DCs)	DC lysate	ST lysate
Alpha-2-M	■	■
Cathepsin D	■	■
Complement C3	■	■
Cathepsin Z	■	
Histone H2A/s	■	■
Transthyretin		■
Aminopeptidase N	■	
Vimentin	■	■
Elongation factor 1-alpha 1		■
Cathepsin B	■	

(G)

Parental proteins (ST)	DC lysate	ST lysate
Fibronectin		■
Fibrinogen gamma chain	■	■
Fibrinogen beta chain	■	■
Ig gamma-2 chain C region		■
Fibrinogen alpha chain	■	■
Complement C3	■	■
Transthyretin		■
Moesin	■	■
Calnexin	■	
Myeloperoxidase	■	

Figure 2 Characterisation of parental proteins for peptides isolated from HLA-DR molecules. (A) Frequency of parental proteins associated to different biological processes, based on the gene ontology database annotations for synovial fluid-pulsed dendritic cells (SF-DCs), synovial tissue-pulsed DCs (ST-DCs) and ST samples. (B, C) Frequency of parental proteins classified according to the subcellular compartments from which they are originated (B), or their tissue specificity (C), based on Uniprot and The Human Protein Atlas (THPA) databases. (D) Frequency of parental proteins reported as targets for serum autoantibodies and/or T-cell responses in patients with RA, according to PubMed, and increased in RA-related tissues or cells, based on data from Uniprot and THPA databases. All frequencies were calculated from a total of 502, 225 and 177 parental proteins, obtained from SF-DCs, ST-DCs and ST, respectively. (E–G) The presence (grey) or absence (white) of the 10 most frequent RA-associated parental proteins for peptides isolated from (E) SF-DCs, (F) ST-DCs, and (G) ST, was evaluated in DCs, SF and ST lysates. RA, rheumatoid arthritis.

figure 12). Finally, we observed that the disease activity score in 28 joints (DAS28) positively correlated with IFN-γ CD4 +T cell responses to the MPO peptide (figure 4D), as well as with the number of peptides eliciting T-cell responses (online supplemental figure 13).

DISCUSSION

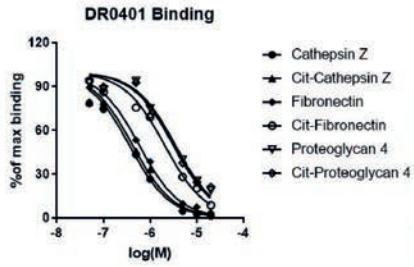
The ST represents the epicentre of RA pathogenesis, a site where infiltrating CD4 +Th1 and Th17 cells lead to the activation of myeloid cells and fibroblasts, and ultimately, to joint destruction.[30] The enrichment of inflammatory APCs and autoantigen-specific memory CD4 +T cells in RA joints,[31 32] together with in vivo evidence of HLA-DR/autoantigen complexes in ST from patients with RA,[33] and in vitro experiments showing the ability of FLS to present synovial autoantigens,[34 35] argue in favour of the synovium as a propitious niche where disease-driving autoantigens could activate self-reactive T cells. One of the main limitations to study T-cell autoreactivity in RA is the paucity of data regarding the epitopes responsible for disease initiation and progression. In this study, we identified multiple HLA-DR-presented NPPs by using MoDCs pulsed ex vivo with synovial material, and directly from ST-resident APCs.

Table 3 Characteristics of peptides selected for T-cell stimulation assays

Parental protein	RA association	Peptide sequence*	Theoretical affinity†	Promiscuity‡	Isolation source	Pool
Alpha-actin-2	Auto-Ab[47]	SGGTTMYPGIADRMQKEITA	LB	12/51	SF-DCs	*
Cit-Alpha-actin-2	Citrullination	SGGTTMYPG**IADrMQKEI**TA	MB	–	SF-DCs	*
Cathepsin Z	↑ ST[48]	SDGTEYWIVRNSWGEPWG	MB	38/51	SF-DCs	*
Cit-Cathepsin Z	Citrullination	SDGTEY**WIVrNSWGE**PWG	HB	–	SF-DCs	*
Fibronectin	Auto-Ab[39]; ↑ ST[49]	APITGYRIVYSPSVEGSS	HB	51/51	ST/ST-DCs	*
Cit-Fibronectin	Citrullination	APITGY**r**IVYSPSVEGSS	HB	–	ST	*
Alpha-2-HS-glycoprotein	Auto-Ab; ↑ SF[50]	SVVYAKCDSSPDSAE	HB	45/51	SF-DCs	†
Aminopeptidase N	↑ SF[51]	INDAFNLASAHKVPV	HB	30/51	ST-DCs	†
Annexin A2	Auto-Ab; ↑ serum[52]	DALNIETAIKTKGVDE	HB	21/51	SF-DCs	†
Calreticulin	Auto-Ab[53]; ↑ SF and plasma[54]	GGGYVKLFPNSLDQT	HB	51/51	SF-DCs	†
Ceruloplasmin	Auto-Ab[50]	VDKEFYLFPTVFDENE	HB	39/51	SF-DCs	†
Clusterin	↑ serum[55]	TVSDNELQEMSNQGSKY	HB	23/51	ST	†
Complement C1q B chain	↑ serum[56]	HVITNMNNNYEPR	HB	16/51	ST	‡
Complement C1r	↑ ST[57]	GDFRYTTTMGVNTY	MB	51/51	ST / ST-DCs	‡
Complement C3	Auto-Ab[50]; ↑ SF[58]	SETRILLQGTPVAQMT	HB	46/51	ST	‡
Elongation factor 1-alpha 1	Auto-Ab[59]	AAGFTAQVIILNHPGQISAG	HB	51/51	ST-DCs	‡
Chaperone BiP	Auto-Ab; ↑ SF and plasma; Auto-T[60]	GVFEVVATNGDTH	HB	16/51	SF-DCs	‡
Ferritin heavy chain	Auto-Ab[61]; ↑ FLS[62]	DDVALKNFAKYFLHQSHE	MB	36/51	SF-DCs	4
Fibrinogen beta chain	Auto-Ab[50]; ↑ SF[63]; Auto-T[14]	TSEMYLIQPDSSVKPY	HB	44/51	ST	4
Ganglioside GM2 activator	↑ SF[64]	TTGNYRIESVLSSSG	HB	51/51	SF-DCs	4
HSP 70–2	Auto-Ab[65]; ↑ SF[63]	EVISWLDANTLAEKD	HB	25/51	ST-DCs	4
HSP 90	Auto-Ab[65]	KELKIDIIPNPQERT	MB	24/51	SF-DCs	4
Gelsolin	Auto-Ab; ↑ SF[50]	DAYVILKTVQLRNGN	HB	51/51	SF-DCs	5
Cit-Gelsolin	Citrullination	DAYVILKTVQL**r**NGN	HB	–	SF-DCs	5
Histone H2B.d	Auto-Ab[66]	MNSFVNDIFERIAGEA	HB	25/51	SF-DCs	5
Cit-Histone H2B.d	Citrullination	MNSFVNDI**FErIAGEA**	HB	–	SF-DCs	5
Proteoglycan 4	↑ ST[67]	THTIRIQYSPARLA	HB	45/51	–	5
Cit-Proteoglycan 4	Citrullination	THT**IrIQYSPAr**LA	HB	–	ST	5
Histone H2A/s	Auto-Ab[66]	TAEILELAGNAARDN	HB	18/51	ST-DCs/ SF-DCs	6
Histone H4	Auto-Ab[68]; ↑ SF[63]	DNIQGITKPAIRR	MB	42/51	SF-DCs	6
ITI heavy chain H4	Auto-Ab[50]; ↑ SF[63]	RPSLVPASAENVNK	HB	41/51	SF-DCs/ ST-DCs	6
Moesin	Auto-Ab[69]; ↑ SF[63]	AELEFAIQPNTTGKQ	HB	41/51	ST	6
Myeloperoxidase	Auto-Ab[70]; ↑ SF[64]	SNEIVRFPTDQLTPDQ	MB	46/51	ST / ST-DCs	6
Osteopontin	↑ ST[71]	GAYKAIPVAQDLNAP	HB	30/51	SF-DCs / ST	7
Phosphoglycerate kinase 1	Auto-Ab[72]; ↑ SF[63]	DKIQLINNMLDKV	HB	39/51	SF-DCs	7
Plasma alpha-L-fucosidase	↑ ST[67]	FDPTWESLDARQLPA	HB	19/51	SF-DCs	7
Plasminogen	Auto-Ab[73]	HSIFTPETNPRAGL	HB	12/51	ST	7
Protein S100-A4	↑ ST[67]	DEAAFQKLMSNLDSNR	HB	25/51	SF-DCs	7
Protein S100-A9	↑ ST[67]	IEHIMEDLDTNADKQ	HB	7/51	SF-DCs	8
Pyruvate kinase PKM	↑ SF[63]	ASDPILYRPVAVALDT	MB	50/51	ST-DCs/ SF-DCs	8
Serotransferrin	Auto-Ab[50]	TIFENLANKADRDQ	MB	50/51	SF-DCs	8
Transthyretin	↑ serum[74]	IAALLSPYSYSTTAVVTNPK	HB	28/51	ST-DCs/SF-DCs/ST	8
Vimentin	Auto-Ab[50]; ↑ SF[64]; Auto-T[10]	VPGVRLLQDSVDFSLADAI	HB	36/51	SF-DCs	8

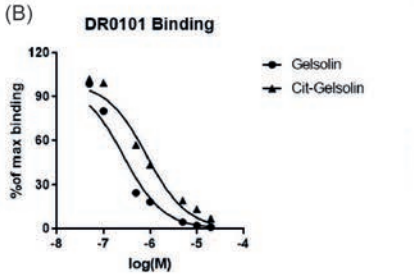
*The 9-mer core of each peptide sequence is underlined and citrulline residues (r) are highlighted in grey.
†Theoretical binding affinities for the corresponding donor SE-positive molecule are shown.
‡Number of HLA-DR molecules to which each peptide is predicted to bind among 51 alleles available in the *Propred* server.
Auto-Ab, autoantibodies; DCs, dendritic cells; FLS, fibroblast-like synoviocytes; HB, high binders; HLA, human leucocyte antigen; LB, low binders; MB, medium binders; RA, rheumatoid arthritis; SF, synovial fluid; ST, synovial tissue.

Human MoDCs are acknowledged as a prototype to study antigen presentation.[36 37] We and others have demonstrated the suitability of using a small number of MoDCs for the characterisation of HLA-DR-associated peptide repertoires and disease-related T-cell epitopes.[28 38] In this study, we obtained abundant non-redundant sequences from MoDCs pulsed with RA SF or ST, which exhibited characteristic features of DCs-derived peptide repertoires in terms of length, presence of nested sets, theoretical affinity, flanking residues properties, intraprotein location and expected degradation pathway.[28] In parallel, we identified 210 peptides from RA ST, which were not significantly different from the MoDCs repertoire, containing a considerable proportion of predicted HB presumably processed through the endo-lysosomal pathway. In addition, we detected an important number of peptides derived from extracellular and tissue-specific proteins, many of which are enriched in RA joints, including previously described autoantigens such as fibronectin[39] and fibrinogen.[14] These findings suggest that HLA-DR-expressing cells in RA ST may correspond to resident or infiltrating cells with antigen uptake, processing and presentation capabilities, as has been shown for autoimmune thyroid glands.[40]



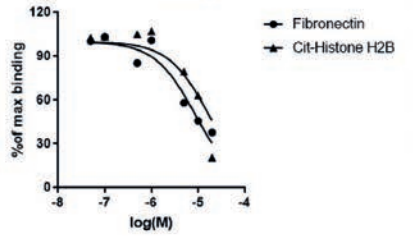
IC50 in µM	Parental protein	Sequence [1]	Donor *HLA-DRB1* haplotype [2]	Predicted binding affinity [3]
>50	Alpha-actin-2	SGGTTMYPGlADRMQKEITA	*0401 / *1101	LB
>50	Cit-Alpha-actin-2	SGGTTMYPGlADrMQKEITA	*0401 / *1101	MB
0.36	Cathepsin Z	SDGTEYWIVRNSWGEPWG	*0401 / *0407	MB
0.44	Cit-Cathepsin Z	SDGTEYWIVrNSWGEPWG	*0401 / *0407	HB
0.57	Fibronectin	APITGYRIVYSPSVEGSS	*0401 / *1101-04	HB
2.12	Cit-Fibronectin	APITGYrIVYSPSVEGSS	*0401 / *1101-04	HB
>50	Histone H2B	MNSFVNDIFERIAGEA	*0401 / *1101	HB
>50	Cit-Histone H2B	MNSFVNDIFErIAGEA	*0401 / *1101	HB
3,57	Proteoglycan 4	THTIRIQYSPARLA	*0401 / *1602	HB
3.04	Cit-Proteoglycan 4	THTIrIQYSPArLA	*0401 / *1602	HB

(B)



IC50 in µM	Parental protein	Sequence [1]	Donor *HLA-DRB1* haplotype [2]	Predicted binding affinity [3]
0.23	Gelsolin	DAYVILKTVQLRNGN	*0101 / *0301	HB
0.74	Cit-Gelsolin	DAYVILKTVQLrNGN	*0101 / *0301	HB

(C)



IC50 in µM	Parental protein	Sequence [1]	Donor *HLA-DRB1* haplotype [2]	Predicted binding affinity [3]
>50	Alpha-actin-2	SGGTTMYPGlADRMQKEITA	*0401 / *1101	MB
>50	Cit-Alpha-actin-2	SGGTTMYPGlADrMQKEITA	*0401 / *1101	MB
8.72	Fibronectin	APITGYRIVYSPSVEGSS	*0401 / *1101-04	HB
>50	Cit-Fibronectin	APITGYrIVYSPSVEGSS	*0401 / *1101-04	HB
>50	Histone H2B	MNSFVNDIFERIAGEA	*0401 / *1101	HB
12.75	Cit-Histone H2B	MNSFVNDIFErIAGEA	*0401 / *1101	HB

Figure 3 Peptide binding competition assays. Citrullinated (cit)-peptides selected for CD4 +T cell stimulation assays, along with their native counterparts, were tested in binding competition assays for shared epitope (SE)-positive molecules expressed by their respective source sample (A, B), or for HLA-DR*1101, the most frequent SE-negative allele among the sample donors for these peptides (C). Binding curves for peptides with IC50 values <50 µM (highlighted in grey in the tables) for HLA-DR*0401 (A), HLA-DR*0101 (B), and HLA-DR1101 (C) are shown. The IC50 values for all analysed peptides are shown in the tables. [1] the predicted 9-mer core of each sequence is underlined and citrulline residues (r) are highlighted in BOLD; [2] SE-positive alleles from the sample donor are highlighted in BOLD; [3] predicted binding affinities for the corresponding donor SE-positive molecule are shown, except for (C), where the theoretical affinity for the HLA-DR*1101 molecule is shown. [4] The native version of the proteoglycan four peptide was not detected among the HLA-DR-associated peptides; however, for these analyses we considered the haplotype of the donor from which its corresponding cit-version was isolated. HLA, human leucocyte antigen; LB, low binders; MB, medium binders; HB, high binders.

Previous studies analysing the RA ST HLA-DR immunopeptidome described a significant number of sequences,[20 22] of which only those derived from *N*-acetylglucosamine-6-sulfatase and filamin-A were reported to activate RA CD4 +T cells.[23] Our results show that peptides deriving from gelsolin, histone H2B, cit-histone H2B, cit-PG4, histone H4 and MPO were able to stimulate RA CD4 +T cells, measured as an increase in the expression of CD40L, which has been shown to be a reliable marker of antigen-activated T cells.[41] In addition, these peptides elicited a higher IFN-γ production by RA CD4 +T cells compared with HC. Interestingly, we showed that T-cell responses to the MPO-derived peptide were correlated with DAS28 score. Supplementary longitudinal studies in a larger patient cohort will be needed to validate the relevance of T-cell responses to these epitopes in disease activity and progression, as well as the relationship with the emergence of autoantibodies against their parental proteins.

To our knowledge, this is the first successful attempt to sequence cit-NPPs from RA samples. This task has proven to be a major challenge in the past given the relatively low amount of peptides retrieved from pHLA and the infrequent occurrence of citrullination in vivo.[22 42] In this study, we obtained both, cit-peptides and their native counterparts, allowing us to manually compare their spectra and validate the presence of citrulline.

It has been proposed that citrullination can contribute to break self-tolerance in RA by enabling peptides to accommodate into the P4 pocket of SE-positive molecules.[5] While this model can be applicable for certain peptides,[6 43] it might not always be the case, as suggested by studies showing that citrulline could also interact with other SE anchor pockets, or be placed at positions available for TCR recognition, thus activating cit-specific T-cell clones.[7–9] In consonance with these reports, our competition binding assays, involving six cit-NPPs and the SE-positive molecule expressed by the corresponding sample donor, revealed that citrullination exerts limited influence, or can even be disadvantageous, for the binding of peptides to HLA-DR*0401 or *0101, as has been shown for α-enolase cit-peptides.[8] Moreover, one cit-peptide only bound to its corresponding SE-negative molecule. These findings, together with a considerable number of predicted promiscuous peptides and HB for SE-negative molecules among our HLA-DR immunopeptidome, suggest that a proportion NPPs, and even some cit-NPPs, could have been isolated from SE-negative molecules, including HLA-DRB4/B3 molecules (although the latter possibility seems less likely, according to our preliminary prediction affinity analysis; data not shown). Our results showing no significant differences in T-cell responses to NPPs between SE-positive and SE-negative

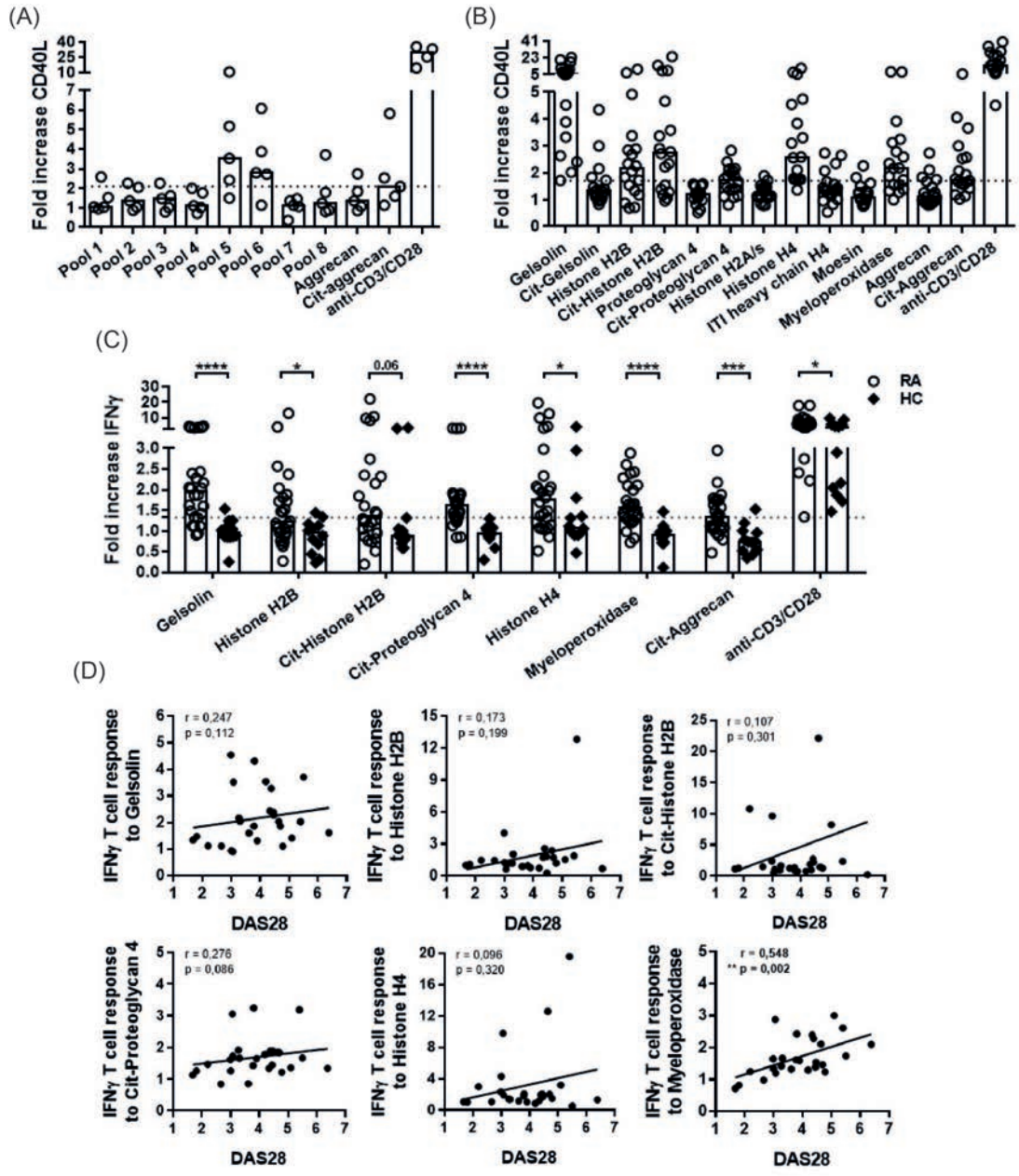


Figure 4 Responsiveness of CD4 +T cells from patients with RA after in vitro stimulation with HLA-DR-associated peptides. (A) Peptides were pooled and used to stimulate PBMCs from patients with RA (n=5). Unstimulated PBMCs and PBMCs stimulated with anti-human-CD3/CD28 beads were used as negative and positive controls, respectively. A previously described autoantigenic aggrecan peptide was included in its native and citrullinated versions. A positive CD4 +CD40L+T cell response was defined as a greater than the median response observed against the cit-aggrecan control peptide in relation to the unstimulated controls. (B) The previous strategy was repeated, using peptides belonging to pools 5 and 6 to stimulate PBMCs from patients with RA (n=18). (C) Peptides with the highest T-cell immunostimulatory capacity in (B) were used for stimulating PBMCs from patients with RA (n=26) and HC (n=12), and IFN-γ production was evaluated. A positive CD4 +IFN-γ+T cell response was defined as greater than the median response observed against the cit-aggrecan control peptide in relation to the unstimulated controls. (D) Correlation between IFN-γ T-cell responses to peptides (fold increase in the percentage of CD4 +IFN-γ+T cells) in patients with RA (n=26) and the disease activity measured by the DAS28 score. *P<0.05; **p<0.01; ***p<001; ****p<0.0001. DAS28, disease activity score in 28 joints; HC, healthy control; HLA, human leucocyte antigen; PBMCs, peripheral blood mononuclear cells; RA, rheumatoid arthritis.

patients with RA, regardless of the presence of citrulline, further support the interpretation that the presentation of some of these peptides may be promiscuous; this assumption should be verified in experiments including a larger set of patients from both groups. Likewise, the chance that these peptides could be presented to CD4 +T cells by other HLA-class II molecules (DP, DQ) requires a more thorough examination.

Altogether, these results provide valuable information about the immunopeptidome displayed by HLA-DR molecules loaded ex vivo and in vivo with RA material, revealing autoantigenic T-cell epitopes that could have an impact in disease initiation and/or progression, both in SE-positive and SE-negative patients. Despite the fact that some of these epitopes have been previously isolated from HLA-class II molecules,[44] none of them have been

previously described as RA T-cell epitopes. Given the exploratory character of this study, we only test the antigenicity of a few peptides. We are currently undertaking a comprehensive screening of T-cell epitopes throughout the whole list of NPPs described herein. We believe that this information could be useful for the development of antigen-specific therapies aimed at restoring self-tolerance in patients with RA, such as those based on antigen-loaded tolerogenic DCs[45] and peptide-specific regulatory T cells.[46]

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Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by—'Comité Ético Científico o de Investigación del Hospital Clínico de la Universidad de Chile', Project Ref. OAIC 735-15 (approval certificate #33), 979-18 (approval certificate #44), 962-18 (approval certificate #21) and 964-18 (approval certificate #24)—'Comité de Ética Científico del Servicio de Salud Metropolitano Oriente', Project Ref. 20-10-2018- 'Comisión de Ética en la Experimentación Animal y Humana de la Universitat Autònoma de Barcelona', Project Ref. CEEAH 3112 (collaboration agreement with 'Biobanco del Centro de Hemoterapia y Hemodonación de Castilla y León', Ref. UAB-DJ-19-01). Participants gave informed consent to participate in the study before taking part.

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TRANSLATIONAL SCIENCE

Deletion of activin A in mesenchymal but not myeloid cells ameliorates disease severity in experimental arthritis

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ABSTRACT

Objective The aim of this study was to assess the extent and the mechanism by which activin A contributes to progressive joint destruction in experimental arthritis and which activin A-expressing cell type is important for disease progression.

Methods Levels of activin A in synovial tissues were evaluated by immunohistochemistry, cell-specific expression and secretion by PCR and ELISA, respectively. Osteoclast (OC) formation was assessed by tartrat-resistant acid phosphatase (TRAP) staining and activity by resorption assay. Quantitative assessment of joint inflammation and bone destruction was performed by histological and micro-CT analysis. Immunoblotting was applied for evaluation of signalling pathways.

Results In this study, we demonstrate that fibroblast-like synoviocytes (FLS) are the main producers of activin A in arthritic joints. Most significantly, we show for the first time that deficiency of activin A in arthritic FLS (ActβA$^{d/d}$ ColVI-Cre) but not in myeloid cells (ActβA$^{d/d}$ LysM-Cre) reduces OC development in vitro, indicating that activin A promotes osteoclastogenesis in a paracrine manner. Mechanistically, activin A enhanced OC formation and activity by promoting the interaction of activated Smad2 with NFATc1, the key transcription factor of osteoclastogenesis. Consistently, ActβA$^{d/d}$ LysM-Cre hTNFtg mice did not show reduced disease severity, whereas deficiency of activin A in ColVI-Cre-expressing cells such as FLS highly diminished joint destruction reflected by less inflammation and less bone destruction.

Conclusions The results highly suggest that FLS-derived activin A plays a crucial paracrine role in inflammatory joint destruction and may be a promising target for treating inflammatory disorders associated with OC formation and bone destruction like rheumatoid arthritis.

Key messages

What is already known about this subject?
⇒ High levels of activin A in serum, synovial fluid and synovial tissue of patients with rheumatoid arthritis (RA) were observed.
⇒ Activin A promotes osteoclast development.

What does this study add?
⇒ Fibroblast-like synoviocytes (FLS) seem to be the main producer of activin A in arthritic joints.
⇒ Deletion of activin A in FLS but not in myeloid cells highly diminishes joint pathology in experimental arthritis displayed by less inflammation and less bone destruction, confirming for the first time a significant role for activin A in arthritis development in vivo.

How might this impact on clinical practice or future developments?
⇒ Blockade of activin A or corresponding signalling pathways may be a promising treatment option for diseases associated with inflammatory bone destruction such as RA.

INTRODUCTION

Rheumatoid arthritis (RA) is a common type of inflammatory arthritis characterised by chronic inflammation, culminating in destruction of the joint. In the pathological state of RA, the synovial lining layer becomes hyperplastic due to influx of macrophage-like synoviocytes and increased cell division of fibroblast-like synoviocytes (FLS).[1–3] The continuous presence of cytokines, chemokines, growth factors and other molecular mediators leads to the activation of FLS resulting in an aggressive tumor-like transformation[4 5] contributing to pannus formation, a highly destructive tissue located at the interface between synovium, cartilage and bone. FLS and macrophages predominate this tissue and mediate the process of joint destruction by expression of degradative enzymes including matrix metalloproteinases and collagenases.[6]

However, bone destruction is mediated by osteoclasts (OCs) located at the interface between the pannus and periarticular bone surface.[7] Many factors such as tumour necrosis factor alpha (TNF-α), interleukin (IL)-1, IL-6, IL-17 and receptor activator of nuclear factor κB ligand (RANKL) produced directly by FLS or within the pannus tissue by infiltrated immune cells are able to enhance the differentiation of cells of the monocyte–macrophage lineage into OC, thereby disturbing the balance of bone remodelling and shifting the remodelling process towards bone resorption.[7–13]

Another factor shown to influence bone destruction in RA by directly promoting OC formation is activin A.[14–19] Activin A belongs to the transforming growth factor beta (TGF-β)-like group of

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the TGF-β superfamily consisting of two disulfide-linked inhibin βA subunits.[20–22] Interestingly, proinflammatory cytokines such as TNF-α, IL-1β and TGF-β have been found to increase levels of activin A in RA.[23 24] In accordance with this, high levels of activin A in serum, synovial fluid and synovial tissue of patients with RA, compared with patients with osteoarthritis (OA), were observed.[25–28] Besides FLS, activin A is produced also by macrophages[29] and not only is induced by proinflammatory cytokines but in turn also stimulates the production of inflammatory mediators like TNF-α, IL-1β, IL-6, nitric oxide and prostaglandin E_2,[25 28 30 31] suggesting an important role of activin A in the pathology of RA. However, the extent to which activin A influences arthritis development and progression in vivo has not been elucidated so far, and even the most significant activin A-producing cell type involved in disease progression has not yet been identified. Therefore, the aim of this study was to investigate whether activin A contributes to joint inflammation and progressive bone loss in experimental arthritis and which activin A-expressing cell type within the inflamed joint is important for disease progression.

METHODS

Detailed experimental procedures are described in the online supplemental material.

RESULTS

Increased levels of activin A in arthritis

Immunofluorescence staining revealed a higher expression of activin A in the synovial tissues of patients with RA compared with those of patients with OA (2.7-fold). Whereas in OA specimens activin A expression appeared mainly in cells of the synovial lining layer, activin A expression in RA was observed throughout the whole synovial tissue (figure 1A). More detailed analysis demonstrated an increase of activin A-expressing macrophages (2.7-fold), FLS (5.6-fold) and neutrophils (2.6-fold), whereby the latter one was not significant (figure 1C). Consistently, a TNFα-dependent chronic arthritis mouse model (hTNFtg) revealed dramatically increased levels of activin A in the hind paws (3.7-fold, figure 1B) and in sera (7.3-fold, figure 1D) compared with wild-type (WT) mice. Moreover, mRNA of the inhibin βA subunit was abundantly expressed in RA FLS as well as in FLS of arthritic mice (figure 1E). Together, these data indicate that under inflammatory conditions, activin A appears to be highly upregulated and that FLS may be the main producers of activin A within the inflamed synovial tissue.

Since an inflammatory environment obviously causes an increase in activin A levels, WT and hTNFtg mouse FLS were stimulated with the proinflammatory cytokines IL-1α, IL-1β, TGF-β, and IL-17A. Indeed, all cytokines were able to significantly enhance the secretion of activin A compared with untreated controls (4.3-fold to 9.7-fold higher) with the exception of IL-17A, where the secretion of WT FLS was identical to the untreated control (figure 1F). Moreover, hTNFtg FLS showed an even stronger increase in activin A secretion on stimulation with proinflammatory cytokines compared with WT FLS (11.6-fold to 17.5-fold higher, figure 1G). Because of the increased number of activin A-producing macrophages in RA and hTNFtg synovial tissue, regulation of activin A by inflammatory cytokines was additionally analysed in bone marrow-derived macrophages (BMDM). In contrast to FLS, a distinct increase in activin A secretion by BMDMs was exclusively observed on stimulation with TGF-β1, and no significant differences in secretion were found between WT (increase by 4.1) and hTNFtg (increase by 6.5) (figure 1I–K). It should be stressed that BMDMs secreted considerably lower amounts of activin A than FLS (pg/mL vs ng/mL range; figure 1H,K), further supporting the hypothesis that FLS are the main producers of activin A within the inflamed synovium.

Activin a highly enhances OC formation and activity

To investigate the impact of activin A on osteoclastogenesis, primary BMDMs isolated from WT mice were differentiated into OC by stimulation with RANKL, activin A or both (figure 2A). Interestingly, concomitant treatment of BMDMs with RANKL and activin A led to enhanced OC differentiation (2.6-fold, figure 2B) associated with a higher number of nuclei per OC (3.6-fold, figure 2C) as well as an increased OC size (2.5-fold, figure 2D), leading to an increased total OC area (6.9-fold, figure 2E) compared with RANKL treatment alone. Of note, activin A alone was not able to induce OC differentiation (figure 2A). Consistently, resorption analysis revealed an increased formation of resorption pits (figure 2F,G) and total resorption area (figure 2H) on calcium phosphate plates on differentiation with both RANKL and activin A simultaneously compared with the RANKL-treated control (2.6-fold and 5.9-fold, respectively). Most interestingly, the additional increase in resorption area per pit by 2.3-fold compared with the RANKL-treated control clearly demonstrate that the observed higher resorption is not only due to higher OC numbers but also additionally based on increased OC activity (figure 2I). Finally, figure 2J exclude that the higher number of OC on stimulation with activin A was dependent on a higher number of BMDMs or available OC precursors provoked by proliferation.

Altogether, these data indicate that activin A strongly enhances the RANKL-mediated osteoclastic resorption due to increased numbers as well as increased activity of in vitro differentiated primary OCs. In order to bridge to humans, the effects of activin A on human OC differentiation were investigated. Consistent with the effects of activin on murine osteoclastogenesis, activin A also enhanced the RANKL-induced differentiation of human peripheral blood mononuclear cells (PBMCs) into OCs by about 5-fold, associated again with an increase in OC size by about 6-fold, indicating that activin A must be considered an important cross-species factor in OC differentiation (figure 2K).

Since both FLS as well as macrophages produce activin A, we next wanted to unravel whether autocrine or paracrine activin A is important for joint destruction in arthritis. To this end, we compared the impact of activin-deficiency in myeloid cells and FLS on the formation of OCs in vitro as well as on arthritis development and progression in vivo.

Lack of activin A in myeloid cells has no influence on OC differentiation

To confirm a myeloid lineage-specific as well as effective deletion of activin A in the $Actβa^{d/d}$ LysM-Cre mice, activin A secretion by FLS (figure 3A) and myeloid cells (figure 3B) was measured by ELISA. As expected, $Actβa^{d/d}$ LysM-Cre FLS showed no changes in activin A secretion compared with FLS from $Actβa^{flox/flox}$ ($Actβa^{f/f}$) mice, whereas a significant, almost complete reduction of activin A secretion by BM cells, BMDMs, pre-OCs (pOCs) and OC of $Actβa^{d/d}$ LysM-Cre mice was observed, indicating that activin A is effectively deleted in cells of the myeloid lineage but not in mesenchymal cells such as FLS.

To analyse whether activin A deficiency in myeloid cells affects osteoclastogenesis in vitro, BMDMs from $Actβa^{f/f}$ and $Actβa^{d/d}$ LysM-Cre mice as well as from $Actβa^{f/f}$ hTNFtg and $Actβa^{d/d}$

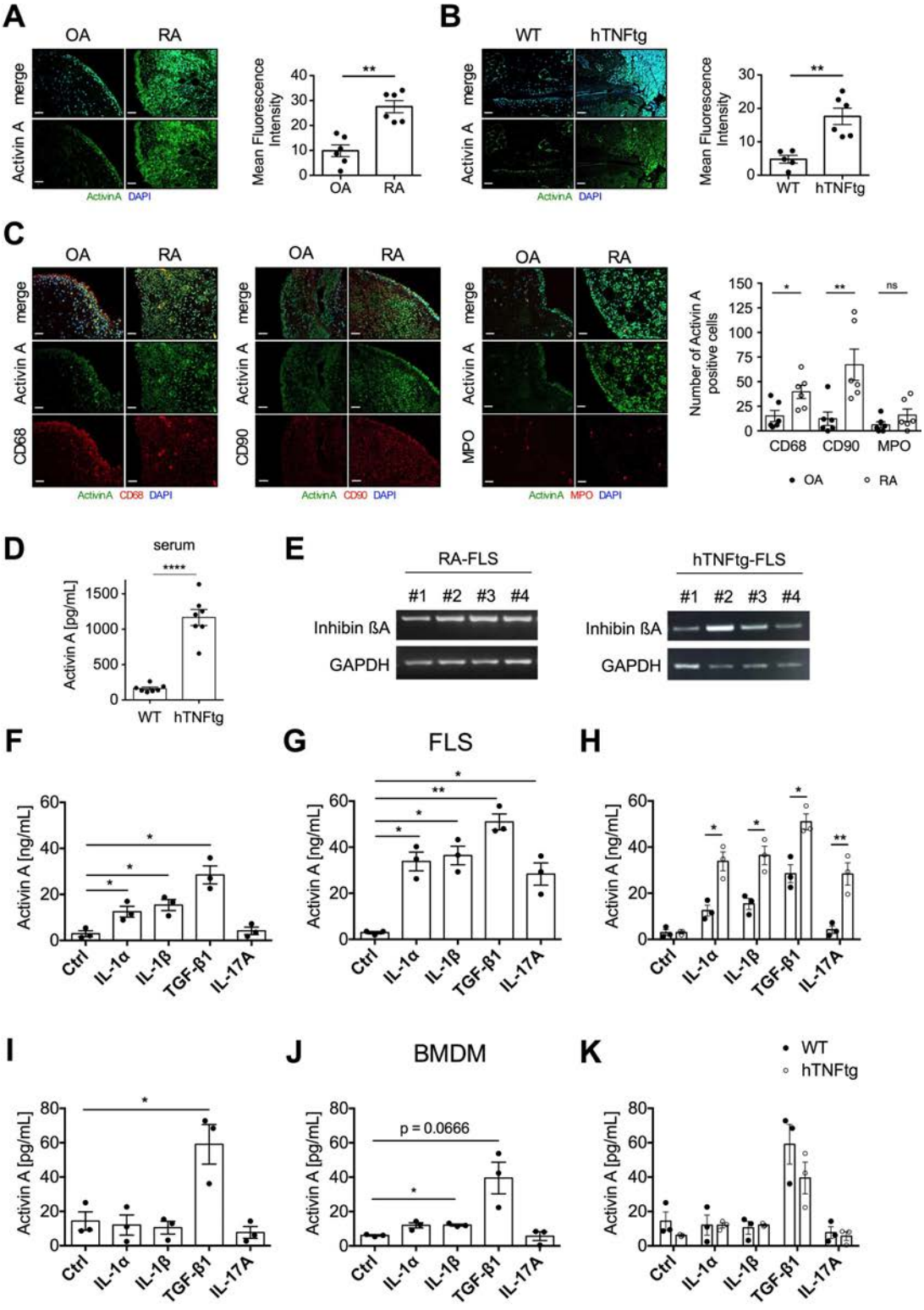

Figure 1 High levels of activin A under inflammatory conditions. (A) representative images of fluorescence stainings of activin A in synovial tissue samples obtained from patients with OA and RA and corresponding quantification (n=6, unpaired t-test**). (B) Representative images of fluorescence stainings of activin A in hind paws of WT and arthritic hTNFtg mice and corresponding quantification (n=5–6, unpaired t-test**). (C) Representative fluorescence costainings of activin A with CD68, CD90 or MPO in synovial tissue samples obtained from patients with OA and RA and quantification of corresponding activin A-positive cells (n=6, unpaired t-test*,**). (D) Activin A concentrations in serum of WT and arthritic hTNFtg mice. Data represent means±SEM (n=7, unpaired t-test****). (E) PCR analysis of inhibin βA-subunit mRNA in FLS of patients with RA (n=4) and of hTNFtg mice (n=4). (F) Secretion of activin A by WT and (G) hTNFtg FLS on stimulation with IL-1α (20 ng/mL), IL-1β (20 ng/mL), TGF-β1 (20 ng/mL) and IL-17A (20 ng/mL) for 48 hours. (H) Comparison of activin A secretion by WT and hTNFtg FLS. (I) Secretion of activin A by WT and (J) hTNFtg BMDMs on stimulation with IL-1α (20 ng/mL), IL-1β (20 ng/mL), TGF-β1 (20 ng/mL) and IL-17A (20 ng/mL) for 48 hours. (K) Comparison of activin A secretion by WT and hTNFtg BMDMs. All data are means±SEM (n=3, paired t-test, comparison WT/hTNFtg unpaired t-test*). *P≤0.05, **P≤0.01, ****P≤0.0001. Ctrl, control; FLS, fibroblast-like synoviocytes; IL, interleukin; ns, not significant; OA, osteoarthritis; RA, rheumatoid arthritis; WT, wild type.

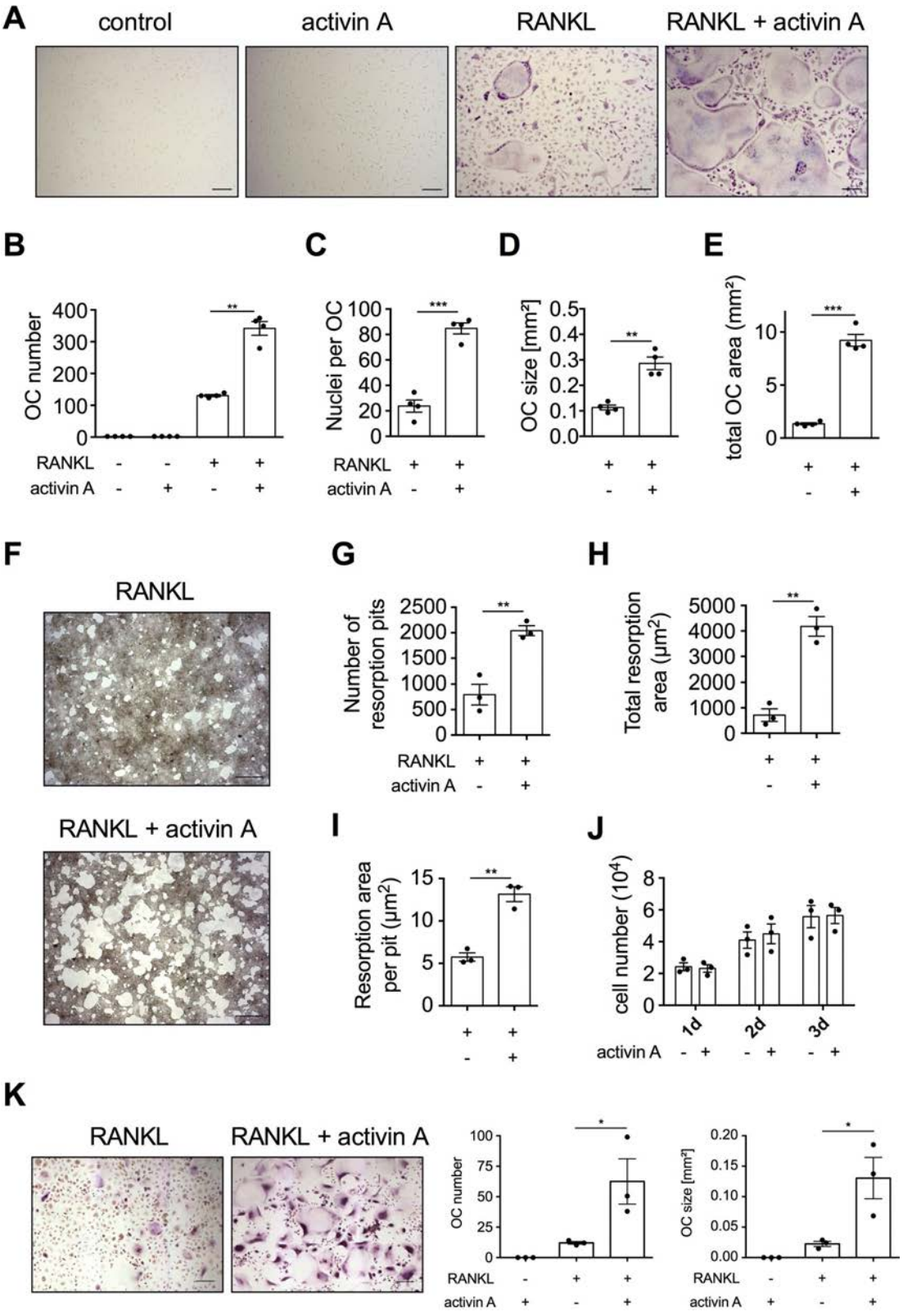

Figure 2 Enhanced RANKL-mediated differentiation and activity of OCs by activin A. (**A**) Representative images of TRAP staining after 4 days of OC differentiation in the presence of 30 ng/mL macrophage colony-stimulating factor (M-CSF, control) together with activin A (30 ng/mL) or RANKL (50 ng/mL) or RANKL plus activin A (scale bar 100 µm). (B) Corresponding OC numbers, (C) number of nuclei per OC, (D) OC size and (E) total OC area per well (n=4). (F) Representative images of resorption pit formation of WT BMDMs after 6 days of OC differentiation using calcium phosphate as substrate on stimulation with RANKL or RANKL plus activin A (scale bar 500 µm). (G) Number of resorption pits, (H) total resorption area and (I) resorption area per pit after 6 days of OC differentiation (n=3). (J) Cell numbers of BMDMs with or without activin A stimulation after 1, 2 and 3 days (n=3). (K) Representative TRAP staining of human OCs after 15 days of differentiation in the presence of M-CSF (30 ng/mL) and RANKL (50 ng/mL) with and without 100 ng/mL activin A (scale bar 200 µm) and corresponding OC number and OC size. All data are means±SEM (t-test). *P≤ 0.05, **P≤0.01, ***P≤ 0.001. OC, osteoclast; RANKL, receptor activator of nuclear factor κB ligand.

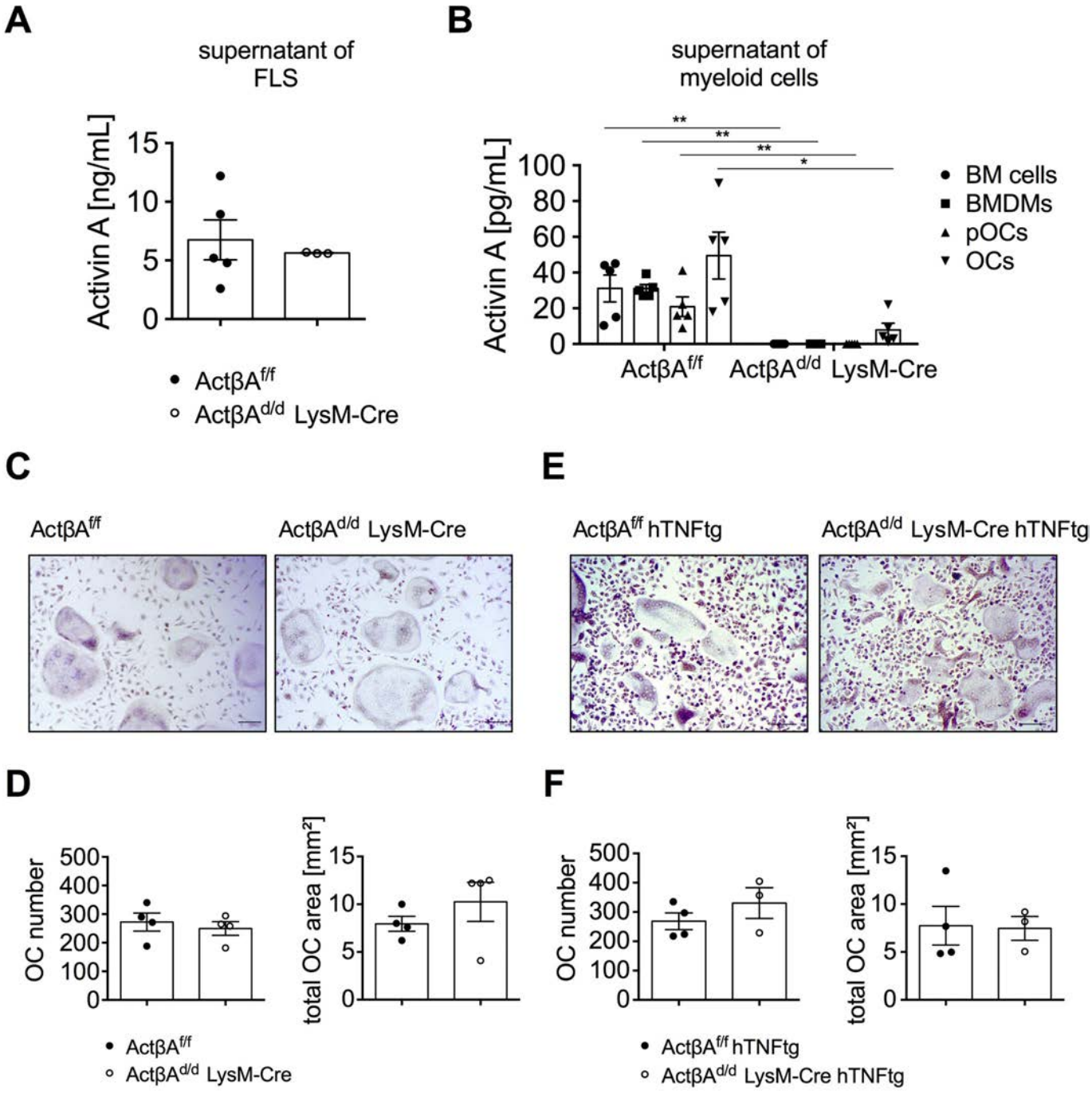

Figure 3 Deficiency of activin A in BMDMs had no impact on OC differentiation in vitro. (A) Secretion of activin A by FLS from ActβA$^{f/f}$ and ActβA$^{d/d}$ LysM-Cre mice after 48 hours. (B) Secretion of activin A by BM cells, BMDMs, pOCs and OCs from ActβA$^{f/f}$ and ActβA$^{d/d}$ LysM-Cre mice after 48 hours. BM cells were not stimulated; BMDMs were stimulated with M-CSF (30 ng/mL) for 3 days; pOCs were stimulated for 3 days with M-CSF (30 ng/mL) followed by stimulation with M-CSF and RANKL (50 ng/mL) for further 2 days. OCs were generated by stimulation of pOCs for a further 2 days with RANKL. All data are means±SEM (n=3–5, Mann-Whitney U test*,**). (C) Representative images of TRAP staining after 5 days of differentiation of BMDMs from ActβA$^{f/f}$ and ActβA$^{d/d}$ LysM-Cre mice (scale bar 100 µm). (D) Corresponding OC number and total OC area per well. All data are means±SEM (n=4, Mann-Whitney U test). (E) Representative images of TRAP staining after 5 days of differentiation of BMDMs from ActβA$^{f/f}$ hTNFtg and ActβA$^{d/d}$ LysM-Cre hTNFtg mice (scale bar 100 µm). (F) Corresponding OC number and total OC area per well. All data are means±SEM (n=4 and 3, respectively; Mann-Whitney U test). *P≤0.05, **P≤0.01. ActβA$^{f/f}$, ActβA$^{flox/flox}$; FLS, fibroblast-like synoviocytes; IL, interleukin; OC, osteoclast; RANKL, receptor activator of nuclear factor κB ligand.

LysM-Cre hTNFtg mice were stimulated with RANKL and subsequently stained for TRAP after 5 days of differentiation (figure 3C,E). Of note, the deficiency of activin A in myeloid cells did not show significant differences in OC number and area neither from non-arthritic (figure 3D) nor from arthritic mice (figure 3F) which leads to the assumption that autocrine activin A is not important for OC differentiation. Because activin levels were rather low, cells were additionally stimulated with TGF-ß1, previously shown to enhance activin secretion in BMDMs (figure 1I,J). However, additional stimulation with TGF-ß1

had no effect on OC formation (online supplemental figure 1). Moreover, to exclude whether residual activin A, due to incomplete deletion in LysM-Cre BMDMs, may interfere with the effects of deletion, cells were additionally treated with activin A antibodies. Indeed, blocking activin A did not influence OC development of ActβA$^{d/d}$ LysM-Cre BMDMs, whereas blocking of myostatin, another member of the TGF-ß superfamily and known to stimulate OC formation, led to decreased OC differentiation (online supplemental figure 4).

Deficiency of activin A in LysM-Cre-expressing cells does not significantly affect disease severity in hTNFtg mice

Taking the involvement of activin A in bone remodelling and inflammation into account, we first asked whether the loss of activin A in cells of the myeloid lineage would influence the development of inflammatory bone destruction in arthritis. Because the complete knockout of activin A is lethal,[32] ActβA$^{f/f}$ mice were used to generate conditional knockouts by breeding these mice with the LysM-Cre mouse line.[33 34] The resulting mice with a cell-specific deletion of activin A (ActβA$^{d/d}$ LysM-Cre) were subsequently cross-bred with hTNFtg mice to generate deleted arthritic mice. hTNFtg mice are overexpressing the hTNFα transgene and thereby develop a chronic destructive arthritis that shares many characteristics with human RA.[35]

As expected, ActβA$^{f/f}$ hTNFtg and ActβA$^{d/d}$ LysM-Cre hTNFtg mice showed an increase in paw swelling (figure 4A) and loss of grip strength (figure 4B) during disease development. However, no significant differences in the clinical symptoms between the two hTNFtg genotypes could be observed during the course of the disease. ActβA$^{f/f}$ and ActβA$^{d/d}$ LysM-Cre showed no signs of arthritis.

Moreover, micro-CT (µCT) and histomorphometry showed no obvious differences in joint destruction as well as OC numbers in 12-week-old arthritic mice with deletion of activin A compared with the hTNFtg mice (figure 4C–E). Quantitative morphometric evaluation confirmed the lack of significant differences in inflammation, bone erosion and OC numbers (figure 4F–H and online supplemental figure 6). However, a tendency towards less inflammation (31.4 %) in the hind paws of activin A-deleted compared with non-deleted hTNFtg mice could be observed. Moreover, evaluation of trabecular and cortical bone parameters in non-arthritic ActβA$^{f/f}$, LysM-Cre and ActβA$^{d/d}$ LysM-Cre mice revealed that activin A also does not influence physiological bone remodelling (online supplemental figure 2). Of note, activin A serum levels were not significantly reduced, although a tendency towards lower levels (about 27%) could be observed in ActßA$^{d/d}$ LysM-Cre hTNFtg mice (online supplemental figure 3A).

Lack of activin A in FLS effectively decreases OC differentiation

In order to analyse the deletion efficiency and specificity of activin A in FLS, secretion of activin A by FLS and myeloid cells was measured via ELISA. As anticipated, ActβA$^{d/d}$ ColVI-Cre FLS showed a significant reduction in activin A secretion by about 85% compared with FLS from ActβA$^{f/f}$ mice (figure 5A). In contrast, activin A secretion by myeloid lineage cells from ActβA$^{d/d}$ ColVI-Cre mice were not significantly reduced compared with those from ActβA$^{f/f}$ mice (figure 5B).

As expected, no differences in OC formation between BMDMs from ActβA$^{f/f}$ and ActβA$^{d/d}$ ColVI-Cre mice,[36] neither of non-arthritc nor of arthritic mice, was observed (figure 5C–F). In contrast, cocultures of BMDMs with FLS from ActβA$^{d/d}$ ColVI-Cre hTNFtg FLS led to the formation of smaller and less OCs compared with the cocultures with ActβA$^{f/f}$ hTNFtg FLS, which generated a higher number of OCs that were also larger in size (figure 5G). Quantitative analysis revealed that the number of OCs and the total OC area were significantly decreased in cocultures with ActβA$^{d/d}$ ColVI-Cre hTNFtg FLS by 25.6% and 36.7 %, respectively, compared with the ActβA$^{f/f}$ hTNFtg FLS, suggesting that activin A is an important paracrine factor for OC formation (figure 5H).

Of importance, FLS do not only express activin A but also myostatin, another member of the TGF-ß superfamily and also a promoting factor for OC differentiation.[37] To clarify the relative contribution of activin and myostatin on OC development, cocultures of arthritic FLS with BMDM in the presence of blocking antibodies against activin A or myostatin were performed. Cocultures with FLS from ActβA$^{f/f}$ hTNFtg mice treated with antibodies against activin A showed a reduction of OC development by about 48%, whereas treatment with antibodies against myostatin showed a tendency towards reduced OC formation (about 20%), pointing to a more important role of paracrine activin A. As expected, the already decreased osteoclastogenesis observed in cocultures with ActβA$^{d/d}$ ColVI-Cre hTNFtg FLS could not be further reduced by anti-activin AB but interestingly, also no further reduction could be seen on treatment with anti-myostatin AB, confirming that activin A is the determining factor in FLS-mediated OC differentiation (online supplemental figure 5).

Taken together, these data indicate that less bone destruction in ActβA$^{d/d}$ ColVI-Cre hTNFtg mice is caused by reduced OC formation due to less activin A secretion by FLS.

Deficiency of activin A in ColVI-Cre-expressing cells highly ameliorates inflammation and bone destruction in hTNFtg mice

Next, we investigated whether a deletion of activin A in mesenchymal cells via ColVI-Cre has an effect on arthritis severity in vivo. To this end, ActβA$^{d/d}$ ColVI-Cre mice were cross-bred with hTNFtg mice to generate conditional deleted arthritic mice. Again, no significant differences in the clinical scores between ActβA$^{f/f}$ hTNFtg and ActβA$^{d/d}$ ColVI-Cre hTNFtg mice was observed. As expected, ActβA$^{f/f}$ and ActβA$^{d/d}$ ColVI-Cre showed no signs of arthritis (figure 6A,B). Evaluation of serum confirmed significantly reduced activin A levels by about 68% in mice with SF-specific activin A deletion compared with non-deleted arthritic mice (online supplemental figure 3A).

Most importantly, µCT and histomorphometry at the age of 12 weeks (figure 6C–E), as well as its quantitative assessment demonstrated a strong reduction of inflammation displayed by less formation of pannus tissue (46.3 %) and a distinct reduction of bone erosion (33.8 %) associated with a significant reduction in the number of OCs (46.2 %) in ActβA$^{d/d}$ ColVI-Cre hTNFtg mice compared with ActβA$^{f/f}$ hTNFtg mice (figure 6F–H and online supplemental figure 6). In consideration of the fact that activin A also stimulates the production of central players in inflammation such as IL-1 and IL-6,[28 30] corresponding serum levels in activin A-deleted and non-deleted arthritic mice were assessed. Indeed, deletion of activin A caused a strong reduction in IL-1 and IL-6 serum levels (51.7% and 47.5%, respectively), confirming an anti-inflammatory effect of activin A via the downregulation of proinflammatory cytokines (online supplemental figure 3B). Assuming that a deletion of activin A in ColVI-Cre-expressing cells within the joint will mainly led to a deletion in FLS, the results strongly indicate that FLS-derived activin A

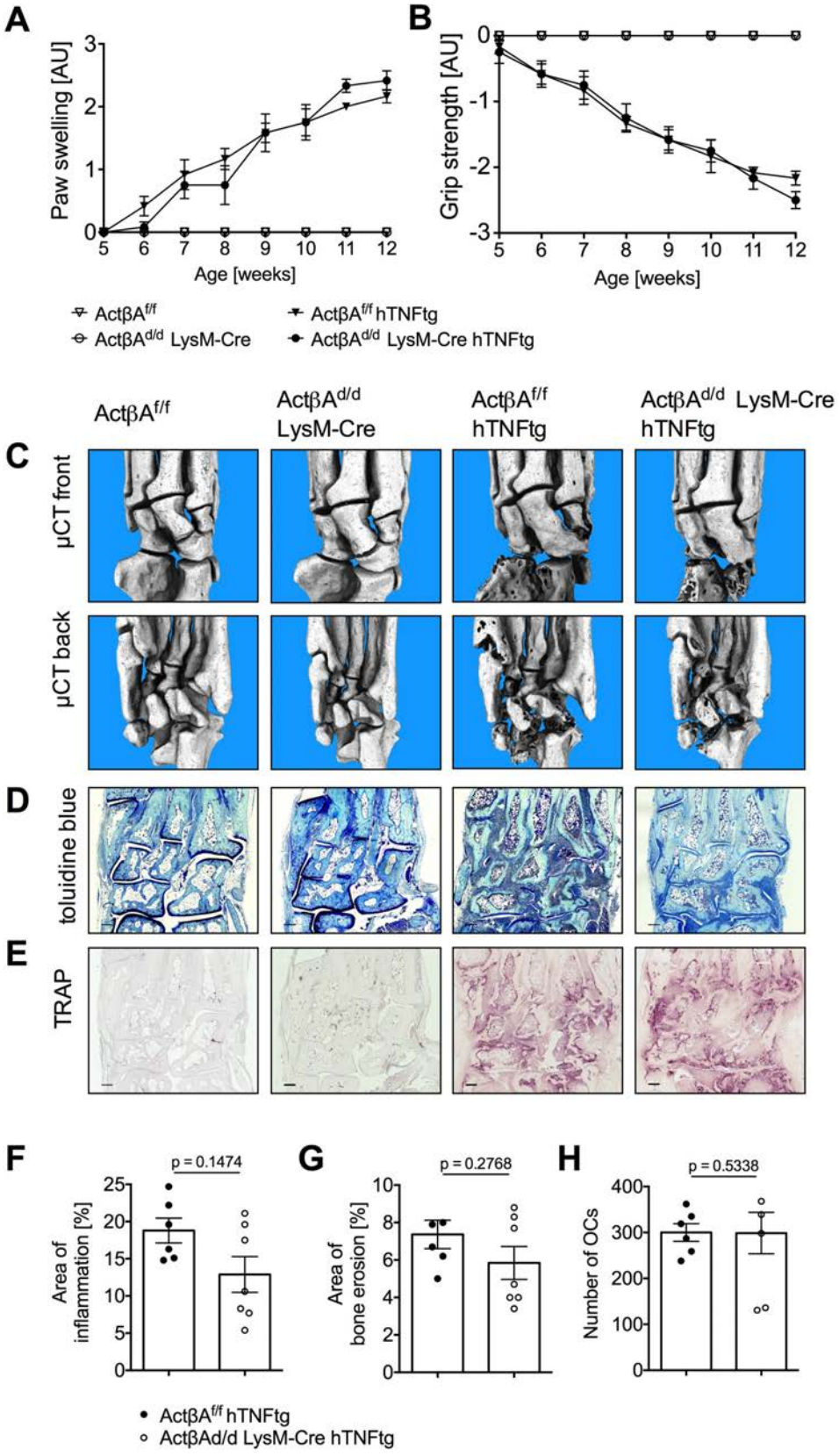

Figure 4 Deficiency of activin A in LysM-Cre-expressing cells does not substantially affect disease severity in hTNFtg mice. (A) Paw swelling and (B) grip strength measured in ActβA$^{f/f}$ (n=6), ActβA$^{d/d}$ LysM-Cre (n=6), ActβA$^{f/f}$ hTNFtg (n=6) and ActβA$^{d/d}$ LysM-Cre hTNFtg (n=6) mice over 5–12 weeks. All data are means±SEM (two-way analysis of variance, Bonferroni's multiple comparison test). (C) Representative images of µCT analysis from the front and back (n=4, each genotype), (D) toluidine blue-stained sections (scale bar 200 µm) and (E) TRAP-stained sections (scale bar 200 µm) from the hind paws of 12-week-old ActβA$^{f/f}$ (n=3), ActβA$^{d/d}$ LysM-Cre (n=3), ActβA$^{f/f}$ hTNFtg (n=6) and ActβA$^{d/d}$ LysM-Cre hTNFtg (n=7) mice. Quantitative histomorphometric assessment of (F) synovial pannus formation, (G) bone erosion and (H) number of OCs in tarsal joints. All data are means±SEM (Mann-Whitney U test). µCT, micro-CT; ActβA$^{f/f}$, ActβA$^{flox/flox}$; OC, osteoclast.

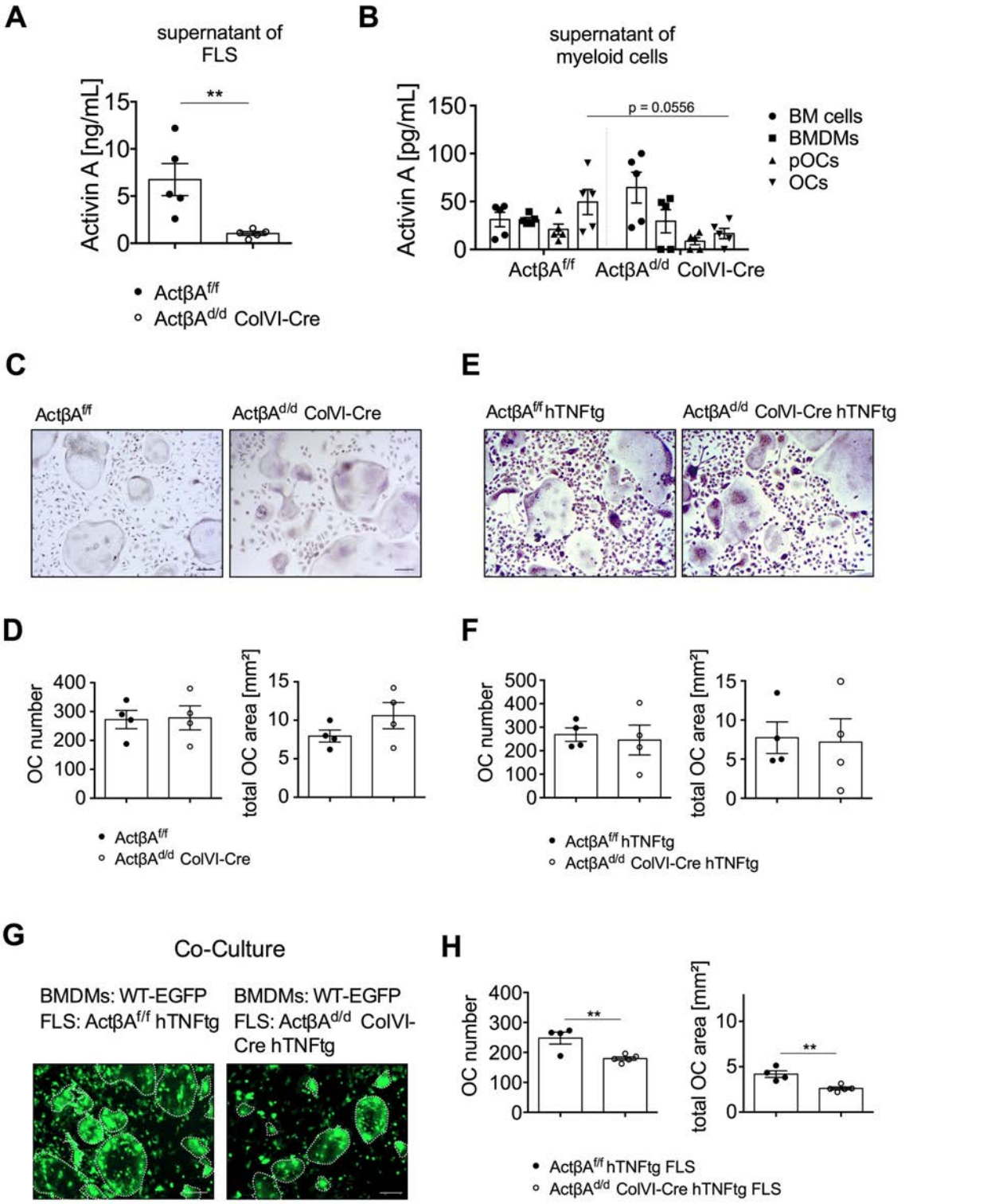

Figure 5 Deficiency of activin A in FLS significantly reduces OC formation. (A) Secretion of activin A by FLS from ActβA$^{f/f}$ and ActβA$^{d/d}$ ColVI-Cre mice after 48 hours. (B) Secretion of activin A by BM cells, BMDMs, pOCs and OCs from ActβA$^{f/f}$ and ActβA$^{d/d}$ ColVI-Cre mice after 48 hours. BM cells were not stimulated; BMDMs were stimulated with M-CSF (30 ng/mL) for 3 days; pOCs were stimulated for 3 days with M-CSF (30 ng/mL) followed by stimulation with M-CSF and RANKL (50 ng/mL) for a further 2 days. OCs were generated by stimulation of pOCs for a further 2 days with RANKL. All data are means±SEM (n=3–5, Mann-Whitney U test**). (C) Representative images of TRAP staining after 5 days of differentiation of BMDMs from ActβA$^{f/f}$ and ActβA$^{d/d}$ ColVI-Cre mice (scale bar 100 µm). (D) Corresponding OC number and total OC area per well. All data are means±SEM (n=4, Mann-Whitney U test). (E) Representative images of TRAP staining after 5 days of differentiation of BMDMs from ActβA$^{f/f}$ hTNFtg and ActβA$^{d/d}$ LysM-Cre hTNFtg mice (scale bar 100 µm). (F) Corresponding OC number and total OC area per well. All data are means±SEM (n=4, Mann-Whitney U test). (G) Representative fluorescence images of WT-EGFP BMDMs cocultured with FLS from ActβA$^{f/f}$ hTNFtg and ActβA$^{d/d}$ ColVI-Cre hTNFtg mice for 5 days to induce OC differentiation in presence of 1 µM PGE_2 (scale bar 100 µm). (H) Corresponding OC number and total OC area per well. All data are means±SEM (ActβA$^{f/f}$ hTNFtg n=4, ActβA$^{d/d}$ ColVI-Cre hTNFtg n=5, unpaired t-test**). **P≤0.01. FLS, fibroblast-like synoviocytes; OC, osteoclast; RANKL, receptor activator of nuclear factor κB ligand.

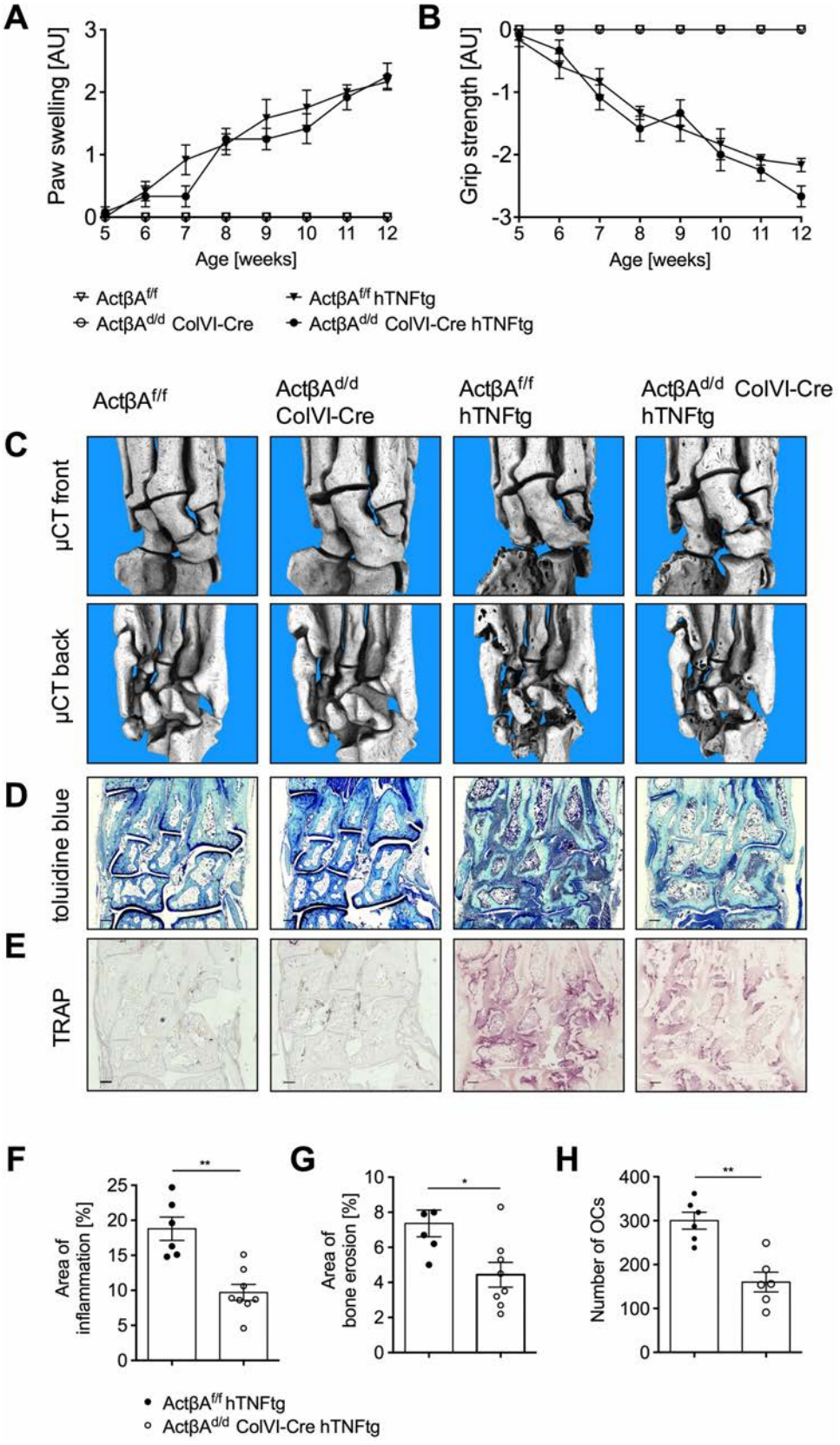

Figure 6 Deficiency of activin A in ColVI-Cre-expressing cells ameliorates disease severity in hTNFtg mice. (A) Paw swelling and (B) grip strength measured in ActβA$^{f/f}$ (n=6), ActβA$^{d/d}$ ColVI-Cre (n=6), ActβA$^{f/f}$ hTNFtg (n=6) and ActβA$^{d/d}$ ColVI-Cre hTNFtg (n=6) mice over 5–12 weeks. All data are means±SEM (two-way analysis of variance, Bonferroni's multiple comparison test). (C) Representative images of μCT analysis from the front and back (n=4, each genotype), (D) Toluidine blue-stained sections (scale bar 200 μm) and (E) TRAP-stained sections (scale bar 200 μm) from the hind paws of 12-week-old ActβA$^{f/f}$ (n=3), ActβA$^{d/d}$ ColVI-Cre (n=3), ActβA$^{f/f}$ hTNFtg (n=6) and ActβA$^{d/d}$ ColVI-Cre hTNFtg (n=6–8) mice. Quantitative histomorphometric assessment of (F) synovial pannus formation, (G) bone erosion and (H) number of OCs in tarsal joints. All data are means±SEM (Mann-Whitney U test). *P≤0.05, **P≤0.01. μCT, micro-CT; OC, osteoclast.

plays an important role in hTNFα-driven RA-like pathogenesis in mice.

Activin A enhances expression of key differentiation genes via Smad2-dependent nuclear translocation of NFATc1

To shed light on the mechanisms by which activin A exerts its effect on OC formation, we focused on potential signalling pathways and the expression of the key markers for OC differentiation.

Western Blot analysis of P-JNK, P-ERK and P-p38 demonstrated that RANKL, but not activin A, induces MAP kinase activation (figure 7A), phosphorylation of NF-κB and degradation of IκBα (figure 7B), indicating that the stimulatory effect of activin A on osteoclastogenesis did not originate from increased MAP kinase and nuclear factor kappa B (NF-κB) signalling. In consideration of the fact that TGF-β-like proteins activate the Smad2/3 signalling pathway, the ability of activin A to activate Smad2 in primary BMDMs was verified. Activin A induced Smad2 phosphorylation after 30 min, while RANKL had no effect on Smad2 activation (figure 7C). Of note, Smad2 phosphorylation was still present on stimulation with activin A for 3 and 4 days (figure 7D). Due to the enhanced osteoclastogenesis associated with activated Smad2 signalling by activin A, it was speculated that activated Smad2 may interact with the key transcription factor in OC differentiation NFATc1, which then could enhance the expression of a set of genes that are essential for osteoclastogenesis. To demonstrate that Smad2 and NFATc1 interact on stimulation with RANKL and activin A, coimmunoprecipitation experiments were performed. As shown in figure 7E, NFATc1 was clearly bound to P-Smad2 on costimulation of BMDMs with RANKL and activin A. Subsequent analyses of the key differentiation makers integrin αv, integrin β3, DC-STAMP, NFATc1 and cathepsin K showed that RANKL induced the expression of all these differentiation markers, whereas activin A alone did not induce any of these marker genes. However, activin A was clearly able to enhance the RANKL-induced expression of all differentiation markers during osteoclastogenesis (figure 7F).

In order to verify whether the stimulatory effect of activin A on OC formation exclusively depends on receptor-mediated Smad2 signalling, a specific ALK4/5/7 inhibitor (SB431542) was used during OC differentiation. TRAP staining showed that treatment with ALK4/5/7 inhibitor strongly reduced the activin A-enhanced OC formation (figure 7G). Quantification revealed a strong inhibition of osteoclastogenesis either on stimulation with RANKL plus activin A compared with untreated differentiation controls, confirming a receptor-mediated and Smad-dependent impact of activin A on OC formation. Finally, recovery experiments showed that the inhibitor was not toxic and OC differentiation increased again after withdrawal of the inhibitor (figure 7H). However, OC differentiation was already reduced on stimulation with RANKL alone, assuming additional autocrine factors. Beside activin A, OC precursors also express myostatin, which similar to activin promotes OC differentiation by activating the Smad pathway. Since both myostatin and activin A signal through a combination of Acvr2b and/or Acvr2a and ALK4/5 or ALK4/7 activating the Smad pathway, the ALK inhibitor will block both the activin and the myostatin-induced Smad2 activation, which very likely cause the inhibition of basal RANKL-induced OC development.

Together, these results led to the conclusion that the stimulating effect of activin A on osteoclastogenesis is mediated by the interaction of P-Smad2 and the key transcription factor of osteoclastogenesis NFATc1, thereby enhancing the expression of osteoclastic genes and subsequently OC development.

DISCUSSION

RA is common type of inflammatory arthritis characterised by chronic inflammation, culminating in joint damage due to cartilage and bone destruction. Identification of molecules and associated signalling pathways involved in these destructive processes has become increasingly important. A family of proteins that affects inflammatory processes and bone metabolism is the TGF-β superfamily, under which activin A has found to be markedly elevated in serum, synovial fluid and synovial tissue of patients with RA[25–28] and to stimulate the production of inflammatory mediators.[29 30] Moreover, activin A is expressed in cells that are involved in bone metabolism such as BM cells, macrophages, OCs and OBs,[26 29 38–42] as well as in FLS that are abundantly present in the inflamed synovium of patients with RA,[1 26 28 43] all suggesting a role of activin A in inflammatory bone remodelling. In accordance with this, we observed highly elevated levels of activin A in synovial tissues obtained from patients with RA as well as from arthritic mice, suggesting that the inflammatory environment leads to an upregulation of activin A expression. Interestingly, in patients with RA, activin A-expressing cells were mostly found in the synovial sublining layer, indicating that within the synovial tissue, FLS are the main producer of activin A.

In more detail, stimulation of FLS and BMDMs with proinflammatory cytokines showed that activin A secretion by FLS was increased on stimulation with proinflammatory cytokines, while activin A secretion by BMDMs was not affected. In addition, activin A secretion by FLS was considerably higher than by BMDMs and even still higher in arthritic FLS compared with WT FLS.

Intriguingly, IL-17A strongly enhanced activin A secretion in hTNFtg but not in WT FLS. In this regard, former studies could show that IL-17-RA and IL-17RC are overexpressed in peripheral whole blood obtained from patients with RA, and these receptors are also highly expressed in the synovium of patients with RA, as well as by RA-FLS,[44 45] probably explaining why arthritic FLS were more susceptible to IL-17A stimulation than WT FLS.

Altogether, these data strongly support the hypothesis that FLS-derived activin A may be an important regulator of inflammatory arthritis.

Since inflammation-induced bone destruction is one main feature of RA, we evaluated the contribution of activin A to OC differentiation in vitro and bone erosion in vivo. Indeed, activin A strongly increased RANKL-induced OC formation and resorptive activity in vitro, which is in line with others.[14–19] Moreover, enhancement of osteoclastogenesis was mediated by the induction of Smad2 phosphorylation, which is consistent with studies from Murase *et al* and Kajita *et al*.[15 19] However, this study showed for the first time that stimulation of primary BMDMs with activin A and RANKL led to an interaction of phospho-Smad2 with NFATc1, the key transcription factor of OC differentiation. Taking additionally the activin A-induced increase of OC differentiation and activity markers into account, we hypothesise that interaction of NFATc1 with phospho-Smad2 led to an increased translocation of NFATc1 into the nucleus, thereby enhancing the expression of OC-specific genes. Since this interaction has also been observed in myostatin-enhanced osteoclastogenesis, it seems to be a common mechanism by which members of the TGF-β family can regulate OC formation.[37]

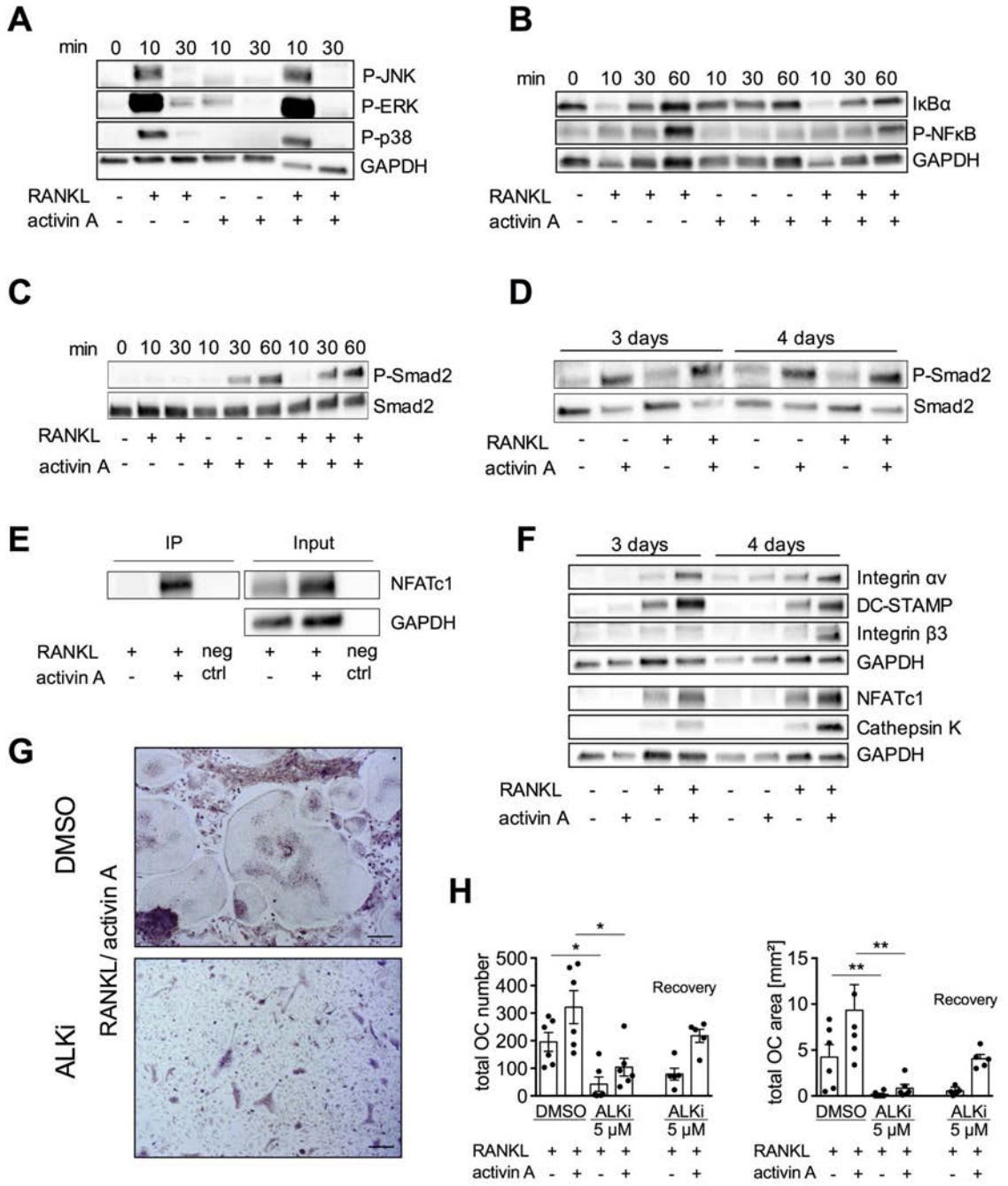

Figure 7 Activin A enhances RANKL-mediated OC formation and expression of OC differentiation markers by activation of Smad2. (A) Representative immunoblots of p-JNK, P-ERK and P-p38 of WT BMDMs stimulated with RANKL (50 ng/mL), activin A (30 ng/mL) or RANKL plus activin A in the presence of M-CSF (30 ng/mL) for 10 and 30 min. Unstimulated BMDMs served as control and glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) as loading control (n=3). (B) Representative immunoblots of IκBα and NF-κB on stimulation of WT BMDMs with RANKL (50 ng/mL), activin A (30 ng/mL) or RANKL plus activin A for 10, 30 and 60 min always in the presence of M-CSF (30 ng/mL). Unstimulated BMDMs were used as control and GAPDH as loading control (n=3). (C) Representative immunoblots of P-Smad2 and Smad2 after stimulation of BMDMs with RANKL (50 ng/mL), activin A (30 ng/mL) or RANKL plus activin A for 10, 30 and 60 min (n=3) and (D) after 3 and 4 days (n=3). (E) Representative immunoblots of coimmunoprecipitation analyses. P-Smad2, coupled to protein G-labelled Dynabeads, was incubated equal amounts of protein lysates of BMDMs stimulated with RANKL, or RANKL plus activin A for 2 days was transferred to the antibody-bead complex. After incubation, pulldown was performed followed by western blot analysis against NFATc1 of the coimmunoprecipitated protein and equal amounts of the total protein lysates (input). Lysis buffer was used as negative control (neg ctrl) (n=3). (F) representative immunoblots of the OC differentiation markers integrin αv, integrin β3, DC-STAMP, NFATc1 and cathepsin K on stimulation of WT BMDMs with activin A (30 ng/mL), RANKL (50 ng/mL) or RANKL plus activin A for 3 and 4 days always in the presence of M-CSF (30 ng/mL). Unstimulated BMDMs served as control. GAPDH was used as loading control (n=3). (G) Representative images of TRAP staining after 4 days of differentiation. (H) Quantification of OC number and area on treatment of BMDMs with an ALK4/5/7 inhibitor together with RANKL (50 ng/mL) or RANKL plus activin A (30 ng/mL) for 4 days. For recovery experiments, ALK4/5/7 inhibitor was removed and BMDMs were stimulated with RANKL or RANKL plus activin A for a further 2 days. The ALK4/5/7 inhibitor was diluted in DMSO; therefore, DMSO-treated BMDMs served as control. All data are means±SEM (n=5–6, Mann-Whitney U test). All experiments were performed in the presence of M-CSF (30 ng/mL). *P≤0.05, **P≤0.01. OC, osteoclast; RANKL, receptor activator of nuclear factor κB ligand.

Therefore, the interaction domains of phospho-Smad2 and NFATc1 might by interesting targets for the treatment of diseases with OC-mediated bone loss such as RA or osteoporosis. Finally, the fact that activin A also stimulates human OC differentiation further confirms the importance of activin A as an osteoclastogenic factor.

Based on the observation that activin A is upregulated within an inflammatory environment, we wondered whether the loss of activin A protects against bone damage during inflammatory destructive arthritis. Since FLS and macrophages appeared to be the main activin A-expressing cells within the inflamed synovium, we assessed the effect of activin A deficiency in ColVI-Cre-expressing and LysM-Cre-expressing cells on disease severity in the hTNFtg mouse model of arthritis.

The deficiency of activin A in myeloid cells (LysM-Cre) does not significantly affect inflammation, bone erosion and OC numbers in hind paws of hTNFtg mice, although a tendency towards a reduced inflammation was observed. The missing impact of this cell-specific deletion on OC formation and bone erosion is probably due to the very small amounts of activin A produced by myeloid cells compared with FLS (pg vs ng range). This assumption is further supported by the fact that the lack of autocrine activin A in BM cells, BMDMs, pOCs and OCs from Actβ$A^{d/d}$ LysM-Cre or Actβ$A^{d/d}$ LysM-Cre hTNFtg mice does not affect OC development.

In contrast, although the clinical scores were not affected by the loss of activin A, histomorphometric analyses clearly showed that deficiency of activin A in ColVI-Cre-expressing cells such as FLS strongly reduces OC formation (about 47%) in the hTNFtg model and thereby attenuating bone erosion (about 40%). These results highly suggest that paracrine, but not autocrine activin A, is important for inflammation-mediated bone erosion by directly acting on OC precursors and thereby regulating OC differentiation and activity. Results from coculture experiments demonstrating that the cell-specific deletion of activin A in FLS leads to less OC differentiation further substantiate the major role of paracrine activin A. However, additional in vivo studies would be helpful to further clarify the relative contribution of FLS-derived myostatin and activin A to OC differentiation in RA.

Moreover, serum levels of proinflammatory cytokines were highly reduced (about 50%), and pannus formation, which also reflects the degree of joint inflammation, was strongly reduced about 48% as well on lack of activin A, both confirming an anti-inflammatory effect of activin A deletion. In this context, activin A was shown to induce cell proliferation of RA-FLS,[26] which potentially may contribute to the formation of pannus tissue within the inflammatory environment. Moreover, it is well known that FLS within the pannus tissue are activated by their proinflammatory environment, resulting in an aggressive phenotype that is maintained even in the absence of inflammatory stimuli.[3 4 46] In this context, activated FLS produce inflammatory cytokines, chemokines, growth factors and degrading enzymes, thereby regulating joint inflammation and destruction. The fact that FLS appeared the be the main producers of activin A in the joint and that the lack of FLS-derived activin A exceedingly ameliorates disease severity confirm the prominent role of FLS in arthritis.

In conclusion, we could show for the first time that activin A significantly regulates disease severity in a mouse model of chronic arthritis. Deletion of paracrine but not autocrine activin A reduces OC formation and bone destruction and most interestingly reduces also joint inflammation in our arthritis model. Thus, inhibition of activin A may be a promising treatment option for arthritis and other diseases associated with inflammatory bone loss.

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Contributors BD and TP conceived the study. VW-K performed most of the experiments. EW, MF, JI, CW and PP were involved in the mouse studies, including histological and micro-CT analyses. DB and JR performed the fluorescence stainings. JR and MD performed the human osteoclast experiments. AK-P and CW participated in data discussion and interpretation. VW-K, BD and TP drafted the manuscript. All authors read, commented on and approved the final manuscript. BD acts as guarantor.

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Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, conduct, reporting or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval Human synovial tissue samples were obtained from patients with a clinical diagnosis of rheumatoid arthritis after informed consent prior to surgery (ethics committee of the Medical Faculty of the Westfalian Wilhelms-University Muenster (2009-447-f-S)). Animal experimental protocols were approved by the Animal Welfare and Ethical Review Committee 'Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen' (84.02.04.2017.A112).

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Data availability statement All data relevant to the study are included in the article. All study data are included in the article and supporting information.

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CLINICAL SCIENCE

Epithelial HIF2α expression induces intestinal barrier dysfunction and exacerbation of arthritis

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ABSTRACT

Objective To investigate how the mucosal barrier in the intestine influences the development of arthritis, considering that metabolic changes in the intestinal epithelium influence its barrier function.

Methods Intestinal hypoxia inducible factor (HIF)-2α expression was assessed before, at onset and during experimental arthritis and human rheumatoid arthritis (RA). Intestinal epithelial cell-specific HIF2α conditional knock-out mice were generated (HIF2α$^{\Delta IEC}$) and subjected to collagen-induced arthritis. Clinical and histological courses of arthritis were recorded; T-cell and B-cell subsets were analysed in the gut and secondary lymphatic organs; and intestinal epithelial cells were subjected to molecular mRNA sequencing in HIF2α$^{\Delta IEC}$ and littermate control mice. The gut intestinal HIF2α target genes were delineated by chromatin immunoprecipitation and luciferase experiments. Furthermore, pharmacological HIF2α inhibitor PT2977 was used for inhibition of arthritis.

Results Intestinal HIF2α expression peaked at onset of experimental arthritis and RA. Conditionally, deletion of HIF2α in gut epithelial cells inhibited arthritis and was associated with improved intestinal barrier function and less intestinal and lymphatic Th1 and Th17 activation. Mechanistically, HIF2α induced the transcription of the pore-forming claudin (CLDN)-15, which inhibits intestinal barrier integrity. Furthermore, treatment with HIF2α inhibitor decreased claudin-15 expression in epithelial cells and inhibited arthritis.

Conclusion These findings show that the HIF2α–CLDN15 axis is critical for the breakdown of intestinal barrier function at onset of arthritis, highlighting the functional link between intestinal homeostasis and arthritis.

WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT?

⇒ Intestinal inflammation and arthritis are closely linked, but mechanistic studies explaining the interaction of these two organs are scarce to date.

WHAT DOES THIS STUDY ADD?

⇒ The study shows that metabolic changes occur in the intestinal epithelium at onset of arthritis that are associated with increased expression of hypoxia-inducible factor (HIF)-2 alpha and the breakdown of intestinal barrier function.

⇒ Inhibition of HIF-2 alpha expression in the intestinal epithelium protects intestinal barrier function and inhibits the onset of arthritis.

HOW MIGHT THIS IMPACT ON CLINICAL PRACTICE OR FUTURE DEVELOPMENTS?

⇒ Interventions that restore or protect intestinal barrier function, such as HIF blockade, may be therapeutically used to inhibit the onset of arthritis.

INTRODUCTION

The intestinal epithelium is covering around 400 m^2 of surface area with a single layer of cells. It acts as physical barrier, tightly controlling the absorption of nutrients while maintaining an effective defence against intestinal microbiota. The epithelium can maintain its selective barrier function through the formation of complex protein networks that mechanically link adjacent cells and seal the intercellular space.[1] These intestinal barriers are subject to rapid turnover and depend on the dynamic assembly and disassembly of intercellular tight junctions (TJs) that demand substantial energy.[2]

Impaired barrier function increases gut permeability, bacterial translocation and systemic inflammation.[3] Furthermore, clinical observations suggest that intestinal barrier changes are associated with arthritis.[4] Hence, it has been shown that TJs, such as the complex Zo-1–occludin, are disassembled in arthritis.[5] Of note, such intestinal barrier changes are highly dependent on oxygen tensions.[6] On hypoxia, tissue is responding with rapid induction of the expression of hypoxia-inducible factors (HIFs). HIF1 and HIF2 are formed by heterodimerisation of their respective alpha subunit with HIF-1β. During normoxia, the alpha subunit of HIF family is continuously hydroxylated by prolyl hydroxylase domain containing enzymes (PHDs), which in turn recruits the von Hippel-Lindau protein (pVHL) for HIFα ubiquitination, mediating its proteasomal degradation.[7] Under hypoxic condition, PHDs are inhibited, resulting in HIFα stabilisation and dimerisation with the constitutively expressed HIF-1β. The heterodimerised HIF1 or HIF2 complex binds to the hypoxia-responsive element (HRE) region and activates expression of target genes involved

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HIFs contribute to the expression of genes encoding TJs, such as claudin (CLDN)-1, allowing the regulation of barrier-adaptive responses within the mucosa.[8 9] With its specific expression in mucosal epithelia and being a major node in intestinal iron absorption,[10] HIF2α is particularly interesting for gut integrity. We therefore hypothesised that the expression of HIF2α in the intestinal epithelium does not only affect barrier function but also affects the development of arthritis. To address this question, we specifically deleted HIF2α in intestinal epithelial cells. We show that intestinal HIF2α expression contributes to the onset and severity of arthritis, indicating that the functional properties of intestinal epithelial cells, that is, HIF2α activation, are critical for the development of arthritis, supporting the concept of a gut–joint axis.

METHODS

Human sample

Serial ileal mucosal biopsies from five healthy controls (undergoing ileocolonoscopy for screening purpose) (four women), five consecutive new-onset treatment-naïve RA active (disease activity score (DAS)28 score >3.2) patients (fulfilling the American College of Rheumatology–European League Against Rheumatism 2010 criteria[11] (all women)) and five patients with pre-RA (defined as having anti-citrullinated protein antibody (ACPA) assessed by testing positive for anticyclic citrullinated peptide 2 IgG autoantibody reactivity but no past or present evidence of joint swelling according to phases C and D of the RA-at risk definition[12] (three women) were analysed (online supplemental table 1). None of these patients had present or past symptoms of a chronic inflammatory bowel disease or coeliac disease. Paraffin-embedded tissues were prepared from all patients to allow histological assessments. Staining for HIF2α (with anti-human HIF2α from Abcam) was performed on paraffin-embedded sections, and the sections were treated with rhodamine red anti-mouse, plus RNasin (200 ng/mL) and counterstained using 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies). Confocal microscopy was used to examine fluorescence staining. The number of HIF2α-expressing cells was determined by counting immunoreactive cells on photomicrographs obtained from three random high-power microscopic fields (×400 magnification).

Mice

C57BL/6 wild-type (WT) mice (6–8 weeks old) and DBA1/J (8 weeks old) were purchased from Janvier Laboratories. To generate small intestine epithelial-specific Hif2α-deficient mice, *Hif2α*$^{flox/flox}$ mice were crossed with Villin-cre mice. *Hif2α*$^{flox/flox}$ mice and Villin-cre mice were previously described.[11] The mice were bred and maintained on a C57BL/6 background and *Hif2α*$^{flox/flox}$ cre-negative or *Hif2α*$^{+/+}$ cre-positive littermates were used as WT controls. Cre-mediated recombination was genotyped by PCR on tail DNA. Sex-matched and age-matched (8–10 weeks) mice were killed using CO_2 for in vitro and ex vivo experiments. Mice were routinely screened for pathogens according to Federation of European Laboratory Animal Science Associations (FELASA) guidelines.

Collagen-induced arthritis (CIA) model

Mice (8–12 weeks of age) were immunised by subcutaneous injection at the base of tail with 100 µL of 0.25 mg chicken type II collagen (CII) in complete Freunds' adjuvant (CFA) (Sigma), containing 5 mg/mL killed *Mycobacterium tuberculosis* (H37Ra). Twenty-one days after the primary immunisation, the mice were boosted with a secondary immunisation with the same amount of CII emulsified in CFA subcutaneous at the base of the tail proximal to the primary injection site. Next, the clinical scores for each paw were evaluated every other day and scored individually on a scale of 0–4, which results in a maximum score of 16. Each paw is scored as follows: 0, no evidence of erythema and swelling; 1, erythema and mild swelling confined to the tarsals or ankle joint; 2, erythema and mild swelling extending from the ankle to the tarsals; 3, erythema and moderate swelling extending from the ankle to metatarsal joints; and 4, erythema and severe swelling encompass the ankle, foot and digits, or ankylosis of the limb.[13] The CIA model was used on *Hif2α*$^{flox/flox}$ Villin-cre+mice, control littermates, WT DBA/1J mice and C57BL/6 mice.

HIF2α inhibitor animal treatments

Eight weeks C57BL/6 mice were purchased from Janvier and induced for CIA model. Six days after the first immunisation, mice were administered by oral gavage with vehicle or HIF2α inhibitor (PT2977, Medchemexpress, 3.6 mg/kg) every other day (three times a week). HIF2α inhibitor was dissolved in 10% dimethyl sulfoxide (DMSO), 40% PEG (Polyethylene glycol) 4000, 5% Tween (Polysorbate) 80 and 45% saline. The mice were scored every other day after the second immunisation. Intestinal permeability was assessed by oral gavage of fluorescein isothiocyanate (FITC)–dextran 4 kDa on day 36. Mice were sacrificed on day 36 for blood collection and further analyses.

Flow cytometry

Single-cell suspensions were prepared from Peyer's patches (PPs), mesenteric lymph nodes (MLNs) and spleen. Red cells were lysed with ammonium–chloride–potassium buffer. Cells were Fc-blocked (CD16/CD32) and stained with antibodies (online supplemental table 2). Analyses of the expression of cell surface molecules on a single-cell level were performed by flow cytometry with Cytoflex (Beckman Coulter) flow cytometer. Dead cells were detected using a LIVE/DEAD Fixable Violet Dead Cell Stain Kit (L34967, Invitrogen) before cell surface staining. For analysis of Th1 and Th17 cells, phorbol-12-myristate-13-acetate (20 ng/mL), ionomycin (1 µg /mL) and monensin (L+PIM) were added to the single-cell cultures 5 hours before fixation and permeabilisation with the Foxp3/Transcription Factor Staining Buffer Set (00/5523/00, eBioscience) according to the manufacturer's instruction. All flow cytometry experiments were gated on viable, single lymphocytes and data were analysed with CytExpert V.2.4 software.

RNA-seq data analysis

RNA-seq experiments were performed by Novogene Europe (Cambridge Science Park, UK). For each sample, a DNA fragment library is generated using the Illumina TruSeq Stranded mRNA kit. The libraries are sequenced single end with 101 bp length on an Illumina HiSeq 2500 platform. TruSeq sequencing adapter sequences are trimmed from the reads and trimmed reads shorter than 60 bp are discarded (cutadapt V.1.18). Read quality is checked after sequencing and after base trimming (fastqc V.0.11.8). The processed reads are mapped to the mouse reference genome GRCm38 with the GENCODE annotation M23 (equals Ensembl 98) using the splice-aware aligner STAR V.2.6.1c, or each non-overlapping exon mapped reads are counted. The sum of all exons yields the number of reads

per gene (samtools V.1.8 and subread V.1.6.1). For analysis of differential expression, it is assumed that the reads per gene correlate with the amount of mRNA for this gene in the sample. The RNA-seq data reported in this paper have been deposited at GEO database, GSE175907 and GSE176266.

Micro-CT (µCT)

Mouse hind paws were fixed in 4% paraformaldehyde (PFA) for at least 4 hours and stored in 70% ethanol before analyses. µCT imaging was performed using the cone-beam Desktop Micro Computer Tomograph 'µCT 40' by SCANCO Medical AG (Bruettisellen, Switzerland). The settings were optimised for calcified tissue visualisation in murine bones at 55 kVp, with a current of 177 μA, 200 ms integration time for 500 projections per 180° and an isotropic voxel size of 8.6 µm. The three-dimensional modelling of the bone was performed with optimised greyscale thresholds of the operating system Open VMS by SCANCO Medical.

Histology

On day 36 post first immunisation of the CIA model, whole paw joints were harvested and fixed in 4% paraformaldehyde, decalcified in EDTA and then embedded in paraffin. Specimens were longitudinally cut into 5 µm sections, then H&E and tartrate-resistant acid phosphatase stainings were performed. For ileum and colon, samples were fixed in 4% paraformaldehyde and embedded in paraffin, sectioned at 5 µm, and stained with H&E or periodic acid–Schiff (PAS) for light microscopy examination. The H&E slides were assigned a histological score for intestinal inflammation according to the criteria supplied by previous publication.[14]

Fluorescence imaging

Epitopes were retrieved from deparaffinised sections using a heat-induced method. Briefly, sections were alternately bathed in boiling sodium citrate buffer (10 mM sodium citrate, pH 6.0) and Tris–EDTA buffer (10 mM Tris base, 1 mM EDTA, 0.05% Tween-20, pH 9.0). Each bathing step was repeated five times for 2 min each. Sections were washed in distilled water and blocked for 1 hour in phosphate-buffered saline (PBS) supplemented with 5% bovine serum albumin) (BSA) or 2% horse serum. Sections were incubated with primary antibodies overnight at 4°C and with secondary antibodies after an intense washing step for 2 hours at ambient temperature. Consecutive staining was performed to minimise cross-reactivity. The antibodies used are listed in online supplemental table 2. Sections were finally mounted in fluorescence antifade mounting medium with DAPI (Vector). For cell and organoid immunofluorescence (IF) staining, we followed the protocols from previous publications.[15 16] Images were acquired on a BZ-X700-All-in-One Fluorescence Microscope (Keyence) or Carl Zeiss LSM 700 Laser Scan Confocal Microscope (Confocal fluorescence microscopy).

Fluorescent in situ hybridisation (FISH) preparation and analysis

FISH was performed as described before.[17] The paraffin-embedded ileum sections were sunk in xylene for deparaffinisation. Then, the sections were rehydrated in serial dilutions of ethanol. Afterwards, sections were incubated in hybridisation buffer (20 mM Tris–HCl, 0.9 M NaCl and 0.1% sodium dodecyl sulfate (SDS) (pH 7.2)) for 10 min at 50°C. Next, sections were incubated with 100 nM bacterial probes (50–>30: GCTGCCTCCCGTAGGAGT, FITC-conjugated; Sigma) in hybridisation buffer in the dark, for 4 hours at 50°C and washed with 20 mM Tris–HCl, 0.9 M NaCl (pH 7.2). After a second washing step, sections were mounted with VECTASHIELD Antifade Mounting Medium with DAPI. Images were acquired using the Keyence microscope.

Chromatin immunoprecipitation (ChIP)

MC38 cells were cultured under normoxic (21% O_2) or hypoxic (1% O_2) conditions for 8 hours. Next, ChIP experiments were performed with ChIP-IT Express kit (53040, Active Motif) according to the manufacturer's protocol. Five-microgram anti-HIF2α antibody and control IgG antibody was used for the immunoprecipitation. Primer sequence for CLDN15 (*Cldn15*) binding site was described previously (online supplemental table 3). HIF2α binding sites were predicted by JASPAR with the consensus core (A/GCGTG) and primers were designed by Primer-BLAST.

Luciferase reporter assay

HRE regions (2 and 3) of *Cldn15* promoter (online supplemental table 3) were amplified by PCR from genomic DNA extracted from splenocytes in C57BL/6 WT mice and cloned into the pGL4.23 firefly reporter vector (Promega). MC38 cells were cotransfected with luciferase reporter construct, HIF2α TM plasmid and Renilla plasmid using Lipofectamine 3000 (Invitrogen). Transfected cells were cultured under normoxic condition for 24 and 36 hours. Cells were then lysed, and luciferase activity was quantified and normalised to the activity of the cotransfected Renilla reporter gene.

Interleukin (IL)-17A and interferon gamma (IFN-γ) detection via ELISA

Serum IL-17A and IFN-γ levels were measured in mice 36 days after CIA induction by ELISA according to the manufacturer's protocol (88-7371-88 Invitrogen and 88-7314-88 Invitrogen, respectively).

Statistical analysis

Data are expressed as mean±SD. Analysis was performed using two-tailed unpaired Student's t-test for single comparison, or analysis of variance (ANOVA) test for multiple comparisons (one-way or two-way ANOVA followed by Tukey's or Bonferroni's multiple comparisons test, respectively). Kaplan-Meier analysis with log-rank test was used to determine the significance of CIA incidence. All experiments were conducted at least two times. P values of 0.05 were considered significant and are shown as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Graph generation and statistical analyses were performed using the Prism V.8 software (GraphPad, La Jolla, California, USA).

RESULTS

HIF2α expression is increased in gut epithelial cells at onset of murine and human arthritis

To investigate the intestinal expression of HIF2α during arthritis, we examined its expression at protein and mRNA level in the small intestine and the colon before, at the beginning and after the onset of experimental (CIA) arthritis (online supplemental figure 1A). HIF2α expression was exclusively elevated in the small intestine at the onset of arthritis (day 29 post first injection (pfi)). HIF2α mRNA and protein expression returned to the initial state in established arthritis (day 36 pfi, online supplemental figure 1B–D). This spurious induction of intestinal HIF2α expression at the onset of arthritis was strain-independent and observed not

only in DBA1 mice but also in C57BL/6 mice subjected to CIA (online supplemental figure 1E–H). In humans, we also found increased HIF2α protein expression in the ileum of patients with rheumatoid arthritis (RA) at onset of their disease compared with healthy individuals or established RA (figure 1A,B). HIF2α expression was particularly strong in the intestinal epithelium (online supplemental figure 1I,J). Taken together, these results suggest an upregulation of HIF2α in epithelial cells of the small intestine at the onset of arthritis.

HIF2α deficiency in intestinal epithelial cells alleviates arthritis

To understand the role of HIF2α transcription factor in the intestinal epithelium during the development of arthritis, we generated mice with specific knockout of HIF2α in the intestinal epithelium (HIF2α$^{\Delta IEC}$) using the *Villin-cre* driven recombination (online supplemental figure 2A). The depletion efficiency was about 90% for HIF2α protein or mRNA after hypoxic challenge in vitro (online supplemental figure 2B-F) or by induction of CIA in vivo (online supplemental figure 2G). To induce HIF2α expression in vitro, hypoxic chambers were used on intestinal organoid cultures isolated from littermate controls (HIF2α$^{F/F}$) or HIF2α$^{\Delta IEC}$ mice. Morphological features of cell death (represented by dark areas in organoids) were less frequent in HIF2α$^{\Delta IEC}$ than in control cultures after 8 hours of hypoxia (1% oxygen) (online supplemental figure 2D,E). These results suggest that HIF2α may promote epithelial cell death under stress conditions, such as hypoxia.

When CIA was induced in HIF2α$^{\Delta IEC}$ mice and littermate controls, intestinal HIF2α deficiency alleviated arthritis, as shown by a delayed disease onset and reduced arthritis scores in HIF2α$^{\Delta IEC}$ mice compared with controls (figure 1C). Moreover, as shown in figure 1D, HIF2α$^{\Delta IEC}$ mice had a significantly lower incidence of arthritis compared with littermate controls. Accordingly, HIF2α$^{\Delta IEC}$ mice presented a lower paw thickness, synovial inflammation, bone erosion and number of osteoclasts (figure 2E–G), confirming attenuated arthritis in HIF2α$^{\Delta IEC}$ mice.

To further characterise the effects of intestinal HIF2α depletion on arthritis, we analysed Th1 cells (IFN-γ+CD4+), Th17 cells (IL-17A+CD4+), regulatory T (Treg) cells (CD25+Foxp3+CD4+) and IgA+ B cells (CD19+B220+IgA+) in the spleen, MLNs and PPs from control and HIF2α$^{\Delta IEC}$ mice with and without CIA. While no difference in IgA+ B cells was found, Th1 and Th17 cells were decreased and Treg cells increased in PPs, MLN and spleen from arthritic HIF2α$^{\Delta IEC}$ mice compared with littermate controls (figure 1I–K and online supplemental figure 3A-D). In the absence of arthritis, no significant difference in the number of lymphocytes was found between HIF2α$^{\Delta IEC}$ and control mice (online supplemental figure 3E-G). In addition, we measured IFN-γ and IL-17A levels in the serum of littermate controls and HIF2α$^{\Delta IEC}$ mice at day 36 pfi showing decreased levels in HIF2α$^{\Delta IEC}$ mice (figure 1H). Thus, loss of HIF2α in intestinal epithelial cells alleviated arthritis by decreasing Th1 and Th17 cell activation.

Intestinal HIF2α deletion ameliorates intestinal inflammation and bacterial invasion at the onset of arthritis

We further explored the changes in the small intestine at the onset of arthritis. H&E staining revealed significant loss of epithelial architecture with infiltration of inflammatory cells in control compared with HIF2α$^{\Delta IEC}$ mice. In accordance, neutrophil infiltration, as shown by myeloperoxidase IF, was higher in control than in HIF2α$^{\Delta IEC}$ mice (figure 2A,D,E). The intestinal epithelium is covered by a thick mucus layer, which contributes to the intestinal barrier. Thus, we checked mucus-producing goblet cells in the small intestine by PAS staining. Although a slight increase of goblet cells was detected on CIA, no difference in goblet cell number was observed between HIF2α$^{\Delta IEC}$ and control mice (figure 2A,F). CIA HIF2α$^{\Delta IEC}$ mice presented decreased mRNA expression level of proinflammatory IL-12 and the neutrophil chemoattractant CXCL1, while the mRNA level of the pro-resolving cytokine IL-10 was strongly elevated in the intestine (figure 2B,C). These findings confirm reduced inflammation and preserved intestinal function in arthritic mice on HIF2α deletion.

As Th1 and Th17 priming requires the interaction with dendritic cells (DCs), we also investigated DCs in the gut of HIF2α$^{\Delta IEC}$ and control mice induced for arthritis. MHCII+, CD103+, CD11b+ DCs were diminished in HIF2α$^{\Delta IEC}$ mice, suggesting less pronounced T-cell activation on HIF2α deletion (figure 2G,H). DCs may be activated by bacterial invasion due to impaired intestinal barrier function in arthritis. Therefore, bacterial invasion was assessed by fluorescence in situ hybridisation (FISH) with the bacterial probe EUB 338.[18] Significantly less bacterial invasion was observed in arthritic HIF2α$^{\Delta IEC}$ mice compared with littermate controls (figure 2I,J), indicating that gut epithelial barrier dysfunction by HIF2α promotes bacterial invasion and immune stimulation.

Reduced CLDN15 expression after loss of HIF2α in intestinal epithelial cells

To determine the HIF2α-mediated pathways in gut epithelial cells, we performed an unbiased analysis of HIF2α-dependent genes by RNA sequencing. Small intestine epithelial cells (IECs) were isolated from naïve and CIA-challenged (36 days pfi) HIF2α$^{\Delta IEC}$ and control mice. Before sequencing, HIF2α deletion efficiency was confirmed by qPCR in IECs (online supplemental figure 2F). Only few genes were differentially expressed in steady state when comparing control with HIF2α-deficient epithelial cells (figure 3A,B). However, in arthritic conditions, 9561 differentially expressed genes (DEGs) were detected (figure 3B). Among these genes, 5113 (18.7%) were upregulated and 4448 (16.2%) were downregulated in HIF2α$^{\Delta IEC}$ IECs. Using Kyoto Encyclopaedia of Genes and Genomes pathway enrichment analysis, cell death-related pathway, TJs, HIF signalling pathway, and T-cell or B-cell immune responses were found to be altered on HIF2α depletion (figure 3C). In addition, expression of specific TJs was altered in the sequencing analysis (figure 3D).

As a next step, we analysed how HIF2α expression in epithelial cells regulates gut barrier function during arthritis. Therefore, FITC–dextran was orally administered in naïve and CIA HIF2α$^{\Delta IEC}$ and control mice and measured afterwards in the serum of mice as an indicator for paracellular transport and mucosal barrier dysfunction. At day 36 pfi, gut permeability was increased in arthritic control mice, while HIF2α$^{\Delta IEC}$ mice were partially protected (figure 3E). This observation may reflect the regulation of paracellular transport by HIF2α in epithelial cells after CIA induction, likely through the regulation of TJs. To test this hypothesis, we performed a targeted array analysis of transmembrane cell–cell adhesion molecules. Functional TJs are formed when CLDNs interact with other transmembrane proteins, cytosolic scaffold proteins and the actin cytoskeleton. We first quantified the expression levels of all CLDN family genes in IECs. Only *Cldn7*, *15*, *3*, *4* and *2* were expressed in IECs (online supplemental figure 4A). This was consistent with

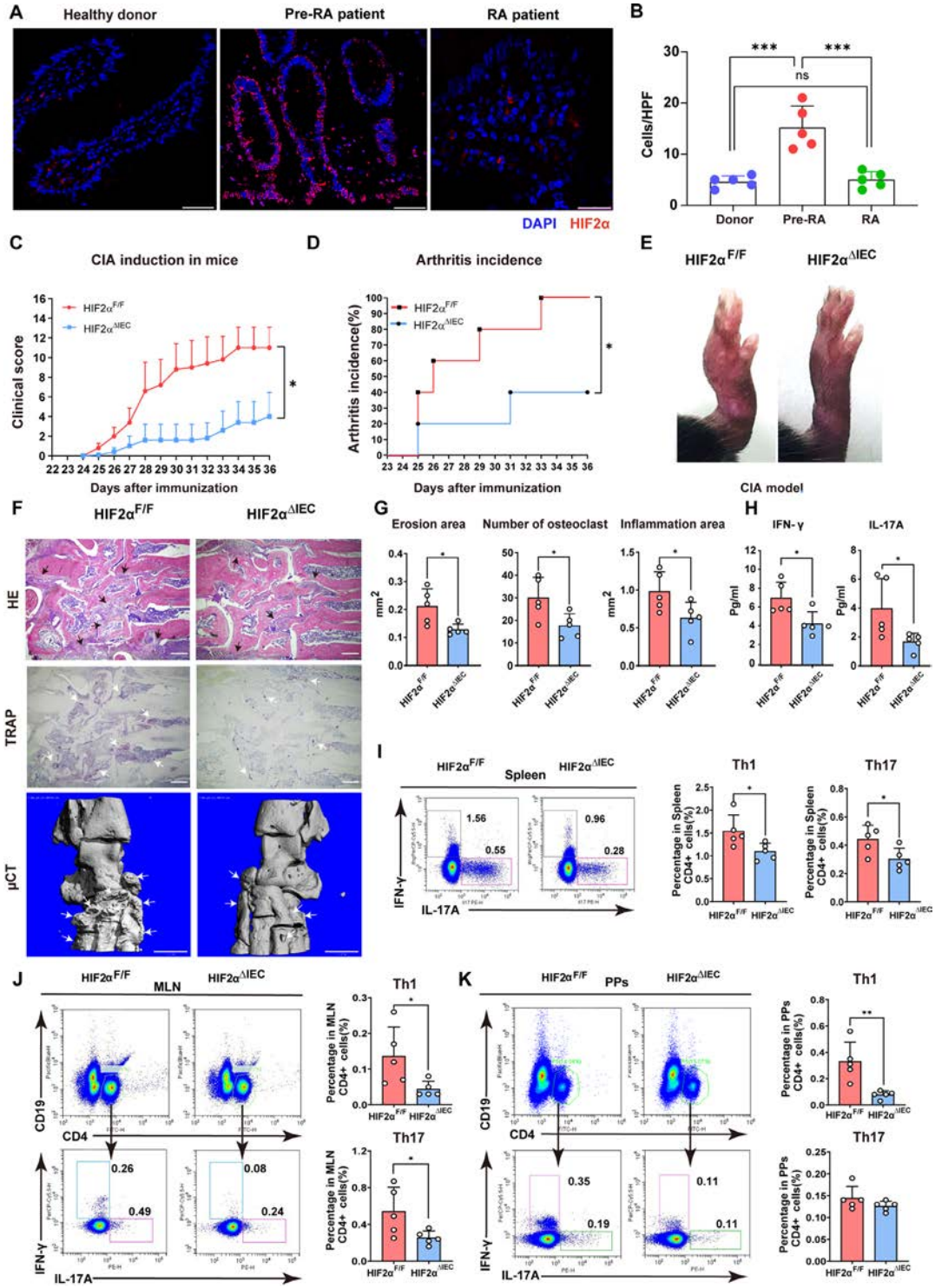

Figure 1 Specific deletion of HIF2α in intestinal epithelial cells alleviates CIA. (A,B) IF staining (A) and quantification (B) for HIF2α (red) and DAPI (blue) in the ileum and in ileal epithelial cells of healthy individuals, patients pre-RA and patients with RA. Scale bar, 25 µm. (C,D) Clinical score (C) and arthritis incidence (D) in mice with HIF2α deletion in small intestine epithelial cells (HIF2α$^{\triangle IEC}$) compared with littermate controls (HIF2α$^{F/F}$) after induction of CIA (n=5). (E) Representative pictures of HIF2α$^{F/F}$ and HIF2α$^{\triangle IEC}$ hind paw at day 36 pfi. (F,G) Representative images of H&E staining, TRAP staining and µCT scans (F) and quantification (G) of erosion area, osteoclast number and inflammation area in the hind paws of CIA (day 36 pfi) HIF2α$^{F/F}$ and HIF2α$^{\triangle IEC}$ mice (n=5). Arrows indicate inflammation (H&E) and bone erosion (TRAP and µCT). H&E and TRAP scale bars, 50 µm. µCT scale bar, 1 mm. (H)IFN-γ, IL-17A serum levels in CIA mice at 36 days post immunisation. (I–K) Representative fluorescence-activated cell sorting (FACS) plots and quantification of Th1 cells (IFN-γ+CD4+) and Th17 cells (IL-17A+CD4+) in the spleen (I), MLNs (J), and PPs (K) of CIA (day 36 pfi) HIF2α$^{F/F}$ and HIF2α$^{\triangle IEC}$ mice (n=5). Data are representative of three independent experiments. Symbols represent individual mice. Data are shown as mean±SD. Statistical significance was determined by Kaplan-Meier analysis with log-rank test* (D) or two-tailed unpaired Student's t-test*,** for single comparisons (C,G–K). *P<0.05, **P<0.01, ***P<0.001. µCT, micro-CT; CIA, collagen-induced arthritis; IF, immunofluorescence; IFN-γ, interferon gamma; IL, interleukin; MLN, mesenteric lymph node; ns, not significant; pfi, post first immunisation; PP, Peyer's patch; RA, rheumatoid arthritis; TRAP, tartrate-resistant acid phosphatase.

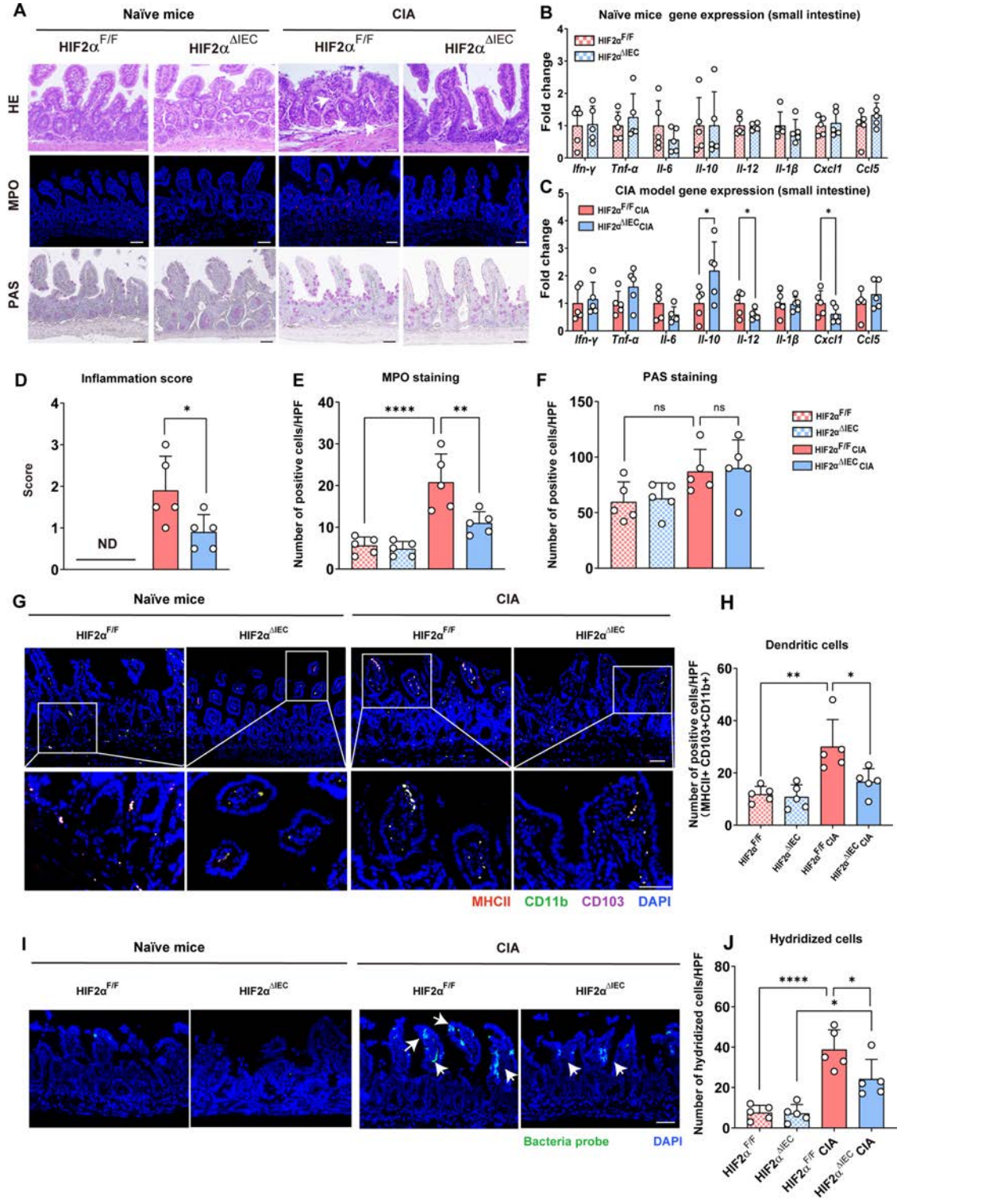

Figure 2 Intestine-specific HIF2α deletion ameliorates inflammation and bacterial load in the intestine. (A) representative images of H&E, MPO and PAS staining in ileum from CIA HIF2α$^{F/F}$ and HIF2α$^{\triangle IEC}$ mice compared with naïve mice. Arrows indicate inflammation area (H&E). Scale bar, 50 µm. (B,C) mRNA expression of *Ifnγ*, *Tnfα*, *Il6*, *Il10*, *Il12*, *Il1β*, *Cxcl1* and *Ccl5* in small IECs from naïve HIF2α$^{F/F}$ and HIF2α$^{\triangle IEC}$ mice (n=5) (B) and mice induced with CIA (n=5) (C). (D–F) Quantification of inflammation score (D), MPO-positive (E) and PAS-positive cells (F) in ileum sections from CIA HIF2α$^{F/F}$ and HIF2α$^{\triangle IEC}$ mice compared with naïve mice (n=5). (G,H) Representative pictures of IF staining for major histocompatibility complex class II (MHCII) (red), CD11b (green), CD103 (purple) and DAPI (blue) (G) and quantification of dendritic cells (H) in the ileum of HIF2α$^{F/F}$ and HIF2α$^{\triangle IEC}$ mice with and without CIA (n=5). Scale bar, 50 µm. (I,J) Representative pictures (I) and quantification (J) of FISH on tissue sections from ileum of HIF2α$^{F/F}$ and HIF2α$^{\triangle IEC}$ mice with and without CIA using the bacteria-specific probe EUB 338-FITC (green) and DAPI (blue) for visualisation of nuclei. Scale bar, 50 µm. Symbols represent individual mice. Data are representative of three independent experiments. Data are shown as mean±SD. Statistical significance was determined by two-tailed unpaired Student's t-test (C) for single comparisons* and one-way analysis of variance (D–F,H) for multiple comparisons.*,**,**** *P<0.05, **P<0.01, ****P<0.0001. CIA, collagen-induced arthritis; FISH, fluorescence in situ hybridisation; IEC, intestine epithelial cell; IF, immunofluorescence; IL, interleukin; MPO, myeloperoxidase; ns, not significant; PAS, periodic acid–Schiff.

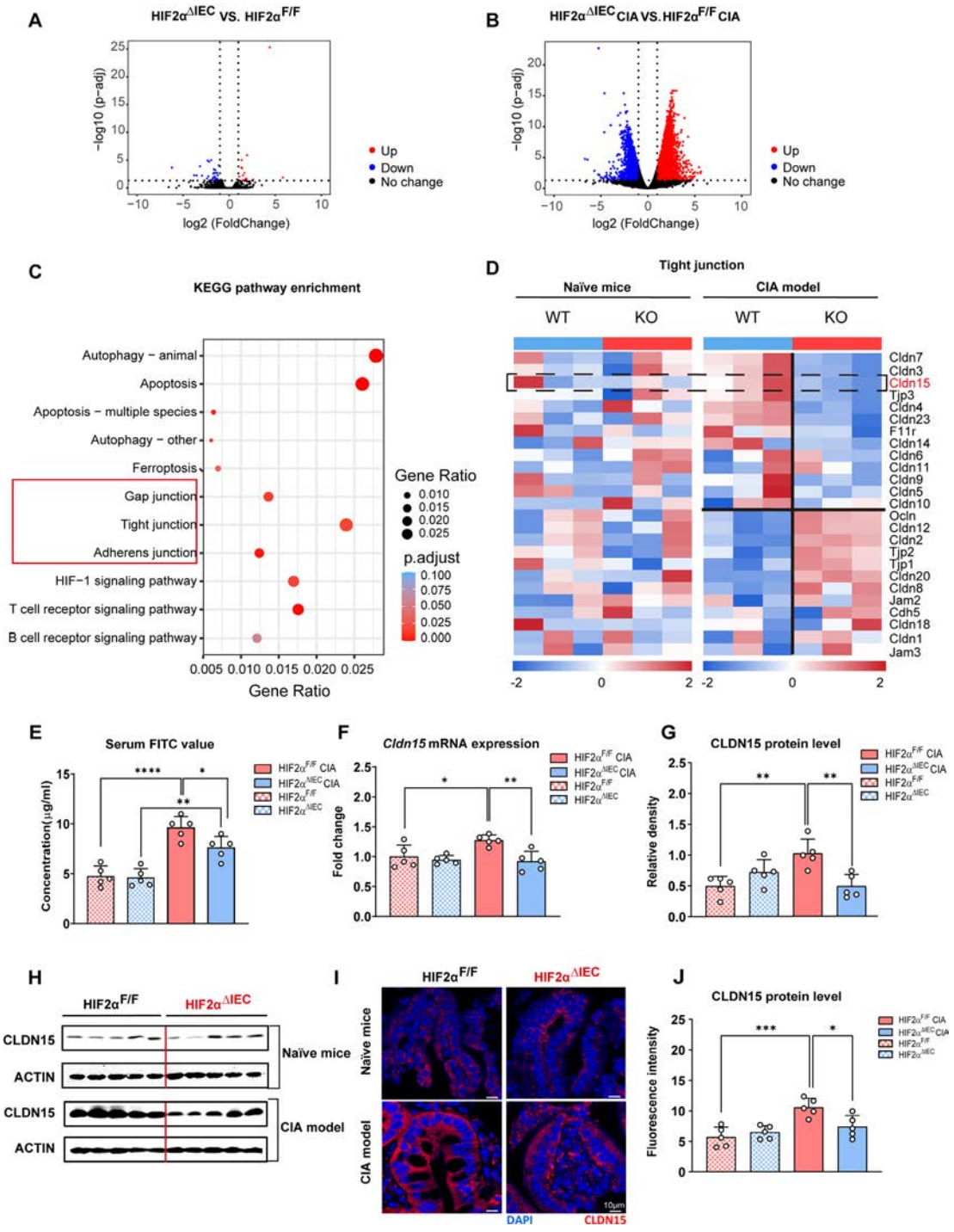

Figure 3 Intestine-specific HIF2α deficiency improves epithelial barrier function and diminishes claudin-15 expression in arthritis. (A,B) Volcano plot displaying DEGs in IECs from HIF2α$^{\triangle IEC}$ mice compared with HIF2α$^{F/F}$ control mice in naïve state (n=3) (A) and in the CIA model at day 36 pfi (n=3) (B). Significantly upregulated genes are depicted in red; downregulated genes are shown in blue. (C) KEGG pathway enrichment analysis of DEGs in IECs from HIF2α$^{\triangle IEC}$ CIA mice as compared with HIF2α$^{F/F}$ CIA mice. Enriched pathways are presented in a bubble plot. The size of the bubbles is proportional to the gene count enriched in the respective pathway, and the colour of the bubbles represents the enrichment significance. (D) Heatmap and hierarchical clustering of tight junction-related DEGs, comparing HIF2α$^{\triangle IEC}$ and HIF2α$^{F/F}$ IECs in naïve state (left) and in the CIA model (right). (E) Spectrophotometric fluorescein in the serum of HIF2α$^{\triangle IEC}$ and HIF2α$^{F/F}$ mice with and without CIA that were labelled with isothiocyanate–dextran (FITC–dextran) 4 hours post oral gavage (n=5). (F) mRNA expression of *Cldn15* in IECs from HIF2α$^{\triangle IEC}$ and HIF2α$^{F/F}$ mice with and without CIA (n=5). (G,H) Western blot quantification (G) and blot (H) of CLDN15 and actin protein in IEC lysates from HIF2α$^{\triangle IEC}$ and HIF2α$^{F/F}$ mice with and without CIA (n=5). (I,J) Representative images of IF staining for CLDN15 (red) and DAPI (blue) (I) and quantification of CLDN15 fluorescence intensity (J) in the ileum of HIF2α$^{\triangle IEC}$ and HIF2α$^{F/F}$ mice with and without CIA (n=5). Data are representative of three independent experiments. Symbols represent individual mice. Data are shown as mean±SD. Statistical significance was determined by one-way analysis of variance (E–G,J) for multiple comparisons. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. CIA, collagen-induced arthritis; DEG, differentially expressed gene; FITC, fluorescein isothiocyanate; IEC, intestinal epithelial cell; IF, immunofluorescence; KEGG, Kyoto Encyclopaedia of Genes and Genomes; KO, knockout; pfi, post first immunisation; WT, wild type.

our RNAseq data, where *Cldn7*, *3*, *15*, *4* and *2* were the top DEGs from the CLDN family in HIF2α$^{\Delta IEC}$ epithelial cells compared with controls (figure 3D). However, quantitative PCR analyses showed no difference in *Cldn3*, *7* and *4, 12, 23* mRNA level between HIF2α$^{\Delta IEC}$ and control IECs (online supplemental figure 4C–E,5H–I). In addition, we checked *Cldn3*, *4*, *7*, *12* and *23* protein levels, where no significant difference was detected between HIF2α$^{F/F}$ and HIF2α$^{\Delta IEC}$ epithelial cells in arthritis (online supplemental figure 4H,I). Thus, we narrowed our research down to CLDN15, 2 and to other important TJs, such as occluding (OCLN) and zonula occludens-1 (Zo-1) protein.[19] Although the mRNA expression of *Cldn2*, *Ocln* and *Zo-1* was specifically increased in HIF2α$^{\Delta IEC}$ IECs on arthritis (online supplemental figure 4B), these changes were not detectable at protein level (online supplemental figure 4F,G). Interestingly, only the mRNA and protein levels of CLDN15 were significantly increased in IECs from CIA control mice, while it remained unchanged on HIF2α depletion (figure 3F–J). CLDN15 mRNA and protein expression in the gut only increased at the early stage of arthritis (day 29 pfi) (online supplemental figure 4J–L), which correlates with HIF2α expression in the small intestine (online supplemental figure 1B–D). C57BL/6 mice also presented an increased CLDN15 expression in the gut (online supplemental figure 5E–G). Moreover, in humans, mRNA expression of CLDN15 was increased in patients pre-RA (figure 4K). Thus, CLDN15 appears to be involved in the HIF2α-mediated disruption of gut barrier function during arthritis.

CLDN15 is a transcriptional target gene of HIF2α

To further delineate the connection between HIF2α and CLDN15, we overexpressed HIF2α in the gut epithelial cell line MC38 using a plasmid expressing a triple mutant (TM) form of HIF2α (HIF2α TM). The plasmid carries the P405A, P530V and N851A mutation of murine HIF2α, leading to the resistance of HIF2α to O_2-induced hydroxylation and proteasome degradation.[20] After 24 hours of normoxic culture, HIF2α TM transfected cells had a markedly increased *Hif2α* and *Cldn15* mRNA expression (figure 4A,B). Similarly, protein expression of HIF2α and CLDN15 was increased in HIF2α TM transfected cells (figure 4C–E). No changes were found in the expression of *Cldn2*, *Zo-1* or *Ocln* (online supplemental figure 5A). To test the impact of the HIF2α-CLDN15 axis on the formation of paracellular connections, we performed transepithelial electrical resistance (TEER) measurements with MC38 cells under different treatments. Overexpression of HIF2α and increased expression of CLDN15 correlated with a reduced epithelial cell barrier function in MC38 cells as observed by decreased resistance in TEER measurements (figure 4F). In contrast, HIF2α shRNA or HIF2α inhibitor (PT2977) reduced CLDN15 expression and even increased the MC38 transepithelial junction (figure 4D–F and online supplemental figure 5B,C).

Since HIF2α is a transcription factor, we checked whether *Cldn15* is a transcriptional target of HIF2α. Analysis of *Cldn15* full-length promoter region revealed three HRE consensus sequences located at −2342 (HRE 1), −1911 (HRE 2) and −1109 (HRE 3) base pair from the transcriptional start site (figure 4G). HIF2α ChIP was performed in MC38 cells under normoxia and hypoxia, where IgG was used as control (figure 4H). HIF2α was actively binding to HRE2 and HRE3 *Cldn15* promoter regions under normoxic conditions, which was further enhanced in hypoxic conditions (figure 4H). Next, HRE2 and HRE3 fragments of CLDN15 promoter were cloned into an expression reporter plasmid for luciferase assay. MC38 cells were cotransfected with CLDN15 reporter plasmids and HIF2α TM plasmid carrying a constitutively active HIF2α. As shown in figure 4I, overexpression of HIF2α resulted in a significant induction of CLDN15 promoter HRE3 and especially HRE2 activity. Moreover, adding an increased amount of HIF2α TM plasmid led to a dose-dependent increase in luciferase activity (figure 4J). Taken together, these data demonstrate a direct HIF2α-mediated transcriptional activation of *Cldn15*. These findings suggest that HIF2α disrupts the gut barrier function by increasing *Cldn15* expression.

Treatment with an HIF2α inhibitor attenuates the severity of arthritis

Based on the aforementioned observations, HIF2α inhibition might ameliorate arthritis by preserving the gut barrier function. Therefore, HIF2α inhibitor (PT2977) was applied by oral gavage into CIA mice starting at day 7 pfi (figure 5A). PT2977 selectively binds to HIF2α hydrophobic cavity and disrupts HIF2α heterodimerisation with HIF1β.[21] As shown in figure 5B, oral administration of PT2977 significantly decreased the incidence of arthritis. Vehicle controls exhibited an earlier disease onset and developed higher arthritis scores than mice treated with PT2977 (figure 5C). PT2977-treated mice presented decreased paw thickness, synovial inflammation, bone erosion and lower osteoclast numbers, confirming attenuation of arthritis (figure 5D–F). Flow cytometric analyses of lymphoid cells from PPs, MLN and spleen showed reduced Th1 and Th17 cells in PPs and MLN, while Treg cells were increased in PPs (figure 5H). Accordingly, IFN-γ and IL-17A levels decreased in the PT2977-treated group (figure 5G). To determine whether the inhibition of HIF2α could rescue from the arthritis-associated intestinal barrier defects, we performed the FITC–dextran analyses at day 36 pfi. Indeed, gut permeability was partly restored after the application of HIF2α inhibitor (figure 5I), which was associated with a reduced mRNA expression of *Cldn15* in IECs (figure 5J,K).

In addition, we performed electron microscopy of gut epithelial cells and IF staining for CLDN15 in the gut of PT2977-treated CIA mice compared with vehicle controls at day 36 pfi. We found a narrower spacing within the TJs and decreased CLDN15 expression in the HIF2α inhibitor-treated group (online supplemental figure 5D and L–M). In accordance, intestinal organoids treated with PT2977 for 48 hours showed reduced *Cldn15* expression (figure 5N). Staining with bacterial probe confirmed the enhanced barrier protection of the gut after HIF2α inhibitor treatment (figure 5O). Collectively, intestinal HIF2α inhibition by PT2977 improves gut barrier function during arthritis and thereby inhibits the development of arthritis.

DISCUSSION

Herein, we show that intestinal expression of the transcription factor HIF2α is increased at the onset of arthritis and induces intestinal barrier dysfunction. Inhibition of intestinal HIF2α alleviates arthritis and regulates epithelial barrier integrity by selectively inhibiting the expression of the pore forming protein CLDN15. Our findings support the concept of the gut–joint axis and illustrate the connection between intestinal hypoxia and arthritis (online supplemental figure 6).

Microscopic gut inflammation has been described in patients with arthritis.[22–24] However, these observations only revealed a coincidence of gut and joint inflammation but did not provide a causal link between gut inflammation and arthritis. Increased expression of chemokines, such as CCR4 and CCR5, has been demonstrated in duodenal tissues from patients with

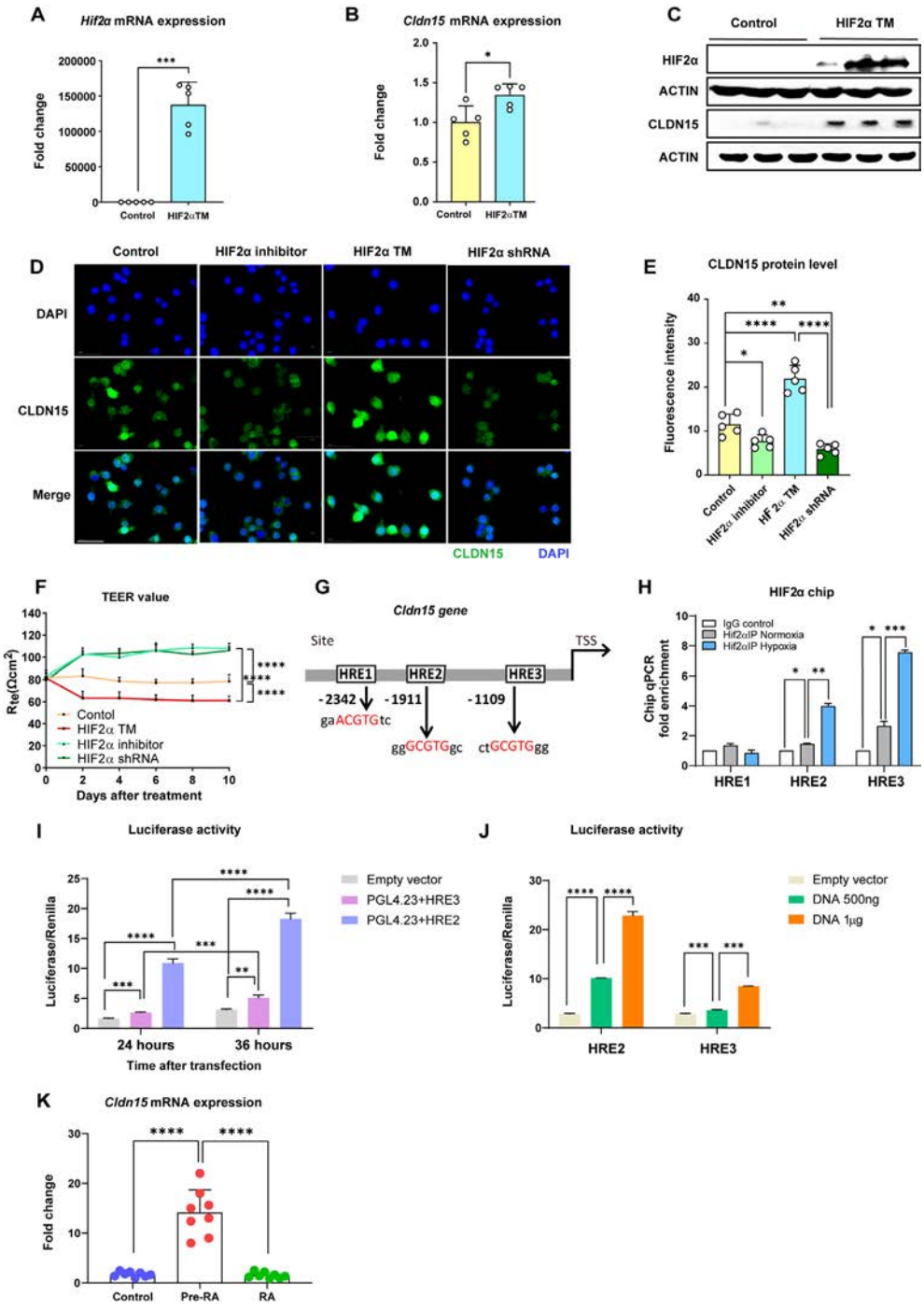

Figure 4 HIF2α directly binds to claudin-15 promoter HRE elements and acts as a transcriptional enhancer. (A,B) mRNA expression of *Hif2α* (A) and *Cldn15* (B) in MC38 cells, transfected with a HIF2α TM overexpression plasmid or an empty plasmid control (n=5). (C) Western blot analysis of HIF2α, CLDN15 and actin in lysates, extracted from MC38 cells, transfected with a HIF2αTM plasmid or an empty plasmid control (n=3). (D,E) Representative images of IF staining for CLDN15 (green) and DAPI (blue) (D) and quantification of CLDN15 fluorescence intensity (E) in MC38 cells that were either untreated, treated with a HIF2α inhibitor or transfected with a HIF2α TM plasmid or HIF2α shRNA plasmid (n=5). Scale bar, 100 µm. (F) TEER changes in M38 cells, transfected with HIF2α TM plasmid, HIF2α shRNA plasmid and empty plasmid control or treated with HIF2α inhibitor (n=6). (G) Schematic illustration of the *Cldn15* promoter sequence including the predicted HRE regions 1, 2 and 3. (H) ChIP-qPCR analysis of HIF2α recruitment to the predicted HRE regions within the *Cldn15* promoter in MC38 cells under normoxic and hypoxic (1% O_2, 8 hours) conditions. Recruitment is expressed as fold enrichment over IgG (n=3). (I) Luciferase reporter gene assay to test the *Cldn15* promoter activity in MC38 cells, 24 or 36 hours after the transfection with HIF2α TM plasmid, Renilla plasmid and pGL4.23+HRE2 or pGL4.23+HRE3 plasmid (n=3–5). (J) Luciferase reporter gene assay of the *Cldn15* promoter in MC38 cells on transfection with different concentrations of HIF2αTM plasmid (n=3–5). (K) RT-PCR quantification of *Cldn15* in the ileum of healthy donors, patients pre-RA and patients with RA (n=8). Data are representative of three independent experiments. Data are shown as mean±SD. Statistical significance was determined by two-tailed unpaired Student's t-test (A,B) for single comparisons*,*** or one-way analysis of variance (E,F,H–K) for multiple comparisons.*,***,***,**** *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. ChIP-qPCR, chromatin immunoprecipitation quantitative PCR; HRE, hypoxia-responsive element; IF, immunofluorescence; RA, rheumatoid arthritis; TEER, transepithelial electrical resistance; TM, triple mutant;TSS, transcription start site.

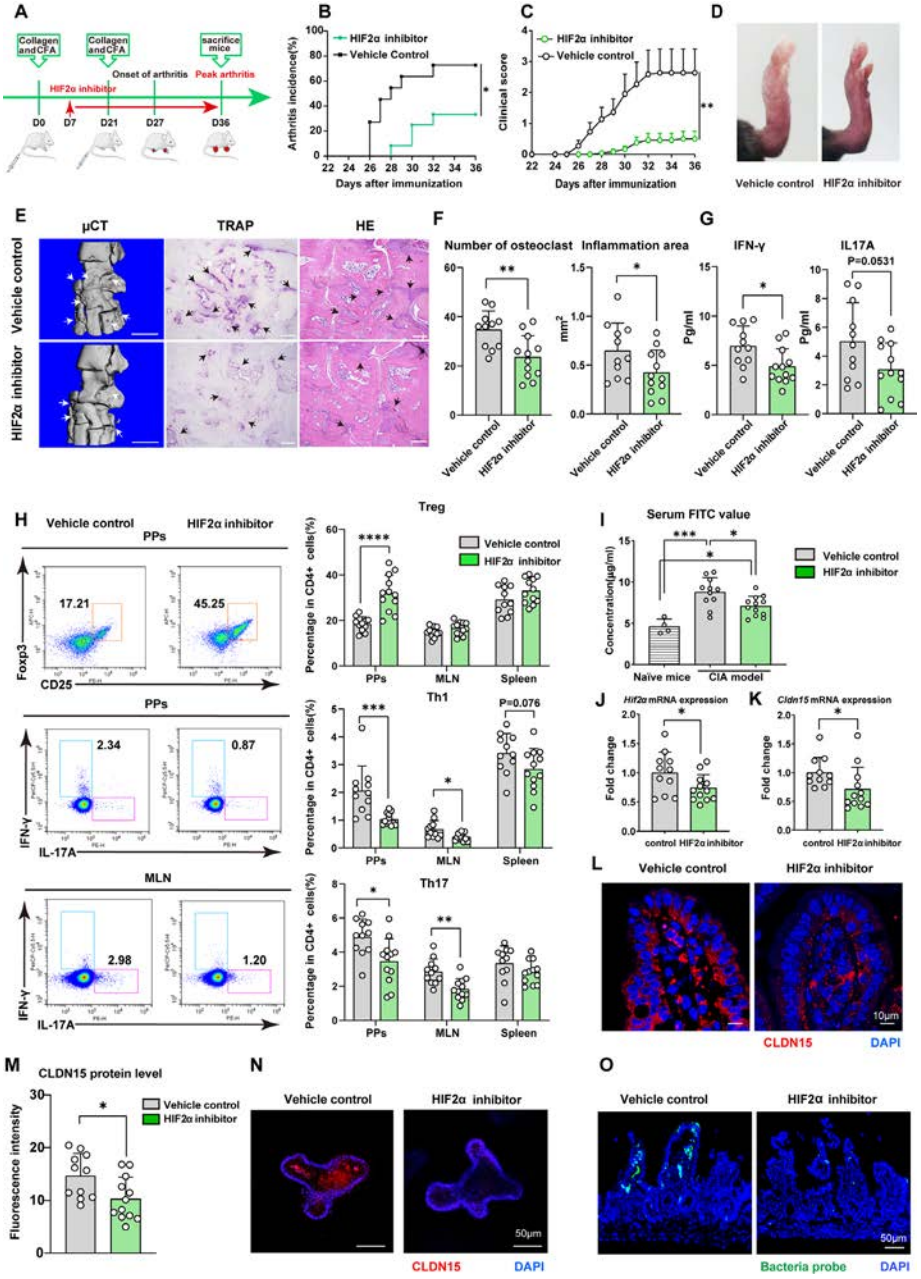

Figure 5 HIF2α inhibition attenuates arthritis by controlling claudin-15-mediated gut barrier dysfunction. (A) Experimental set-up of CIA in C57BL/6 mice (immunisation at days 0 and 21, analysis at day 36) and the treatment with HIF2α inhibitor (starting from day 7 to day 36 pfi). (B,C) Arthritis incidence (B) and clinical score (C) in CIA C57BL/6 mice gavaged with HIF2α inhibitor compared with vehicle control (n=11–12). (D,E) Representative pictures of thickness (E), μCT scans, TRAP and H&E staining (E) in the hind paws of CIA C57BL/6 mice gavaged with HIF2α inhibitor compared with vehicle control. Arrows indicate inflammation (H&E) and bone erosion (TRAP and μCT). μCT scale bar, 1 mm. HE and TRAP scale bars, 50 μm. (F) Quantification of osteoclast number and inflammation area in the hind paws in the aforementioned groups (n=11–12). (G) IFN-γ, IL-17A serum levels in CIA mice at 36 days post immunisation treated or not with HIF2α inhibitor (n=11–12). (H) Representative FACS plots and quantification of Treg cells (CD4+CD25+Foxp3+ cells), Th1 cells (IFN-γ+CD4+cells), and Th17 cells (IL-17A+CD4+ cells) in the PPs, MLNs and spleen of the aforementioned groups (n=11–12). (I) Evaluation of intestinal permeability in CIA C57BL/6 mice treated with vehicle control or HIF2α inhibitor (n=11–12) compared with naïve C57BL/6 mice (n=4) by FITC-labelled dextran. (J, K) mRNA expression of *Hif2α* (J) and *Cldn15* (K) in IECs from CIA C57BL/6 mice treated with vehicle control or HIF2α inhibitor (n=11–12). (L,M) Representative pictures (L) of IF staining for CLDN15 (red) and DAPI (blue) and quantification (M) of CLDN15 fluorescence intensity in the intestine of CIA C57BL/6 mice gavaged with HIF2α inhibitor compared with vehicle control (n=11–12). Scale bar, 10 μm. (N) Representative pictures of if staining for CLDN15 (red) and DAPI (blue) in small intestinal-derived organoids treated with vehicle control or HIF2α inhibitor for 48 hours. Scale bar, 50 μm. (O) Representative pictures of FISH on tissue sections from ileum of CIA C57BL/6 mice treated with vehicle control or HIF2α inhibitor using the bacteria-specific probe EUB 338-FITC (green) and DAPI (blue) for visualisation of nuclei. Scale bar, 50 μm. Symbols represent individual mice. Data are shown as mean±SD. Statistical significance was determined by Kaplan-Meier analysis with log-rank test (B), two-tailed unpaired Student's t-test (C,F–H,J,K,M) for single comparisons*/**/***/**** within a tissue and one-way analysis of variance (I) for multiple comparisons.*/*** *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. μCT, micro-CT; CIA, collagen-induced arthritis; FISH, fluorescence in situ hybridisation; FITC, fluorescein isothiocyanate; IEC, intestine epithelial cell; IF, immunofluorescence; IFN-γ, interferon gamma; IL, interleukin; MLN, mesenteric lymph node; pfi, post first immunisation; PP, Peyer's patch; TRAP, tartrate-resistant acid phosphatase.

RA, suggesting immune cell activation in the small intestine.[25] Subclinical intestinal inflammation has also been shown in a small cohort of patients with early RA.[5] Intestinal inflammation in these patients was characterised by an increased number of infiltrating immune cells, such as T cells, B cells and CD68+ macrophages.[5] In addition, gut dysbiosis has been associated with experimental arthritis and RA, which might contribute to the subclinical gut inflammation and promote the activation of specific innate and adaptive immune responses.[26] In our study, we intervene into the arthritis-associated gut barrier disruption by deleting HIF2α in intestinal epithelial cells. This intervention not only preserved the intestinal barrier function, but also ameliorated the course of arthritis, indicating that arthritis onset might be controlled by mucosal sites.[27]

Previous studies have indicated that HIFs contribute to intestinal inflammation. However, so far, studies mainly focused on intestinal inflammation models, such as inflammatory bowel disease, eosinophilic esophagitis and radiation-induced intestinal toxicity, but did not examine a potential axis between the gut and the joints.[6 28 29] For instance, chronic activation of HIF2α in intestinal epithelial cells was shown to induce experimental colitis, while inhibition of intestinal HIF2α decreased colitis.[30] On the other hand, in radiation-induced injury, HIF2α activation was shown as a protective factor by regulating angiogenesis and crypt regeneration.[31] Hence, HIF2α is involved in both intestinal inflammation and resolution of injury. Therefore, we considered that HIF2α expression in intestinal epithelial cells may regulate gut barrier and thereby influence the onset of arthritis.

HIF1α and HIF2α influence the expression of genes that are involved in barrier function, such as mucins (eg, Muc-3), intestinal trefoil factor (TTF3) and P-glycoprotein (P-GP, Mdr-1 gene product).[32 33] Except for mucin, also intestinal TJs are key components of the gut barrier and were shown to be defective at onset of human RA.[5 34] HIFs were described to regulate the expression of certain components of this TJ barrier, such as CLDN-1.[28] The CLDN protein family consists of 24 members that determine the properties of paracellular barriers. There are sealing, but also pore forming CLDNs.[35] While the former increase barrier function, the latter ones decrease it. By screening TJ targets, CLDN15 expression was linked to HIF2α expression in IECs. Nevertheless, several CLDN family members are coexpressed and interact with each other. There is therefore still the possibility that other cell junction proteins participate in the gut–joint axis in RA. Nonetheless, expression of other CLDN family members, such as cldn2, 3, 4, 7, 12, 15, 23, was not affected. Our work showed that HIF2α acts as a transcriptional enhancer for CLDN15. CLDN15 is a pore-forming CLDN that creates paracellular channels,[7] which are indispensable for paracellular sodium permeability and glucose absorption in the small intestine.[36] CLDN15 expression was significantly increased in intestinal epithelial cells on induction of arthritis, while it was specifically reduced when HIF2α was missing or inhibited in IECs. Therefore, we assume that induction of CLDN15 during onset of arthritis leads to increased sodium permeability and thereby decreases the intestinal barrier function. However, the increased sodium uptake by IECs on HIF2α- CLDN15 activation needs to be examined in future studies. Furthermore, we also analysed CLDN15 expression in human biopsies. In patients with pre-RA, CLDN15 mRNA expression was significantly increased, resembling the findings observed for HIF2α expression. These results suggest that human CLDN15 is correlated to HIF2α expression and to onset of the disease. However, further studies are required to understand the potential role of CLDN15 in human disease, especially the possible link between HIF2α- CLDN15 on iron malabsorption and regulation of epithelial cell death.[11]

Intestinal dysbiosis has been shown to be associated with the development of experimental arthritis and human RA.[26] Of note, several products of commensal gut bacteria downregulate HIF2α, indicating that microbial homeostasis in the gut influences HIF2α expression and thereby controls intestinal barrier function. DAP, a polyamine, produced by several bacterial species, has the capacity to suppress intestinal HIF2α activity. Also reuterin, a product of *Lactobacillus reuteri*, can inhibit HIF2α. Moreover, short-chain fatty acids, which have barrier-maintaining and anti-inflammatory properties,[37–39] inhibit HIF2α expression in the small intestine.[40] Non-steroidal anti-inflammatory drugs may also reduce HIF1α and HIF2α expression and thereby reduced hypoxia-induced angiogenesis.[41 42] Specific pharmacological reagents can be used to suppress HIF2α. For testing the effect of potential therapeutic inhibition of HIF2α, we made use of the fact that HIF2α (but not HIF1α) contains a ligand-binding cavity, which can be targeted by small molecular drugs. Several highly specific small molecule inhibitors have been identified, such as PT2977 specific for HIF2α.[43 44] A phase II study is currently ongoing to evaluate PT2977 for the treatment of renal cell carcinoma. PT2977 acts as a systemic HIF2α inhibitor and may therefore affect other tissues to modulate the severity of arthritis. HIF2α is highly expressed in the synovium and synovial overexpression of HIF2α leads to arthritis.[44] However, we did not observe a significant difference in HIF2α expression in CIA synovium with or without the treatment of PT2977 likely because we administrated the inhibitor by oral gavage (data not shown). Our study demonstrated that PT2977 administration effectively reduces the incidence and severity of arthritis, intestinal infiltration with Th1 and Th17 cells, CLDN15 expression and gut leakiness.

In summary, these data suggest that intestinal HIF2α expression not only controls barrier function, but also the onset of arthritis, emphasising that an impaired gut barrier is a critical driver for arthritis.

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Contributors JW and AB designed the study and wrote the manuscript; JW and PL performed the in vitro experiments; JW, PL, IS, ZL, RS, XM and SC performed the in vivo experiments; FC and GG generated the human experiments; JX analysed the RNA-seq data; DA and KK contributed to the discussion and manuscript preparation; GS and AB supervised the study, wrote the manuscript and are responsible for the overall content .

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Patient and public involvement Patients and/or the public were not involved in the design, conduct, reporting or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by the institutional review board of the Azienda Ospedaliera Universitaria L. Vanvitelli of the University della Campania L. Vanvitelli (Naples, Italy). The patients provided written informed consent. Participants gave informed consent to participate in the study before taking part.Animal protocols were approved by the Institutional Animal Care and by local ethics committee of Regierung von Mittelfranken, Germany.

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TRANSLATIONAL SCIENCE

Concise report: a minimal-invasive method to retrieve and identify entheseal tissue from psoriatic arthritis patients

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ABSTRACT

Objectives To establish a minimally invasive biopsy technique for the analysis of entheseal tissue in patients with psoriatic arthritis (PsA).

Methods Human cadavers were used for establishing the technique to retrieve tissue from the lateral humeral epicondyle enthesis (cadaveric biopsies). After biopsy, the entire enthesis was surgically resected (cadaveric resections). Biopsies and resections were assessed by label-free second harmonic generation (SHG) microscopy. The same technique was then applied in patients with PsA with definition of entheseal tissue by SHG, staining of CD45+immune cells and RNA extraction.

Results Entheseal biopsies from five cadavers allowed the retrieval of entheseal tissue as validated by the analysis of resection material. Microscopy of biopsy and resection sections allowed differentiation of entheseal, tendon and muscle tissue by SHG and definition of specific intensity thresholds for entheseal tissue. In subsequent entheseal biopsies of 10 PsA patients: the fraction of entheseal tissue was high (65%) and comparable to cadaveric biopsies (68%) as assessed by SHG microscopy. Furthermore, PsA biopsies showed immune cell infiltration and sufficient retrieval of RNA for further molecular analysis.

Conclusion Entheseal biopsy of the lateral epicondyle is feasible in patients with PsA allowing reliable retrieval of entheseal tissue and its identification by SHG microscopy.

Key messages

What is already known about this subject?

⇒ Enthesitis is a hallmark of psoriatic arthritis, however, direct assessment of entheseal tissue in humans has been challenging to date. Second harmonic generation (SHG) microscopy allows to define differences in collagen structure in tissues.

What does this study add?

⇒ This study describes a new reliable and well-tolerated biopsy approach for harvesting entheseal tissue in humans in vivo by performing biopsies of the lateral epicondyle enthesis. Furthermore, SHG assessment of the retrieved tissue allows to define the quality of the biopsy by visualising the content of entheseal tissue in the sample.

How might this impact on clinical practice or future developments?

⇒ This method allows to perform reliable molecular and cellular analyses of entheseal tissue in humans, which is needed for a better understanding of diseases such as psoriatic arthritis.

INTRODUCTION

Enthesis is a specialised interface tissue that connects tendons and ligaments with bone.[1 2] Enthesitis, the inflammation of these insertion sites, represents a hallmark feature of spondyloarthritis, including psoriatic arthritis (PsA). Enthesitis has been suggested as an early event in PsA, with the Achilles tendon, the plantar fascia and the lateral epicondyle being the most commonly involved sites.[3] So far, most of the data on enthesitis in PsA are based on clinical assessment of tenderness as well as MRI or ultrasound examinations.[4] These approaches, however, do not allow molecular analysis of entheses, which will ultimately require acquisition of entheseal tissue.

Good quality sampling of human synovial tissues has been instrumental for enhancing the understanding of rheumatoid arthritis.[5 6] While synovial tissue is rather easily accessible and based on well-defined anatomical structures, assessment of entheses is technically challenging. Hence, it is currently unknown, which entheseal structure in humans would qualify for a feasible biopsy and how correct sampling of entheseal structures could be ascertained within such biopsy material. These technical challenges have led to substantial lack of knowledge on human entheseal tissues. To overcome these hurdles, we developed a guided biopsy approach of entheses, which does not require the concurrent sampling of bone tissue and includes a second harmonic generation (SHG) microscopy-based sampling control to identify and confirm the presence of entheseal tissue within the collected sample.

Table 1 PsA patient characteristics

Patient characteristics	
	Patients (n=10)
Female sex; n (%)	4 (40)
Age (years); mean±SD	53.7±9
Tender joint count mean±SD	7.7±6.7
Swollen joint count mean±SD	2.4±3.6
DAPSA mean±SD	22±11
LEI mean±SD	1.5±1
SPARCC mean±SD	4.1±2
PD signal before biopsy; n (%)	10 (100)
Treatment before biopsy	
Non-steroidal anti-inflammatory drugs; n (%)	4 (40)
Prednisolon; n (%)	1 (10)
Methotrexate; n (%)	5 (50)
VAS after biopsy; mean±SD	2±1
Haematoma after biopsy; n (%)	2 (20)
Wound infection; n (%)	0 (0)
Normal function after 14 days; n (%)	10 (100)

DAPSA, Disease Activity Psoriatic Arthritis; LEI, Leeds Enthesitis Index; PD, Power Doppler; PsA, psoriatic arthritis; SPARCC, Spondyloarthritis Consortium Canada; VAS, visual analogue scale.

MATERIALS AND METHODS

Minimally invasive entheseal biopsy

We obtained elbows from five cadavers from the Institute of Anatomy and recruited 10 PsA patients who fulfilled the classification criteria for PsA.[7] All patients with PsA showed clinical signs of enthesitis at the elbow (without synovitis), including stiffness and pain elicited on local pressure and/or exercise and had a positive Power Doppler (PD) signal in the ultrasound (table 1).

All patients with PsA were naïve to biological disease-modifying anti-rheumatic drugs (bDMARDs) and had active enthesitis under treatment with conventional (c)DMARDs. Extent of enthesitis was assessed by Leeds Enthesitis Index and Spondyloarthritis Research Consortium of Canada. Biopsy was done by Blakesley forceps in 60° flexed position after identifying the lateral epicondyle, the olecranon and the radial head by ultrasound. In patients with PsA, 2 mL of 1% mepivacaine was injected prior to biopsy while skin incision was closed by two stitches with non-absorbable suture material after biopsy. The instrument was inserted until bone contact, then slightly withdrawn to obtain the biopsy (5 mm) from the extensor tendon insertions of the lateral epicondyle. In cadavers, the whole enthesis including adjacent tendon, muscle and bone was surgically resected after the biopsy for further analysis. Ethical approval from the local institutional review board (University of Erlangen-Nürnberg, #30_19B) and written informed consent was obtained from all participants. Further methodological information is provided in online supplemental file.

RESULTS

Ultrasound-guided biopsy of the lateral epicondyle enthesis in cadavers

We first established ultrasound-guided biopsy of the lateral epicondyle in five human cadaveric specimens (online supplemental file). Ultrasound examination using B-mode of the region defined the lateral epicondyle enthesis (figure 1A,B). Anatomical landmarks (lateral humeral epicondyle, radial head and olecranon) were marked on the skin (figure 1C). An incision (5 mm) was made along the line between the lateral epicondyle and the radial head followed by preparation of subcutaneous tissue and fascia (figure 1D). A Blakesley forceps was inserted through the incision (figure 1E) and one 5 mm biopsy of the tendon plate of the extensor muscles (digitorum communis, digitus minimus and carpi radialis) was taken (figure 1F). Retrieved tissue was later examined by histochemistry and SHG (=cadaveric biopsy specimens).

Definition of the anatomic environment of the entheses

After biopsy, the subcutaneous tissue and the fascia were split up until the enthesis (figure 1G), making the biopsy site clearly visible (figure 1H,I). The joint capsule was not affected indicating extra-articular localisation (figure 1I). For histologic validation, the entire entheseal region including adjacent bone, tendon and muscle was then removed (=cadaveric resection specimens).

Identification of entheseal tissue by SHG

Histological analysis of cadaveric resection specimens showed the adjacent bone (blue in Safranin O) and muscle (red in Trichrome) and the enthesis between bone and tendon (figure 1J). Induction of SHG by multiphoton microscopy allows visualising the composition of structural proteins, that is, collagens.[8] SHG intensity (SHG-I) of each tissue (bone, enthesis, tendon and muscle) was measured in different regions of interests and normalised to the highest intensity value (internal control=100%) (figure 1K,M). Tendons always had the highest SHG-I (mean±SD: 91%±13%) followed by bone (80%±12%), while muscles emitted the lowest SHG-I (12%±5%). Entheses showed a unique intermediate signal (31%±6%), which was statistically different to other tissues (figure 1L,M). No difference was observed between calcified and uncalcified entheseal areas (figure 1N). As SHG allows identifying entheseal tissue, we used SHG to validate the content of entheseal tissue in cadaveric biopsy specimen (figure 1O-Q-O-Q). To identify optimal cut-offs to differentiate enthesis from muscle and tendon, we used 16-bit pixel depth images with a high resolution and a dynamic intensity range from 0 till 65 535 (=2^{16} units on a grey scale). Based on the observed data, we were able to identify optimal cut-offs to differentiate muscle from enthesis (5507 95% CI 4227 to 6962 units on the 16-bit grey scale) and enthesis from tendon (12 318, 95% CI 11 159 to 13 817 units on the 16-bit grey scale) (figure 1P) with high accuracy (0.93 and 0.95, respectively). We identified 68% of cadaveric biopsy sample being entheseal tissue (figure 1Q).

Biopsies of the lateral epicondyle enthesis in patients with PsA

Based on the data retrieved from the biopsies of the entheses in cadavers, we addressed the feasibility to biopsy lateral epicondyle enthesis in 10 patients with PsA (table 1) using the same approach (figure 2A–F). Prior to biopsy, diagnosis of enthesitis was confirmed by clinical examination and PD Ultrasound. The aforementioned anatomical structures were defined by ultrasound. After incision, a 5 mm biopsy of the tendon plate of the extensor muscles was taken (figure 2E). All incisions healed well without complications and stitches could be removed after 7–10 days. Range of motion of the elbow was examined 14 days after biopsy with normal function in all participants. Only two patients developed mild haematoma, with no functional limitations. Standard histochemistry did

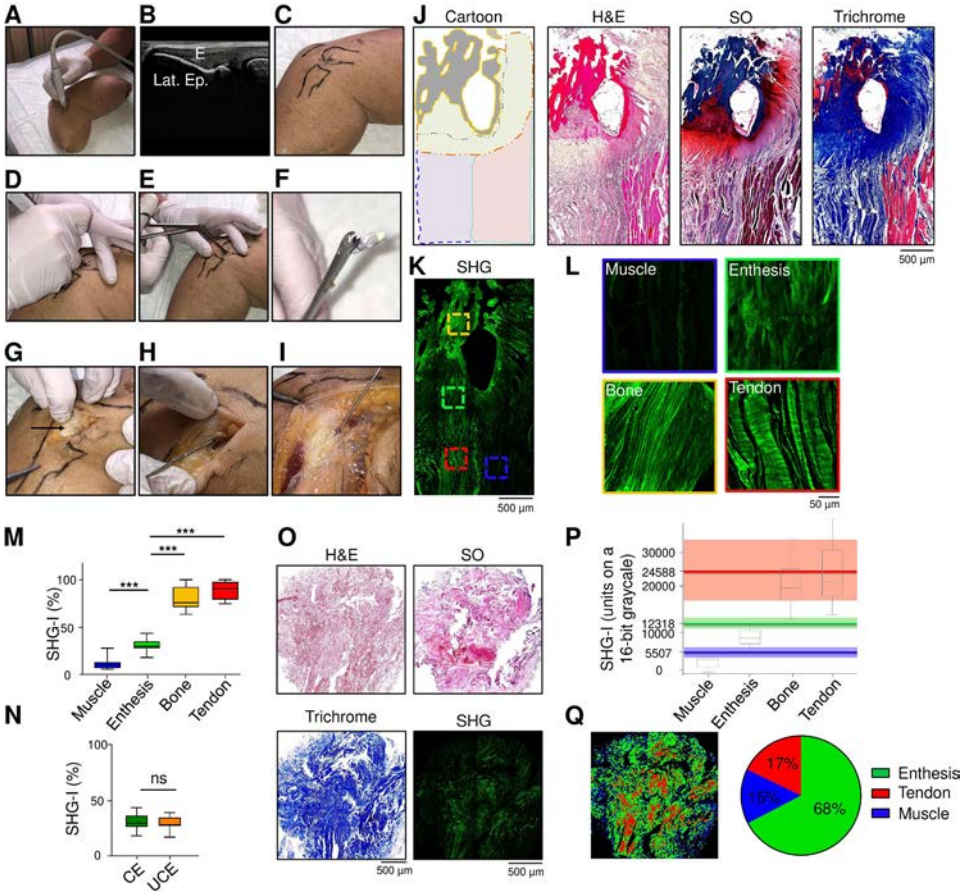

Figure 1 Minimally invasive ultrasound-guided biopsy. (A) Ultrasound (US) identification of the extensor tendon enthesis. (B) B-mode US image of the lateral epicondyle enthesis (E) of the lateral epicondyle (Lat. Ep.). (C) Anatomical landmarks (lateral humeral epicondyle, radial head and olecranon) on the skin. (D) Skin incision (5 mm) between the radial epicondyle and the radial head. (E) Insertion of Blakesley forceps. (F) Biopsy of the enthesis. (G) Longitudinal skin incision to directly visualise the biopsy area and for later resection of the entire enthesis. (H) Identification of the percutaneous biopsy site. (I) Identification of the percutaneous biopsy site outside the joint capsule. (J) Evaluation of a representative entheseal resection sample showing the complete enthesis: a cartoon illustrating the different tissues and their extent (B: bone; BM: bone marrow; CE: calcified enthesis; UCE: uncalcified enthesis; T: tendon; M: muscle); H&E, safranin O and trichrome stains.[18] (K–L) Evaluation of a entheseal resection sample by second harmonic generation (SHG) microscopy and corresponding magnifications for each tissue (muscle, enthesis, bone and tendon). (M) Quantification of SHG intensity in entheseal resection samples: for each sample, the signal intensities of muscle, enthesis, bone and tendon were separately measured (five cadaveric samples; three assessments/subregion each) and normalised to the highest value within the sample (=100%). Data are shown as mean±SEM, ***p<0.0001 was determined by ordinary one-way analysis of variance (ANOVA) test. (N) SHG intensity quantifications of calcified (CE) versus uncalcified (UCE) areas of the enthesis. Data are shown as mean±SEM, ns (p>0.05) was determined by Student's t-test. (O) Staining of a representative cadaveric entheseal biopsy with H&E, safranin O and trichrome stains and evaluation of a cadaveric entheseal biopsy by SHG microscopy. (P) Observed intensity values and estimated thresholds with 95% bootstrap confidence intervals to discriminate different tissue types: (Red) cut-off and range for bones and tendons (24588, 95% CI 16 776 to 31905), (green) range for entheses (12318, 95% CI 11 159 to 13817), (blue) range for muscle (5507 95% CI, 4227 to 6962)[19] . (Q) Fractions of tissues in cadaveric entheseal biopsies expressed as percentages. ***p<0.0001 was determined by ordinary one-way ANOVA test.

not allow differentiating entheseal, tendonal and muscular components (figure 2G). In contrast, substantial differences in SHG-I corresponding to muscular (lowest), entheseal (medium) and tendonal (highest SHG) tissues were found (figure 2G,H). Based on the defined cut-offs, we could show that 65% of the tissue sample consisted of entheseal tissue (figure 2I), which was in line with our previous observations from cadaveric biopsies. In contrast to cadaveric biopsies, all PsA specimens showed infiltration with CD45+ immune cells (figure 2J,K). In addition, samples were validated with respect to their suitability to retrieve enough RNA for molecular analysis. In situ hybridisation of deparaffinised sections as well as whole specimen digestion and subsequent measurements of expression levels of target genes by real-time PCR revealed a high RNA integrity (figure 2L,M).

DISCUSSION

To date, very little is known about the cellular and molecular composition of human entheses. McGonagle and colleagues were the only who have undertaken the effort to perform biopsies from peripheral human entheses in patients with SpA.[9] They performed Yamshidi needle-based biopsies of plantar and patellar entheses in five patients with SpA and showed infiltration by T cells and macrophages as well as increased vascularisation.[10] Other, even more invasive studies were done in surgery material from the spine of patients with non-SpA and SpA, showing infiltration by immune cells, fat deposition and expression of inflammatory cytokines, such as IL-23, IL-17 and tumour necrosis factor alpha (TNFα).[9 11–13] While these studies indicate that access to entheseal tissues might improve our

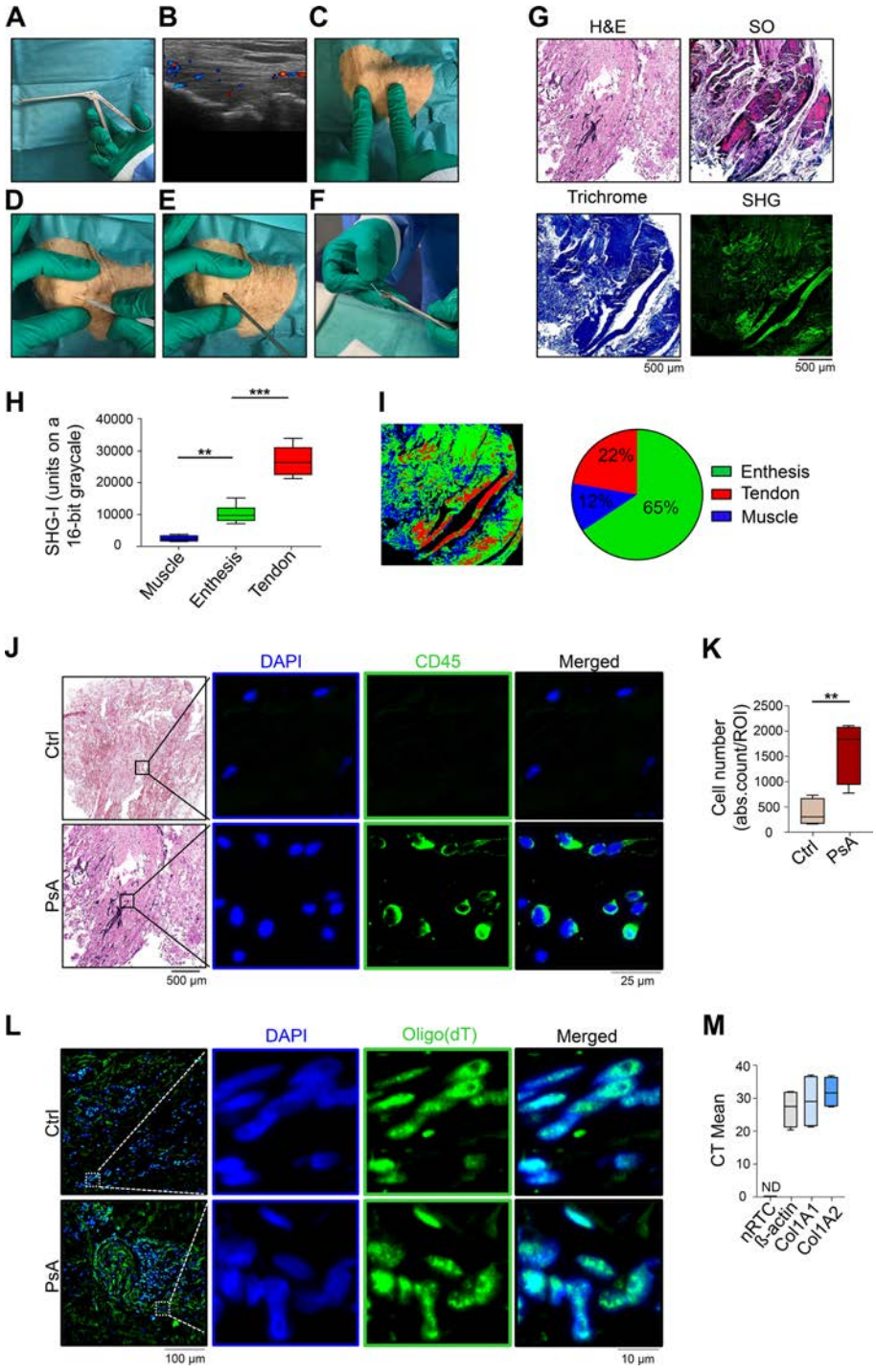

Figure 2 Evaluation of entheseal biopsies from psoriatic arthritis patients. (A) Blakesley forceps adopted for the biopsy retrieval; (B) Power Doppler ultradsound image showing enthesitis of the lateral humeral epicondyle; (C) palpatory identification of the anatomical structures (lateral humeral epicondyle, radial head and olecranon); (D) skin incision (5 mm) between the radial epicondyle and the radial head. (E) Insertion of Blakesley forceps. (F) Biopsy of the enthesis. (G) Staining of entheseal biopsies from patients with psoriatic arthritis (PsA) by H&E, safranin O and trichrome and evaluation of PsA entheseal biopsies by SHG microscopy. (H) Individual intensity values for each tissue in an established intensity range between 0 and 65 535 units. (I) Fractions of tissues in PsA entheseal biopsies expressed as percentages. Data are shown as mean±SEM, ***p<0.0001 was determined by ordinary one-way analysis of variance (ANOVA) test. (J) Representative H&E stainings from cadaveric (Ctrl) and PsA entheseal biopsies; inserts show the region for fluorescence stainings for leukocytes (anti-CD45, green) and total cells (DAPI, blue). (K) Cellular numbers in cadaveric biopsy specimens (Ctrl) and PsA patients; (L) in situ hybridization from cadaveric (Ctrl) and PsA patient biopsy specimen; inserts show the region for mRNA (Oligo-dT, green) and nuclei (DAPI, blue) detection. (M) RNA expression of target genes expressed as CT values in PsA patient biopsy specimen, NRT (non-RT control).

current understanding of SpA and PsA, the lack of a standardised and feasible biopsy procedure for peripheral entheses and the absence of a quality control procedure to confirm presence of entheseal tissue in the biopsy represent serious complications.

Herein, we present a safe, well-tolerated, minimally invasive, US-guided biopsy approach to retrieve entheseal material from the lateral epicondyle, which is one of the most common sites of enthesitis in PsA.[3] While conventional histology is often used for

evaluation of ex vivo biopsies, visual distinction of fibrocartilage from tendon is challenging if not impossible if the available tissue volume is low. In addition, entheses lack tissue-specific markers separating them from tendon and cartilage. Of note, entheses display a specific feature of collagen assembly, where type-II collagen fibres display a distinct fibrous organisation.[14 15] SHG can visualise fibrillar collagen assembly, which intrinsically emits a strong SHG signal.[16] Therefore, SHG has been used for qualitative and quantitative analyses of collagen in various diseases, such as cancer.[17] In this study, we defined SHG-I signatures that allow to differentiate entheseal from tendon and muscle tissue and, therefore, allow identification of entheseal tissue in small biopsies. Furthermore, in vivo biopsies are useful for the immunohistochemical detection of immune cells as well as for extraction of sufficient amount of RNA for expression studies.

In summary, this standardised entheseal biopsy procedure with the adjoined method to assure tissue quality can be carried out by rheumatologists and is useful to study the molecular and cellular changes of human entheses in diseases and the specific effects of anti-inflammatory treatments in enthesitis.

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Contributors MLP, MGR, CX, GS and AR designed the study; MLP, MGR, CX, SR, KT, HL, MV, MSAS, DS, JR, AS, LB, AK, AR acquired data; MLP, MGR, CX, SR, KT, AS, LB, GS and AR interpreted data; MLP, DS, JR, LB, AK provided materials; MLP, MGR, CX, GS and AR prepared the manuscript. Author acting as guarantor: AR.

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Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Consent obtained directly from patient(s)

Ethics approval This study involves human participants and was approved by Ethical Committee of the Friedrich-Alexander-University (FAU) Erlangen-Nürnberg. Participants gave informed consent to participate in the study before taking part.

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CLINICAL SCIENCE

Biological impact of iberdomide in patients with active systemic lupus erythematosus

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ABSTRACT

Objectives Iberdomide is a high-affinity cereblon ligand that promotes proteasomal degradation of transcription factors Ikaros (*IKZF1*) and Aiolos (*IKZF3*). Pharmacodynamics and pharmacokinetics of oral iberdomide were evaluated in a phase 2b study of patients with active systemic lupus erythematosus (SLE).

Methods Adults with autoantibody-positive SLE were randomised to placebo (n=83) or once daily iberdomide 0.15 mg (n=42), 0.3 mg (n=82) or 0.45 mg (n=81). Pharmacodynamic changes in whole blood leucocytes were measured by flow cytometry, regulatory T cells (Tregs) by epigenetic assay, plasma cytokines by ultrasensitive cytokine assay and gene expression by Modular Immune Profiling.

Results Iberdomide exhibited linear pharmacokinetics and dose-dependently modulated leucocytes and cytokines. Compared with placebo at week 24, iberdomide 0.45 mg significantly (p<0.001) reduced B cells, including those expressing CD268 (TNFRSF13C) (−58.3%), and plasmacytoid dendritic cells (−73.9%), and increased Tregs (+104.9%) and interleukin 2 (IL-2) (+144.1%). Clinical efficacy was previously reported in patients with high *IKZF3* expression and high type I interferon (IFN) signature at baseline and confirmed here in those with an especially high IFN signature. Iberdomide decreased the type I IFN gene signature only in patients with high expression at baseline (−81.5%; p<0.001) but decreased other gene signatures in all patients.

Conclusion Iberdomide significantly reduced activity of type I IFN and B cell pathways, and increased IL-2 and Tregs, suggesting a selective rebalancing of immune abnormalities in SLE. Clinical efficacy corresponded to reduction of the type I IFN gene signature.

Trial registration number NCT03161483.

Key messages

What is already known about this subject?

⇒ Iberdomide is a high-affinity cereblon ligand which promotes proteasomal degradation of Ikaros (*IKZF1*) and Aiolos (*IKZF3*) and is currently in development for the treatment of patients with systemic lupus erythematosus (SLE), multiple myeloma and lymphoma.

⇒ In a phase 2a trial in patients with active SLE, iberdomide significantly reduced B cells and plasmacytoid dendritic cells (pDCs) and showed trends of improvements in SLE disease severity.

What does this study add?

⇒ In this larger phase 2b study, iberdomide significantly improved lupus disease activity and reduced hallmarks of the immunopathogenesis of SLE by decreasing B cells, pDCs and myeloid dendritic cells, and by increasing interleukin 2 and regulatory T cells.

⇒ In patients with a high type I interferon (IFN) gene signature at baseline, iberdomide treatment reduced the IFN gene signature score by as much as 81% from the median at baseline, an effect that coincided with an improved SLE Responder Index-4 clinical response rate.

How might this impact on clinical practice or future developments?

⇒ This study confirmed the mechanism of action of iberdomide in vivo in patients with SLE and identified the high type I IFN gene signature as a predictive biomarker for evaluation as a selection tool in future clinical studies of iberdomide.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune inflammatory disorder arising from the interaction of a genetically determined immune phenotype with environmental factors.[1 2] Disease susceptibility is influenced by genes related to immune response pathways and major histocompatibility complex classes I and II. Dysregulated immune responses lead to B cell hyperactivity and production of pathogenic autoantibodies. Immune complexes containing nucleic acids are potential stimuli of the innate immune system, leading to type I interferon (IFN) production in SLE.

Ikaros (*IKZF1*) and Aiolos (*IKZF3*) are zinc finger transcription factors involved in immune cell development and homeostasis.[3–5] Ikaros is required for development of B cells and plasmacytoid dendritic cells (pDCs), which are important producers of IFN-α. Ikaros also represses interleukin 2 (IL-2) transcription.[6] Aiolos is a B cell modulator and is required for maturation of plasma cells. *IKZF1*

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and *IKZF3* mRNA and proteins are overexpressed in the cells of patients with SLE.[4 5 7–10] Genetic variants in the *IKZF1* and *IKZF3* loci are associated with an increased risk of developing SLE.[2 10] In particular, the *IKZF1* polymorphism rs4917014 was identified as a trans-expression quantitative trait locus (eQTL) increasing expression of type I IFN response genes (*HERC5*, *IFI6*, *IFIT1*, *MX1* and *TNFRSF21*).[9]

Iberdomide (CC-220) is a high-affinity cereblon ligand, which promotes ubiquitination and proteasomal degradation of Ikaros and Aiolos.[4 5 11] The binding affinity of iberdomide to cereblon is higher than that of other related cereblon binders, such as lenalidomide or pomalidomide. In vitro studies have shown a potent effect of iberdomide in reducing Ikaros and Aiolos protein levels in B cells, T cells and monocytes from healthy donors. In peripheral blood mononuclear cells from patients with SLE, iberdomide inhibited autoantibody production and B cell differentiation. Iberdomide also increased T cell-derived IL-2 production in the whole blood of healthy volunteers owing to an iberdomide-mediated decrease in the repressive activity of Ikaros and Aiolos.[5] In a pilot phase 2 trial of ascending doses of iberdomide in patients with SLE, strong correlations were observed between iberdomide exposure and reductions in the numbers of B cells and pDCs.[12]

A phase 2 randomised, controlled trial evaluated the efficacy and safety of iberdomide compared with placebo over 24 weeks in patients with active SLE. As reported elsewhere,[13] the primary efficacy endpoint of SLE Responder Index-4 (SRI-4) response was met with 54% of patients receiving iberdomide 0.45 mg once daily having achieved an SRI-4 response versus 35% in the placebo group (stratified difference: 19.4%; 95% CI 4.1 to 33.4; p=0.01) at week 24. Furthermore, the treatment effect of iberdomide 0.45 mg compared with placebo for SRI-4 response was greater in the prespecified biomarker-defined subsets of patients with high expression of *IKZF3* at baseline (64% vs 33%; p=0.011) and high expression of type I IFN at baseline (60% vs 33%; p=0.006). As understanding of SLE pathophysiology increases, the precise biological impact of therapeutic agents is of great interest and may be useful in identifying biomarkers of clinical response. Therefore, the effects of iberdomide on immunologic biomarkers in patients with active SLE were further evaluated in this phase 2 study.

PATIENTS AND METHODS

Study design

The study design of the phase 2, multinational, randomised, placebo-controlled, double-blind study has been reported.[13] Briefly, patients with active SLE were randomised (2:2:1:2) to receive oral iberdomide (0.45 mg, 0.3 mg or 0.15 mg) or placebo once daily for 24 weeks while continuing standard-of-care medications.

Patients

Eligible patients were adults (≥18 years of age) with a diagnosis of SLE for at least 6 months, a Systemic Lupus Erythematosus Disease Activity Index 2000 score ≥6 points and positive for autoantibodies associated with SLE. Stable doses of corticosteroids (≤20 mg prednisone or equivalent daily) were allowed. Exclusion criteria were active, severe or unstable neuropsychiatric lupus disease, antiphospholipid syndrome or history of thrombosis, estimated glomerular filtration rate <45 mL/min/1.7 m^2 or proteinuria >2000 mg/d, or active lupus nephritis, which may require induction therapy.

Pharmacokinetic assessments

One predose blood sample was collected at week 4, week 12 and week 24 for pharmacokinetic analysis. Iberdomide concentration was determined by a validated assay.[14] A population pharmacokinetic analysis was performed (see online supplemental methods), and individual oral clearance values were used to calculate area under the concentration–time curve.

Pharmacodynamic assessments

Blood samples were collected at baseline, week 4, week 12 and week 24 for analysis of whole blood leucocytes, plasma proteins and whole blood gene expression. Flow cytometry (Covance, Indianapolis, Indiana, USA) was used to analyse B cells (CD19+ and CD20+), T cells (including CD4+ and CD8+), plasmablasts, pDCs and myeloid dendritic cells (mDCs). T helper 17 (Th17) cells, regulatory T cells (Tregs) and T follicular helper (Tfh) cells were measured by epigenetic assays (Epiontis ID; Epiontis GmbH, Berlin, Germany), an approach that correlates strongly with flow cytometry.[15–17]

Plasma cytokines, IL-2, IL-10, IL-17A, IL-17F and the B lymphocyte stimulator (BLyS; *TNFSF13b*) were determined by the ultrasensitive Singulex assay (Erenna; EMD Millipore, Burlington, Massachusetts, USA). The DxTerity Autoimmune Profiler (DxTerity, Rancho Dominguez, California, USA) was used to analyse whole blood stabilised through direct collection into DxCollect tubes for subsequent gene expression using chemical ligation probe amplification technology for generating PCR products. The resultant PCR amplicons were then separated by capillary electrophoresis on the ABI 3500xL Dx Genetic Analyzer (ThermoFisher Scientific, Waltham, Massachusetts, USA) for the following gene modules: B cell (*CD19*, *BACH2* and *CD22*), type I IFN (*IFI27*, *IFI44*, *IFI44L* and *RSAD2*[18]), Ikaros (eQTL) type I IFN (*HERC5*, *IFI6*, *IFIT1*, *MX1* and *TNFRSF21*[9]) and T cell exhaustion (*CTLA4*, *IL7R*, *LAG3*, *PDCD1* and *ABCE1*[19]). Samples were also tested for *IKZF1* (Ikaros) and *IKZF3* (Aiolos) gene expression levels.

Cut-off values for each gene expression module were determined a priori based on an independent training data set from the peripheral blood samples of 96 patients with SLE who were receiving standard-of-care medications but not biologics (DxTerity). An exploratory analysis was conducted on study data for the type I IFN and Aiolos signatures using a bootstrapping and aggregating of thresholds from trees procedure (see online supplemental methods).[20]

Given that greater clinical treatment effect was observed in patient subsets with elevated expression of type I IFN and Aiolos modules, we analysed biomarkers in these subsets at baseline and as median per cent change from baseline.

Statistical analyses

Pharmacokinetic analyses were performed for all patients who were randomised and received ≥1 dose of iberdomide with ≥1 quantifiable plasma concentration. Pharmacodynamic analyses included patients with a baseline value and a value at the time point reported. Data were reported as adjusted mean per cent changes from baseline. Treatment comparison of adjusted means was based on multiple imputation in conjunction with a regression model that used M-estimation, had the absolute value or change from baseline at a given time point as the response variable and adjusted for treatment group, baseline value and stratification factors. There was no correction for multiple comparisons.

RESULTS

Patients

A total of 288 patients received treatment. As reported elsewhere, baseline patient demographics and disease characteristics were balanced between treatment groups.[13] The proportions of patients with expression of specific gene modules were generally similar between the treatment groups (online supplemental table 1). High Aiolos gene expression was more common in the iberdomide 0.3 mg and 0.45 mg dose groups, and type I IFN module high expression was more common in the 0.45 mg group.

Pharmacokinetics

Iberdomide exhibited linear pharmacokinetics (online supplemental figure 1A). Exposure increased in a dose-related manner over the dose range of 0.15–0.45 mg once daily, with a 3-fold dose increase resulting in an approximately 2.5-fold increase in the area under the concentration–time curve at steady state. Age, body weight, creatinine clearance, race, sex, ethnicity and disease status did not have a clinically significant effect on iberdomide exposure. There were no differences in iberdomide pharmacokinetics between patients with low and high type I IFN signature or Aiolos expression at baseline (online supplemental figure 1BC).

Pharmacodynamics

At week 24, iberdomide significantly decreased CD19+ and CD20+ B cells and increased CD8+ cytotoxic T cells from baseline in a dose-dependent manner compared with placebo (figure 1). Iberdomide had no effect on the numbers of CD4+ Th cells or natural killer cells. The difference in adjusted mean per cent change from baseline to week 24 in B cells expressing CD268 (*TNFRSF13C*, encoding BLyS receptor) for iberdomide 0.45 mg compared with placebo was −58.3% ($p<0.001$) and for post-switched memory B cells was −40.8% ($p<0.001$). Significant treatment differences for iberdomide 0.45 mg were also noted for pDCs (−73.9%; $p<0.001$) and mDC 1 cells (−36.8%; $p=0.004$), Tregs (104.9%; $p<0.001$) and Tfh cells (+32.6%; $p<0.001$) at week 24 (figure 1). No significant changes were noted for plasmablasts or plasma cells, which were not significantly elevated at baseline, or Th17 cells.

Iberdomide increased IL-2 levels from baseline compared with placebo (figure 2). Iberdomide treatment resulted in a dose-dependent increase in IL-2, reaching +144.1% for the 0.45 mg dose ($p<0.001$), +91.7% for the 0.3 mg dose and +75.2% for the 0.15 mg dose versus placebo. No dose-dependent changes in IL-10, IL-17A, IL-17F, IL-21 or BLyS were noted.

Iberdomide decreased expression of gene modules representing the type I IFN, Ikaros eQTL type I IFN gene signature and B cell pathways and increased expression of Ikaros and Aiolos genes (figure 3). A dose–response relationship was noted for the B cell gene module but not for the type I IFN module.

The distribution of patient subsets by gene expression at baseline is shown in figure 4 with the type I IFN signature showing a biphasic distribution. Greater SRI-4 responses were noted in subsets having a high level of Aiolos and type I IFN gene expression at baseline (figure 5). In an exploratory analysis, the subset of patients in the 0.45 mg group with the highest expression of the type I IFN signature (baseline type I IFN gene signature >0.615) was found to have an SRI-4 response rate treatment difference of 54% at week 24 versus placebo (figure 6). Response rate plots (figure 7) showed that as the baseline IFN gene signature increased in magnitude, the week 24 SRI-4 response increased for iberdomide 0.45 mg up to 100% but decreased for placebo.

Analysis of changes in pharmacodynamic markers showed that patients with a high baseline type I IFN signature manifested a significant reduction in the IFN signature as a result of active treatment, whereas those with low baseline IFN signature did not. In contrast, both groups exhibited a significant reduction in B cells and pDCs and significant increases in IL-2 and Tregs (online supplemental figure 2). Baseline Aiolos expression had no impact on changes in type I IFN signature or any other pharmacodynamic parameter (online supplemental figure 3).

DISCUSSION

Pharmacodynamic analyses showed that iberdomide treatment reduced activity of the B cell and type I IFN pathways. These effects were evident in reductions in total B cells and B cells expressing the gene for the BLyS receptor and in switched memory B cells. Elevated BLyS levels have been documented in patients with SLE and shown to correlate with disease activity.[21] BLyS also induces plasmablast differentiation and drives autoantibody production in SLE.[4 21] Among patients with elevated anti-dsDNA antibodies at baseline, higher doses of iberdomide (0.3 mg and 0.45 mg) resulted in dose-dependent reductions versus placebo at week 24. Although there was no change in plasma cells in the blood, there may have been a change in plasma cell production of autoantibodies and/or plasma cells located in tissues. Treatment with iberdomide was associated with a significant, dose-dependent reduction in pDCs and mDCs, which are primary sources of type I IFNs.[1]

In patients with SLE, levels of IL-2 have been reported to vary.[4 22] A reduction in IL-2 production from T cells has been associated with impaired Treg development. Iberdomide has been shown to increase IL-2 production from T cells[5] and, in this clinical trial of SLE patients, iberdomide increased serum levels of IL-2 and expanded the Treg population in the blood. Ikaros is a repressor of IL-2 gene transcription,[6 23] and, therefore, reduction of Ikaros protein would be expected to result in transcriptional de-repression and an increase in IL-2 production. Because IL-2 is a major driver of Treg expansion and maintenance,[24] the observed increase in Tregs (up to +104.9%) could be explained by the increase in IL-2 (+144.1%). Besides the increase in IL-2, there were no dose-dependent effects of iberdomide on the other cytokines measured (IL-10, IL-17A, IL-17F, IL-21 and BLyS). No effect of iberdomide was observed on IL-17 plasma levels or Th17 cells, consistent with a lack of effect on the Th17 immune response in patients with lupus. These effects confirm the unique mechanism of action of iberdomide, suppressing dendritic cells and the type I IFN response, reducing B cells and anti-dsDNA antibodies, and augmenting IL-2 and Tregs, consistent with the role of Ikaros and Aiolos in immune homeostasis and with prior studies in healthy volunteers and patients with SLE.[4 5] The increase in *IKZF1* and *IKZF3* gene expression by iberdomide may be explained by the negative feedback each transcription factor can have on its own expression.[25]

The majority of patients enrolled in this trial had elevated expression of genes in the type I IFN and Ikaros pathways, which are typical of the SLE population.[26] Dysregulation of the type I IFN pathway can contribute to clinical features, immune dysregulation and laboratory manifestations in SLE.[27] However, the strongest association to gene expression changes is found with autoantibodies, which are influenced by patient ancestry.[28] In addition, patients with active SLE have decreased Treg numbers and function, as excess IFN prevents normal activation and expansion of Tregs in response to inflammation.[26] In the current study, a correlation analysis of baseline variations in

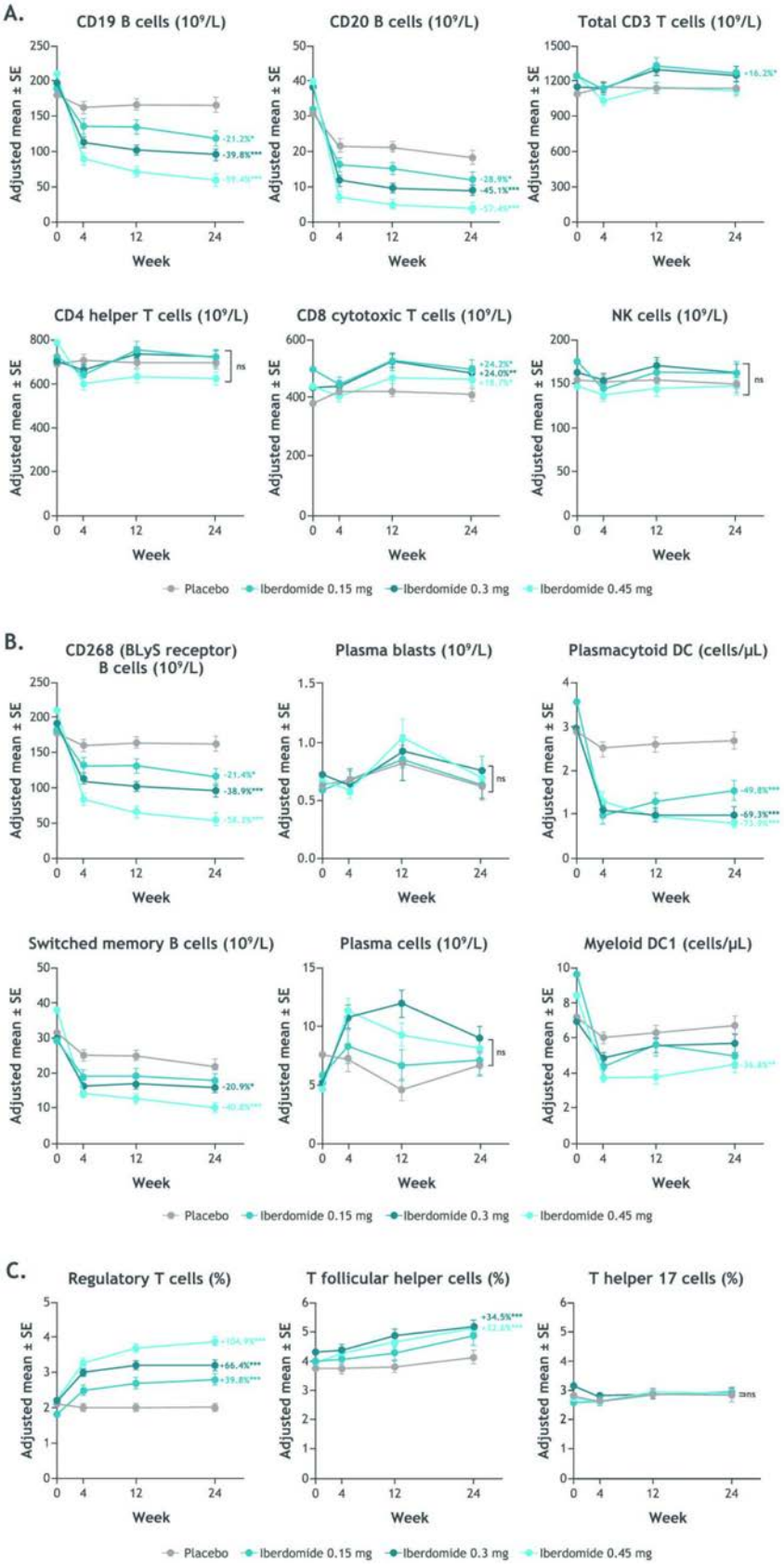

Figure 1 Time course of change from baseline during iberdomide treatment in whole blood leucocyte counts and selected B cells, T cells and NK cells by flow cytometry (Covance, Indianapolis, Indiana, USA) (A), CD268, plasma blasts, switched memory B cells DC subset counts and plasma cells by flow cytometry (B) and Tregs, Tfh cells and Th17 cells by epigenetic assay (Epiontis ID, Epiontis GmbH, Berlin, Germany) (C). *p≤0.05; **p≤0.01; ***p≤0.001 vs placebo. Values shown are the treatment comparison vs placebo of adjusted mean per cent change from baseline. See online supplemental table 2 for numeric data. BLyS, B lymphocyte stimulator; DC, dendritic cell; NK, natural killer; Tfh, T follicular helper; Th17, T helper 17; Tregs, regulatory T cells.

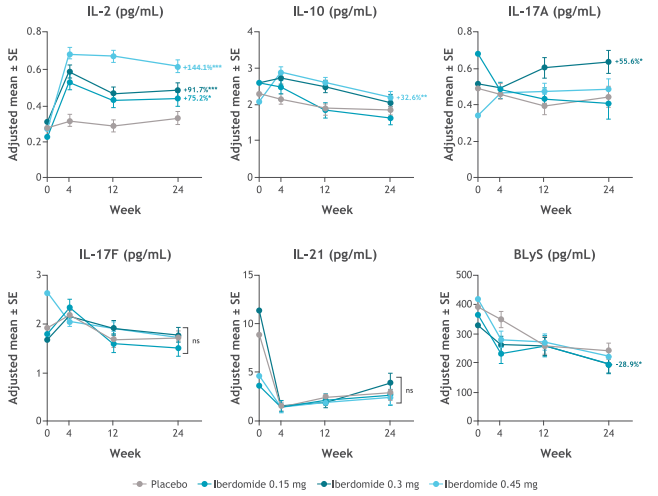

Figure 2 Change from baseline in plasma cytokines during iberdomide treatment by ultrasensitive cytokine assays (Erenna, EMD Millipore, Burlington, Massachusetts, USA). *p≤0.05; **p≤0.01; ***p≤0.001 vs placebo. Values shown are the treatment comparison vs placebo of adjusted mean per cent change from baseline. See online supplemental table 3 for numeric data. BLyS, B lymphocyte stimulator; IL, interleukin.

gene expression with clinical features found that the type I IFN gene module was directly proportional to SLEDAI and Cutaneous Lupus Erythematosus Disease Area and Severity Index (CLASI) score, and was higher in patients on oral corticosteroids or azathioprine. This is consistent with previous literature associating the type I IFN gene signature with more severe disease and use of corticosteroids and immunosuppressants.[29] Baseline Aiolos (*IKZF3*) gene expression was not proportional to SLEDAI

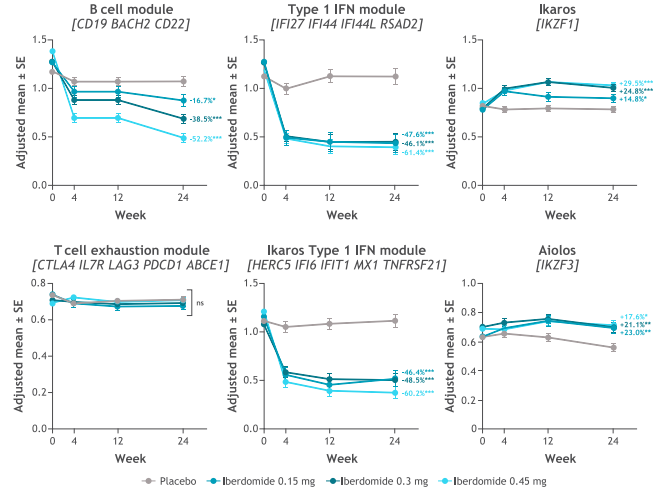

Figure 3 Change from baseline in whole blood gene expression during iberdomide treatment by multiplex PCR-based chemical ligation probe amplification target capture on the ThermoFisher ABI 3500xL DX Genetic Analyzer (DxTerity CLIA-certified laboratory)[a]. *p≤0.05; **p≤0.01; ***p≤0.001. [a]B cell module: *CD19*, *BACH2* and *CD22*; type I IFN module: *IFI27*, *IFI44*, *IFI44L* and *RSAD2*[18]; Ikaros type I IFN module: *HERC5*, *IFI6*, *IFIT1*, *MX1* and *TNFRSF21*[9]; and T cell exhaustion module: *CTLA4*, *IL7R*, *LAG3*, *PDCD1* and *ABCE1*.[19] Values shown are the treatment comparison vs placebo of adjusted mean per cent change from baseline. See online supplemental table 4 for numeric data. IFN, interferon.

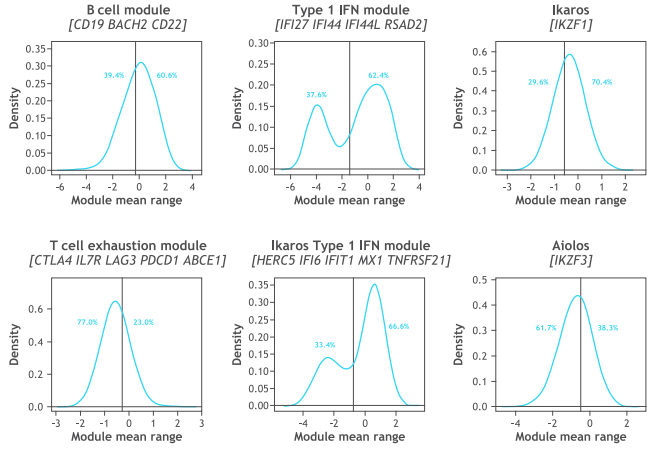

Figure 4 Patient subsets based on peripheral blood gene expression at baseline. The cut-offs were set a priori based on an independent training data set (96 samples from patients with SLE, data not shown). The type I IFN module and the Ikaros type I IFN (eQTL) module had bimodal distributions and the cut-offs were set at the antimode: type I IFN module (*IFI27*, *IFI44*, *IFI44L* and *RSAD2*)=−1.38; Ikaros type I IFN module (*HERC5*, *IFI6*, *IFIT1*, *MX1* and *TNFRSF21*)=−0.76. The distributions of Ikaros, Aiolos and B cell module were unimodal, and the cut-offs were set at the median: Ikaros (*IKZF1*)=−0.58; Aiolos (*IKZF3*)=−0.49; B cell module (*CD19*, *BACH2* and *CD22*)=−0.3; T cell exhaustion module (*CTLA4*, *IL7R*, *LAG3*, *PDCD1* and *ABCE1*)=−0.51. eQTL, expression quantitative trait locus; IFN, interferon.

or CLASI score and was not different in any subgroups based on medication (data not shown).

As previously reported,[13] iberdomide decreased anti-dsDNA antibodies among patients with high levels at baseline (≥8 IU/mL), with 0.45 mg decreasing levels by 61.2% (p=0.008) and 0.3 mg decreasing levels by 56.1% (p=0.027) compared with placebo. The clinical efficacy of iberdomide in patients with active SLE in this phase 2 study was greater among subgroups who had high expression of the type I IFN or Aiolos gene signature at baseline.[13] Moreover, exploratory analysis indicates that the highest cut point for the type I IFN subgroup (representing 31% of the total study population) was associated with the most enhanced relationship with response, providing a treatment difference of 54% versus placebo. At the extreme high IFN gene signature

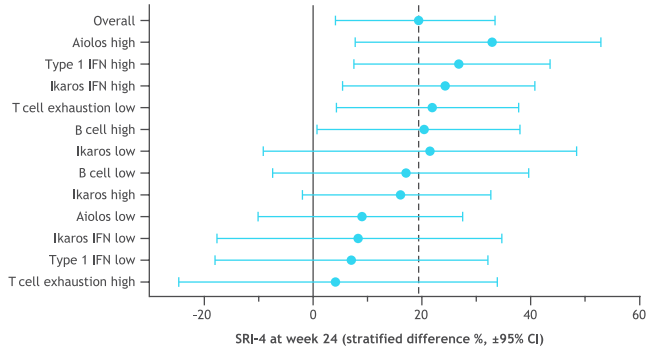

Figure 5 Clinical efficacy treatment comparison (week 24 SRI-4 response rate, iberdomide 0.45 mg—placebo) within prespecified patient subsets defined by gene expression at baseline. Gene module score cut-offs were set as described in figure 5. See online supplemental table 5 for numeric data. IFN, interferon; SLE, systemic lupus erythematosus; SRI-4, SLE Responder Index-4.

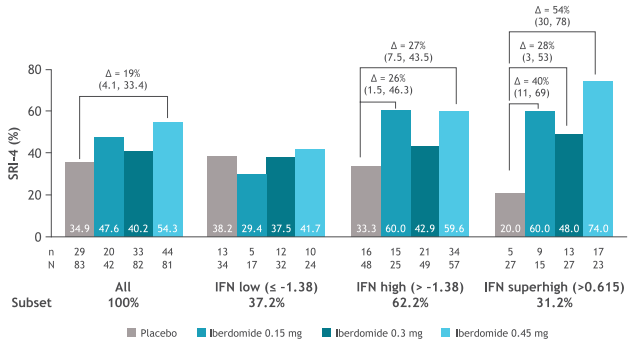

Figure 6 SRI-4 response rate at week 24 in the patient subsets defined by type I IFN gene signature at baseline. Δ=stratified difference from placebo (95% CI); n=number of responders; N=number of patients per subset within each treatment group. IFN, interferon; SLE, systemic lupus erythematosus; SRI-4, SLE Responder Index-4.

(expressed by 14% of patients), 100% (11/11) of patients had an SRI-4 response to iberdomide 0.45 mg, suggesting that the SRI-4 clinical response rate to iberdomide is proportionate to the baseline expression level of the type I IFN gene signature. Iberdomide significantly decreased the type I IFN gene signature only in the IFN-high patient subgroup, which corresponded to stratified treatment differences for SRI-4 in the IFN-high group ranging from 25.6% to 26.8% versus placebo. Iberdomide did not significantly reduce the type I IFN gene signature in the IFN-low patient population, with no significant differences in SRI-4 from placebo in this subgroup. In other studies, the relationship between IFN gene signature and disease activity has varied, a finding that is likely a result of disease and gene expression heterogeneity as well as differences in the methods used to define gene signatures across studies. In several cross-sectional

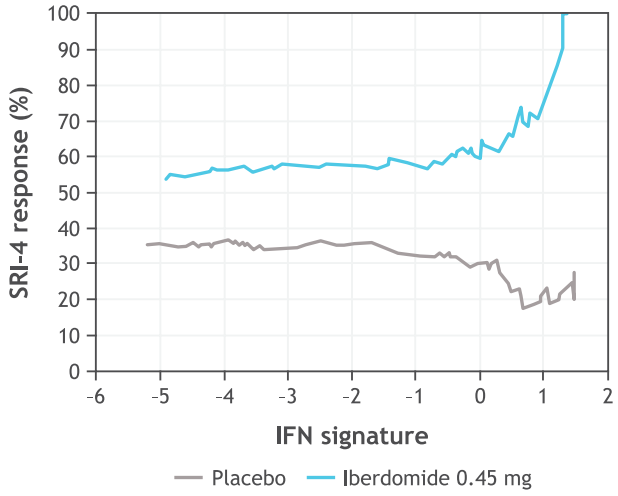

Figure 7 Relationship between baseline type I IFN signature and SRI-4 response rates at week 24 comparing placebo and iberdomide 0.45 mg treated SRI-4 cumulative response rates across the range of baseline type I IFN signature values (*IFI27*, *IFI44*, *IFI44L* and *RSAD2*). In exploratory analysis using bootstrapping and aggregating of thresholds from trees, the type I IFN signature optimal cut point was at 0.615 (interaction p=0.0037), SRI-4 at 0.45 mg=74% vs placebo=20%, OR=11.3 (2.9–43.8). this 'IFN-Superhigh' cut point captured 90/288 (31%) patients. At the extreme IFN >1.31 (top 14% of patients), in the iberdomide 0.45 mg group, 11/11 (100%) patients had an SRI-4 response. IFN, interferon; SLE, systemic lupus erythematosus; SRI-4, SLE Responder Index-4.

gene expression studies, the type I IFN gene signature has identified a distinct subset of lupus patients who have greater disease severity and a worse clinical prognosis.[30 31] In a recent longitudinal study, the type I IFN gene signature was prognostic for early development of lupus nephritis after adjusting for age at SLE diagnosis, gender and race (HR: 3.36).[32]

The pharmacodynamic and pharmacokinetic analyses were conducted based on 24 weeks of iberdomide treatment. Longer-term treatment or discontinuation effects were not evaluated. Patients continued to receive standard-of-care medications, including corticosteroids with no mandatory tapering, but the results of iberdomide pharmacodynamic analyses were as predicted, suggesting that background treatment did not impact results. Other factors, including concomitant medication use (antimalarials and immunosuppressants) and ancestral diversity, may impact our findings. Additional analyses of the pharmacodynamic effects could examine the influence of baseline disease characteristics and other response measurements. Results of exploratory cut point analyses require validation in future studies.

The most common adverse events with iberdomide (urinary tract infection, upper respiratory tract infection, neutropenia, influenza, nasopharyngitis and diarrhoea)[13] might be related to the modulatory effects of iberdomide on innate or adaptive immunity.

In conclusion, iberdomide showed significant improvement in the treatment of patients with active SLE.[12 13] Predominant pharmacologic activity was observed on the type I IFN and B cell/plasma cell pathways, leading to reductions in B cells, pDCs and autoantibody levels. Increased levels of Tregs and IL-2 suggest immune system rebalancing. An elevated type I IFN gene signature was associated with improved response and the largest change from baseline to week 24 in the gene signature expression. These findings may provide an opportunity to implement precision medicine to evaluate therapy on a molecular basis and potentially identify biomarkers associated with response to iberdomide for evaluation in future clinical studies.

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Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval Study procedures were conducted in accordance with the general ethical principles of the International Conference on Harmonisation guidelines and the Declaration of Helsinki for Good Clinical Practice. All patients provided written informed consent. The study was approved by an institutional review board at each site.

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TRANSLATIONAL SCIENCE

Longitudinal analysis of ANA in the Systemic Lupus International Collaborating Clinics (SLICC) Inception Cohort

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ABSTRACT

Objectives A perception derived from cross-sectional studies of small systemic lupus erythematosus (SLE) cohorts is that there is a marked discrepancy between antinuclear antibody (ANA) assays, which impacts on clinicians' approach to diagnosis and follow-up. We compared three ANA assays in a longitudinal analysis of a large international incident SLE cohort retested regularly and followed for 5 years.

Methods Demographic, clinical and serological data was from 805 SLE patients at enrolment, year 3 and 5. Two HEp-2 indirect immunofluorescence assays (IFA1, IFA2), an ANA ELISA, and SLE-related autoantibodies were performed in one laboratory. Frequencies of positivity, titres or absorbance units (AU), and IFA patterns were compared using McNemar, Wilcoxon and kappa statistics, respectively.

Results At enrolment, ANA positivity (≥1:80) was 96.1% by IFA1 (median titre 1:1280 (IQR 1:640–1:5120)), 98.3% by IFA2 (1:2560 (IQR 1:640–1:5120)) and 96.6% by ELISA (176.3 AU (IQR 106.4 AU–203.5 AU)). At least one ANA assay was positive for 99.6% of patients at enrolment. At year 5, ANA positivity by IFAs (IFA1 95.2%; IFA2 98.9%) remained high, while there was a decrease in ELISA positivity (91.3%, p<0.001). Overall, there was >91% agreement in ANA positivity at all time points and ≥71% agreement in IFA patterns between IFA1 and IFA2.

Conclusion In recent-onset SLE, three ANA assays demonstrated commutability with a high proportion of positivity and titres or AU. However, over 5 years follow-up, there was modest variation in ANA assay performance. In clinical situations where the SLE diagnosis is being considered, a negative test by either the ELISA or HEp-2 IFA may require reflex testing.

Key messages

What is already known about this subject?

⇒ Cross-sectional data of small cohorts suggest significant variation in the performance of antinuclear antibody (ANA) assays from different manufacturers leaving clinicians uncertain about the use or value of ANA testing in making a diagnosis.

What does this study add?

⇒ In a longitudinal analysis of well-characterised patients with incident systemic lupus erythematosus (SLE), almost all SLE patients early in disease had highly positive ANAs and no patients had tested within the normal range over 5 years of follow-up with all three assays.

⇒ As the disease evolved over 5 years of follow-up, there was decreased frequency of positive ANAs (above the normal range) and decreased ANA titres or absorbance units by some assays.

How might this impact on clinical practice or future developments?

⇒ In a patient without an established diagnosis of SLE and in whom the clinical suspicion for SLE is moderate to high, both immunofluorescence assay and ELISA should be performed if one or the other provides results in the normal range.

INTRODUCTION

Antinuclear antibody (ANA) testing is an integral approach to accurately diagnose and classify systemic lupus erythematosus (SLE).[1] A systematic review and meta-regression of indirect immunofluorescence assays (IFA) reported high sensitivity (97.8%) for SLE diagnosis at a titre of ≥1:80.[2] This presaged the decision to include a positive ANA at that titre on HEp-2 cell IFA 'or an equivalent positive test on other diagnostic platforms' occurring at least once as an entry criterion for the 2019 European League Against Rheumatism/American College of Rheumatology (EULAR/ACR) SLE Classification Criteria.[3 4]

Previous longitudinal examinations of ANA and SLE-related autoantibodies suggest that a patient's ANA status can change from positive to within the normal range and vice versa during the disease course.[2 5–16] However, these studies have typically been limited to small, single centre cohorts with incomplete disease characterisation, short follow-up, and/or using outdated assays with conflicting results. The factors influencing changes in ANA have also not been thoroughly studied. Taken together, this has left clinicians with uncertainty about the value and interpretation of ANA testing in making a diagnosis of, or classifying SLE. In addition, the clinically actionable value of repeat ANA testing once a diagnosis of SLE is established requires clarification.[17 18]

Much of the confusion and debate on the clinical utility of ANA testing in SLE is related to reported variations in HEp-2 IFA assay performance in cross-sectional cohorts,[19–22] and some have questioned whether the ANA IFA should continue to be the 'gold-standard' screening test.[23–25] For instance, in a cross-sectional study, Pisetsky *et al* tested the same sera using different ANA assays (e.g., IFA, enzyme-linked immunosorbent assay (ELISA) and multiplex bead assay)[21] and reported that the frequency of an ANA test within normal reference range in SLE patients with disease duration ranging from 0.1 to 33.4 years varied from 4.9% to 22.3%. Further, it has been proposed that the IFA could be replaced or complemented by newer generation solid phase multianalyte immunoassays (SPMAI) such as ELISA and/or addressable laser bead immunoassays.[24–26] A recent systematic review and meta-regression analysis of ANA testing in >13 000 SLE patients with disease duration ranging from 0 to 17 years reported that only ~2.5% of these patients had an IFA ANA <1:80,[2] although a higher prevalence of ANA within the normal reference range has been reported in other cohorts including the Systemic Lupus International Collaborating Clinics (SLICC) Inception Cohort (6.2% were <1:160 at inception).[27]

The primary goal of this study was to gain a more thorough understanding of ANA detection and its clinical value by comparing the performance of three currently available ANA assays in a longitudinal analysis (at least 5 years) of a large multinational SLE inception cohort.

METHODS

Study population

Between 1999 and 2011, SLICC (https://sliccgroup.org)[28] enrolled 1827 patients fulfilling the 1997 Updated ACR SLE Classification Criteria for definite SLE[29] within 15 months of diagnosis from 31 medical centres in 11 countries. Sera, clinical and demographic data were collected at enrolment and annually thereafter. Of the 1827 patients, 1432 (78.4%) were followed for ≥4 years; of these 1432 patients, we included the 805 patients who provided an enrolment and two additional serum samples within 5 years of enrolment, with the third sample being ≥4 years after enrolment. Permission from the SLICC Biological Material and Data Utilisation Committee was obtained to access the required data and biobanked serum samples.

ANA and autoantibody testing

Aliquots of sera were obtained from the 805 patients in the SLICC Inception Cohort at three time points: (1) enrolment (sample #1), (2) 2–4 years after enrolment (sample #2) and (3) 4–10 years after enrolment (sample #3). Hereafter, samples #1–3 are referred to as enrolment, year 3 and year 5, respectively. Samples were stored at −80°C until required for immunoassays and analysed centrally at MitogenDx Laboratory (Calgary, Canada). Three US Food and Drug Administration-approved and Conformitè Europëenne (CE) marked ANA tests were used, including two HEp-2 IFA, IFA1 (Bio-Rad Laboratories, Hercules, USA) and IFA2 (NovaLite, Werfen, San Diego, USA), and an ELISA (Werfen, San Diego, USA). In accordance with the manufacturers' directions, a positive test was defined as a titre of ≥1:80 for IFA1 and IFA2 (titre <1:80 is considered normal range) and ≥20 absorbance units (AU) for ELISA. IFA1, IFA2, and ELISA were tested on the full patient cohort (n=805) sera from all three time points. IFA results (titres and patterns) were initially read by an automated digital IFA microscope and then checked manually by a technologist with 30 years of experience. ANA IFA patterns were classified according to the new International Consensus on ANA Patterns (ICAP) recommendations (http://www.anapatterns.org/index.php).[30] Quality control was performed by repeating all ANA results that were within the normal range and a random selection of ANA-positive samples to ensure intertest reliability. SLE-related autoantibodies (online supplemental table 1) were also performed on each patient at enrolment, year 3 and 5.

Clinically defined samples

Demographic and clinical data (online supplemental table 2) at enrolment included age, sex, disease duration, race/ethnicity, nephritis (fulfilling the ACR criterion for renal disease or based on a renal biopsy), ACR Classification Criteria, Systemic Lupus Erythematosus Disease Activity Index—2000 (SLEDAI-2K), SLICC/ACR Damage Index (SDI) and medication use (current and ever use of glucocorticoids, antimalarials, and immunosuppressives, including biologics). We also collected longitudinal data on nephritis, SLEDAI-2K, SDI and medications.

Statistical analysis

Demographic, clinical and serological characteristics were described using summary statistics. Changes over time in demographic and clinical features were described using differences in means or proportions, with 95% CIs. As our analysis used a subgroup of the larger SLICC cohort based on sera availability, we compared the enrolment characteristics of the 805 patients included in this study with the 627 patients who were followed for ≥4 years but were not included because three serial serum samples were unavailable. We also compared the characteristics of the 781 patients providing the third serum sample 4–7 years after enrolment with the 24 patients providing the third serum sample 8–10 years after enrolment.

We assessed the frequency of ANA positivity and titre or AU at each time point. Using the paired McNemar's test, we calculated changes in ANA positivity between enrolment and year 5 for each test and the intertest agreement in ANA positivity between tests at each time point. A histogram with a curve of best fit line was used to plot the changes in distribution of titres and AU over time and were compared using the Wilcoxon signed rank test for paired data. We examined the frequency of each ANA pattern and how many patients retained their HEp-2 IFA pattern over the three serial samples. ANA patterns were further categorised into three groups using ICAP nomenclature: (1) isolated nuclear (AC 1–14, 29), (2) isolated cytoplasmic and/or mitotic (CMP, AC 15–28) and (3) mixed nuclear and CMP patterns. Agreement between IFA1 and IFA2 ANA titres and patterns was assessed using the weighted and unweighted kappa (κ) statistic, respectively. Established SLE-related autoantibody profiles of patients with an ANA result within the normal range on IFA1, IFA2, or ELISA alone, on two of three assays, and on all three assays at

Table 1 Patient characteristics at enrolment and year 5 (n=805)

Characteristic	Enrolment	Year 5	Difference* (95% CI)
Demographic and clinical			
Mean age at dx, years (SD)	35.2 (13.6)		
Female, %	88.7		
Mean disease duration, yrs (SD)	0.58 (0.49)		
Mean number of ACR Criteria without ANA (SD)	3.9 (1.0)		
Ethnicity, %			
Asian	24.3		
African	13.5		
White	52.3		
Hispanic	6.3		
Other ethnicities†	3.5		
Nephritis‡	28.9	36.6	**7.7 (5.7 to 9.7)**
Mean total SLEDAI-2K (SD)§	5.4 (5.3)	3.0 (3.5)	**−2.3 (−2.7 to -1.9)**
Mean total SDI (SD)¶	0.34 (0.74)	0.86 (1.25)	**0.52 (0.43 to 0.62)**
Medications			
Current, %			
Glucocorticoids	69.6	56.8	**−12.8 (−16.5 to to -9.1)**
Antimalarials	70.1	79.4	**9.3 (5.9 to 12.7)**
Immunosuppressants	41.0	50.8	**9.8 (6.1 to 13.5)**
Ever, %			
Glucocorticoids	81.5	87.3	**5.8 (4.1 to 7.6)**
Antimalarials	76.6	91.1	**14.4 (11.9 to 17.0)**
Immunosuppressants	43.9	66.3	**22.5 (19.5 to 25.5)**
Autoantibodies, %			
dsDNA**	34.2	29.1	**−5.1 (−8.7 to -1.6)**
Ribosomal P	24.3	20.0	**−4.3 (−7.8 to -0.9)**
Ro52/TRIM21	37.5	37.4	−0.1 (−3.4 to 3.2)
SSA/Ro60	42.5	42.0	−0.5 (−3.7 to 2.7)
SSB/La	20.7	16.3	**−4.5 (−7.5 to -1.5)**
Sm	22.7	14.7	**−8.1 (−11.1 to -5.0)**
U1RNP	28.2	23.0	**−5.2 (−8.5 to -2.0)**
Histones	31.3	22.7	**−8.6 (−12.1 to -5.0)**
Cardiolipin IgG/IgM††	20.5	16.4	**−4.1 (−7.7 to -0.6)**
β2GP1 IgG/IgM††	19.8	12.9	**−6.9 (−9.8 to -4.0)**
Lupus anticoagulant‡‡	20.6	16.7	−3.9 (−9.8 to 2.0)

Bold values indicate that the difference was statistically significant p<0.05.
*Difference between enrolment and year five visit.
†Other ethnicities include: Native North American, Native Hawaiian or other Pacific Islanders.
‡Nephritis defined as fulfilling the ACR criterion for renal disease or if a renal biopsy was performed prior to cohort entry.
§Complete data available for n=793 patients.
¶Complete data available for n=380 as the disease needs to be present for at least 6 months before the SDI can be calculated.
**Complete data available for n=798 patients.
††Complete data available for n=800.
‡‡Complete data available for n=282.
ACR, American College of Rheumatology; ANA, antinuclear antibodies; CI, confidence interval; dsDNA, double-stranded DNA; dx, diagnosis; IgG/M, immunoglobulin G/immunoglobulin M; RNP, ribonucleoprotein; SD, standard deviation; SDI, SLICC/ACR Damage index; SLEDAI-2K, systemic lupus erythematosus disease activity index-2000; Sm, Smith; TRIM21, Tripartite Motif Protein (TRIM) 21; β2GP1, β2-glycoprotein-1.

enrolment and year 5 were examined to understand which autoantibodies were not being captured by the ANA screening assays. Statistical analysis was performed using Stata V.15.1 (StataCorp).

RESULTS

Study population

Eight hundred and five SLE patients were included. The mean time from disease diagnosis to enrolment was 0.58 years (SD 0.49); the mean time between the enrolment and the year 3 sample was 2.8 years (SD 0.8) and between the enrolment and the year 5 sample was 5.0 years (SD 1.1). Patients had a mean age at diagnosis of 35.2 years (SD 13.6), 88.7% (714/805) were female and 47.7% (384/805) were of race/ethnicity other than White (table 1). From enrolment to year 5, the prevalence of lupus nephritis increased by 7.7% (95% CI 5.7% to 9.7%), mean SLEDAI-2K decreased by -2.3 (95% CI -2.7 to -1.9), and mean SDI increased by 0.52 (95% CI 0.43 to 0.62). There were significantly fewer patients on glucocorticoids (69.6% vs 56.8%, difference −12.8% (95% CI −16.5% to −9.1%)) and more patients on antimalarials (70.1% vs 79.4%, difference 9.3% (95% CI 5.9% to 12.7%)) or immunosuppressants (41.0% vs 50.8%, difference 9.8% (95% CI 6.1% to 13.5%)). The frequency of most SLE-related autoantibodies decreased at year 5.

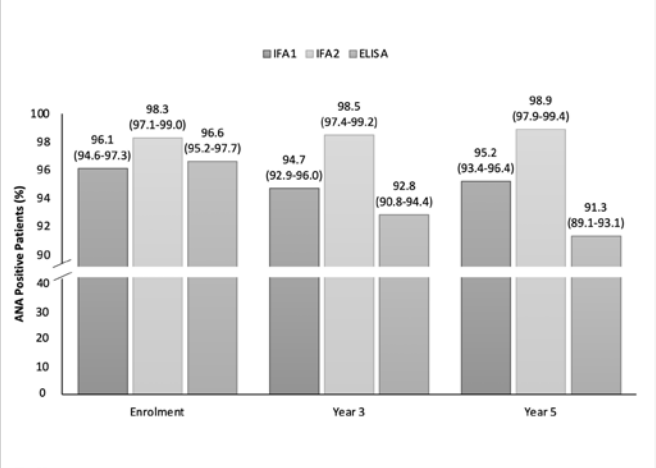

Figure 1 ANA positivity among IFA1 (n=805), IFA2 (n=805) and ELISA (n=805) at enrolment, year 3 and year 5. There is a break in the y-axis between 40% and 90% to enhance the readability of the graph from 90% to 100%. ANA, antinuclear antibody; IFA, indirect immunofluorescence assay.

The enrolment characteristics of the 805 patients included in our study were similar to the 627 patients who provided ≥4 years of data but did not have three available serial serum samples (online supplemental table 3). However, there was a higher proportion of Asian (18.8% (95% CI: 15.3% to 22.2%)) and lower proportion of Hispanic participants (−20.6% (95% CI −24.5% to −16.8%)) in the study cohort compared with the cohort not providing serial samples. The enrolment characteristics of the 781 patients whose year 5 sample was collected between years 4 and 7 were similar to the 24 patients whose year 5 sample was collected between years 8 and 10 (online supplemental table 4).

ANA positivity and agreement among different assays over time

At enrolment, the frequency of ANA positivity by IFA1, IFA2 and ELISA was high (96.1% (95% CI 94.6% to 97.3%), 98.3% (95% CI 97.1% to 99.0%) and 96.6% (95% CI 95.2% to 97.7%)), respectively) (figure 1) and 99.6% (802/805) of patients had ≥1 positive ANA of ≥1:80. An additional five (0.6% incremental effect), three (0.5%), and two patients (0.4%) at enrolment, year 3 and year 5 visits, respectively, were ANA positive on the ELISA, but within the normal range for both IFA1 and IFA2. There was no significant change in ANA positivity at enrolment compared with year five for IFA1 or IFA2. However, ANA positivity by ELISA decreased significantly from enrolment to year 5 (difference −5.3% (95% CI −7.4% to −3.3%), p<0.001) such that 91.3% (735/805) of patients were positive by year 5. Notably, 1.2% (10/805) of subjects were within the normal range at all three time points by ELISA compared with 0.9% (7/805) by

Table 2 ANA intertest percentage agreement among IFA1 (n=805), IFA2 (n=805) and ELISA (n=805)

	Enrolment (%)		Year 3 (%)		Year 5 (%)	
	IFA1	IFA2	IFA1	IFA2	IFA1	IFA2
IFA2	96.4% (94.9–97.6)		95.2% (93.4–96.5)		95.5% (93.9–96.8)	
ELISA	94.8% (93.0–96.2)	95.7% (94.0–97.0)	91.2% (89.0–93.0)	92.5% (90.5–94.3)	91.4% (89.3–93.3)	91.2% (89.0–93.0)

ANA, antinuclear antibodies; ELISA, enzyme-linked immunosorbent assay; IFA, indirect immunofluorescence assay.

IFA1 and 0.1% (1/805) by IFA2. At all time points, no patients were classified as being within the normal range if all three of the assays were considered.

Overall, the intertest agreement for positivity between any pair of assays was >91% (table 2). In cases where there was disagreement between IFA1 and IFA2, there was significant asymmetry (McNemar's test) such that most disagreements were due to more patients with an ANA by IFA1 within the normal range and a positive ANA by IFA2 (−IFA1/+IFA2) rather than a positive ANA by IFA1 and an ANA within the normal range by IFA2 (+IFA1/−IFA2) for all three time points (online supplemental table 5). Regarding the disagreements between IFA1 and ELISA, there was no significant asymmetry until year 5 when there were more cases of disagreement due to +IFA1/−ELISA compared with −IFA1/+ELISA. For disagreements between IFA2 and ELISA, there was significant asymmetry across all time points with more cases of +IFA2/−ELISA than −IFA2/+ELISA.

ANA titres or absorbance units among different assays over time

At enrolment, the median ANA titre or AU for IFA1, IFA2 and ELISA were 1:1280 (IQR 1:640–1:5120), 1:2560 (IQR 1:640–1:5120) and 176.3 AU (IQR 106.4 AU–203.5 AU), respectively (figure 2). The distribution of ANA titres or AU was skewed to the left for all assays at enrolment (higher proportion of patients with very high ANA titres or AU). Only a small proportion had ANA titres of 1:80 to 1:160 at enrolment (IFA1 10.4% (84/805) and IFA2 8.1% (65/805)). The median titres or AU at year five were significantly lower compared with enrolment for IFA1 (1:640 (IQR 1:320–1:2560), paired Wilcoxon signed rank p<0.0001, a change in one dilution step) and ELISA (157.3 AU (IQR 66.14 AU– 200.65 AU), p<0.0001)). There was good agreement between IFA1 and IFA2 titres at enrolment, 84.9% (95% CI 82.2% to 87.3%) agreement, k=0.49 (95% CI 0.45% to 0.53%); at year 3, 81.1% (95% CI 78.2% to 83.7%) agreement, k=0.39 (95% CI 0.35% to 0.43%) and at year 5, 82.0% (95% CI 79.1% to 84.6%) agreement, k=0.41 (95% CI 0.37% to 0.45%).

ANA patterns among different assays over time

The most common ANA IFA pattern was an isolated nuclear staining pattern for IFA1 (62.1%–68.7%) and IFA2 (59.3%–62.1%) at all visits (table 3). The top three individual IFA patterns for both IFA1 and IFA2 were AC-1 (homogeneous), AC-4 (nuclear fine specked) and AC-5 (nuclear large speckled) (online supplemental figure 1). There was fair-to-moderate agreement between IFA1 and IFA2 ANA IFA staining patterns at enrolment, (74.0% (95% CI 70.7% to 77.0%) agreement, κ=0.46 (95% CI 0.39 to 0.53)), year 3, (71.4% (95% CI 68.0% to 74.6%), κ=0.39 (95% CI 0.33 to 0.46)) and year 5 (71.0% (95% CI 67.7 to 74.2), κ=0.39 (95% CI 0.33 to 0.46)).

ANA patients within the normal range and seroconversion

At enrolment and year 5, 8 and 20 patients were within normal range by IFA1 and ELISA, 3 and 4 patients by ELISA and IFA2, and 8 and 6 patients by IFA1 and IFA2 (table 4). When examining the autoantibody profiles of patients whose ANA were within normal range at enrolment or year 5, depending on the assay 38.7%–53.8% had no detectable SLE-related autoantibodies. Anti-Ro52/TRIM21 and anti-SSA/Ro60, the former not detectable by HEp-2 IFA and the latter does not have a clearly established IFA pattern, were the most frequent autoantibodies detected when the ANA test was within normal range. Seroconversion from ANA positive to normal range (titre <1:80) from enrolment to year 5 was observed in 4.8% (39/805) of patients using IFA1, 1.1% (9/805) using IFA2, and 8.7% (70/805) using ELISA. The median titre of ANA at enrolment prior to seroconversion was low (IFA1 1:160 (IQR 1:80–1:640)), IFA2 1:320 (IQR 1:160–1:2560), and ELISA 61.5 AU (IQR 20 AU–158 AU)).

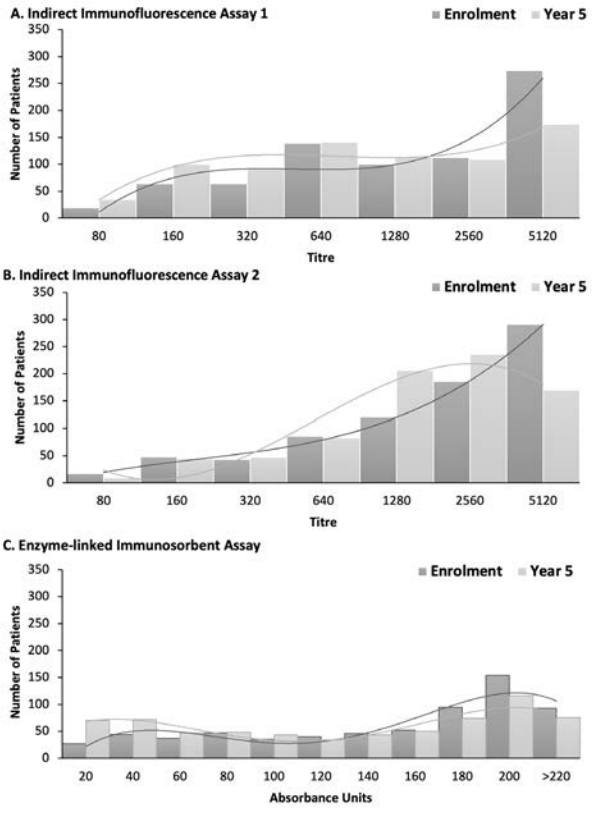

Figure 2 Distribution of ANA titres at enrolment and year 5 visit for (A) IFA1 (n=805), (B) IFA2 (n=805) and C) ELISA (n=805). Lines represent the curve of best fit. ANA, antinuclear antibody; ELISA, enzyme-linked immunosorbent assay; IFA, indirect immunofluorescence assay.

Table 3 ANA patterns over time with indirect immunofluorescence assay (IFA) 1 (n=805) and IFA2 (n=805)

Pattern	Enrolment n (%)	Year 3 n (%)	Year 5 n (%)	Same ANA pattern over 5 years n (%)
IFA1 patterns				
Nuclear	481 (62.1)	519 (68.1)	526 (68.7)	305 (37.9)
Cytoplasmic ± Mitotic	17 (2.2)	18 (2.4)	21 (2.7)	1 (0.1)
Mixed	276 (35.7)	225 (29.5)	219 (28.6)	81 (10.1)
IFA2 patterns				
Nuclear	491 (62.1)	477 (60.4)	472 (59.3)	273 (33.9)
Cytoplasmic ± mitotic	9 (1.1)	6 (0.8)	4 (0.5)	0 (0.0)
Mixed	291 (36.8)	308 (38.8)	320 (40.2)	114 (14.2)
IFA1 and 2 agreement (k)				
Agreement (95% CI)	74.0 (70.7–77.0)*	71.4 (68.0–74.6)*	71.0 (67.7–74.2)*	
Kappa (95% CI)	0.46 (0.39–0.53)	0.39 (0.33–0.46)	0.39 (0.33–0.46)	

*P<0.0001 using unweighted kappa (k) statistics.
ANA, anti-nuclear antibodies; IFA, indirect immunofluorescence assay.

Among those who were originally anti-dsDNA positive at enrolment (n=273, 34.2%), the frequency of ANA positivity was high at enrolment irrespective of the ANA assay (99.3%–100.0%). At year 5, frequency of ANA positivity for these same patients, irrespective of their anti-dsDNA status at year 5, declined slightly using for the IFAs (IFA1 −2.2%, IFA2 −1.1%) and −4.8% for the ELISA (data not shown).

DISCUSSION

To our knowledge, this is the largest longitudinal, multinational study (805 patients and 2415 serum samples) that compared the performance of different ANA assays in a well-characterised inception cohort of SLE patients. Our study was designed to overcome the limitations of prior reports that studied smaller cohorts and were historical and/or cross-sectional in nature. These data are timely given ANA test positivity is an entry criterion for the 2019 EULAR/ACR classification criteria for SLE.[31 32] We found that, regardless of the assay, almost all patients with recent onset SLE (802/805) had a positive ANA at enrolment on ≥1 assay, all were ANA-positive on ≥1 assay at least once across the 5 years, and the mean ANA titres or AU were high. However, over the 5 years, some variation between ANA assay performance was detected, including a statistically significant decrease in ELISA ANA positivity and reduction in titres or AU for IFA1 and ELISA.

It has been suggested that the variation in performance between different ANA assays may be related to differences in laboratory techniques, equipment, interobserver consistency and reagents.[25 33] However, in our study, all ANAs were performed and interpreted at one central laboratory by a highly experienced (30 years of experience) technician. Even after controlling for the impact of inter-laboratory and interobserver variation, we still identified some significant inter-assay disagreements. Disagreement between ELISA and IFA is likely primarily due to factors intrinsic to the test platforms themselves. Unlike the IFA, the ELISA contains extracts of cell homogenates augmented by purified proteins derived from native and/or synthetic, recombinant sources.[34] The composition of the different ELISA ANA preparations is diverse and dependent on the manufacturer as to which key target autoantigen(s) associated with autoimmune diseases are included and at what concentrations.[34] ELISAs may also have decreased detection of ANA because of poor autoantibody binding, as some antigens may also bind to other targets in the same mixture, resulting in a masking effect. Furthermore, many autoantibody targets are components of macromolecular complexes where key epitopes may be hidden or masked.[34] A thorough study of the affinity and avidity of the various autoantibodies would add useful understanding to the use of ANA ELISAs.

Prior studies of more established SLE patients reported that as high as 30% have an ANA below the positive threshold.[35] Over time, we observed a decrease in ANA positivity with ELISA, a decrease in ANA titres or AU with IFA1 and ELISA, and decreased detection of specific autoantibodies. We postulate that factors such as disease activity and medication exposure influence ANA.[36–39] However, the extent to which therapeutic interventions can alter ANA production, especially by long-lived plasma cells, remains to be proven and the expression of other autoantibodies can occur following diagnosis, possibly attributed to epitope spreading continuing despite therapy.[39]

Our study addresses important questions raised about the ANA in the 2019 EULAR/ACR SLE classification criteria,[3 4 40] which require an 'ever positive' ANA of ≥1:80 by HEp-2 IFA or an equivalent test on another platform as an entry criterion for classification. For example, it is important to note that all subjects had at least one positive ANA at the 1:80 threshold over the 5 years of follow-up. The new criteria also state that a solid phase assay of at least equivalent performance can be used in place of the HEp-2 IFA, although a precise definition of 'equivalent performance' was not specified. Our results show that although some inter-assay disagreement exists between these three assays, >91% of recent-onset SLE patients will have a positive ANA using either HEp-2 IFA or ELISA, although titres or AU decreased by year 5 for IFA1 and the ELISA. As expected from previous reports,[20 41] ELISA had the highest proportion of SLE patients with an ANA within the normal <1:80 reference range, and therefore, the ELISA used as a screening test may benefit from judicious reflex testing to the HEp-2 IFA. In turn, since the HEp-2 IFA can be negative when the ELISA is positive, the reciprocal reflex approach could be considered.

Importantly, consistent with other studies and emerging recommendations on ANA testing,[20 41] we demonstrated that a combination of two different ANA assays reduced the proportion of SLE patients with ANAs in the normal range; particularly when IFA2 was combined with ELISA. A combination of all three assays resulted in no patients who had an ANA within normal range at enrollment and two subsequent follow-up visits. This helps shed light on the question of the value of ANA testing to follow the clinical course of SLE, but more detailed follow-up studies evaluating disease activity and flares at follow-up visits

Table 4 Autoantibodies detected in patients with an ANA that was within the normal range on IFA1, IFA2, ELISA, either alone, on two or all three assay at enrolment and year 5*

	ELISA		IFA1		IFA2		IFA1 and ELISA		ELISA and IFA2		IFA1 and IFA2		All three assays	
% Autoantibodies	Enrolment (N=27)	Year 5 (N=70)	Enrolment (n=31)	Year 5 (N=39)	Enrolment (N=14)	Year 5 (N=9)	Enrolment (N=8)	Year 5 (N=20)	Enrolment (N=3)	Year 5 (N=4)	Enrolment (N=8)	Year 5 (N=6)	Enrolment (N=3)	Year 5 (N=3)
None detected	44.4	45.7	38.7	53.8	42.9	44.4	62.5	65.0	66.7	50.0	50.0	50.0	66.7	66.7
dsDNA†	7.7	5.7	6.7	5.1	0.0	11.1	0.0	0.0	0.0	0.0	0.0	16.7	0.0	0.0
Ribosomal P	3.7	11.4	6.5	10.3	7.1	11.1	0.0	10.0	0.0	25.0	0.0	16.7	0.0	33.3
Ro52/ TRIM21	11.1	21.4	22.6	20.5	21.4	11.1	0.0	20.0	0.0	25.0	0.0	0.0	0.0	0.0
SSA/Ro60	7.4	12.9	25.8	10.3	21.4	11.1	0.0	5.0	0.0	0.0	12.5	0.0	0.0	0.0
SSB/La	7.4	7.1	0.0	5.1	0.0	0.0	0.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0
Sm	3.7	4.3	6.5	2.6	0.0	11.1	0.0	0.0	0.0	25.0	0.0	0.0	0.0	0.0
U1RNP	3.7	7.1	0.0	5.1	0.0	0.0	0.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0
Histones	0.0	10.0	0.0	2.6	7.1	11.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

*Patients who were within the normal range for ANA at enrolment are not necessarily the same patients at year 5 and vice versa.
†dsDNA was measured at enrolment for only 26 patients on ELISA, 13 on IFA2, and two on both who tested within the normal range for ANA.
ANA, antinuclear antibodies; dsDNA, double-stranded DNA; ELISA, enzyme-linked immunosorbent assay; IFA, indirect immunofluorescence assay; RNP, ribonucleoprotein; Sm, Smith; TRIM21, Tripartite Motif protein (TRIM) 21.

in the context of ANA testing are still required. Healthcare providers should be aware of the technical issues for ANA assays used in their jurisdictions and recognise that different ANA assays or simply following manufacturer's recommended reference ranges might not be optimal in applying ANA testing results.[42 43] Additional longitudinal studies comparing other ELISAs and SPMAI such as other multiplex bead immunoassays and emerging ANA technologies are needed.

Our study has some important strengths. To our knowledge, this is the largest study of ANA status in SLE patients with data collected longitudinally and in a protocolised fashion over a mean follow-up of 5 years. All ANA testing was conducted in an accredited central laboratory with stringent quality control. However, we acknowledge some important limitations. First, there may be a potential selection bias for SLE patients who were ANA positive to be enrolled into the SLICC cohort compared with patients in conventional clinical care. Second, as enrolment could occur up to 15 months after diagnosis (although mean disease duration at enrolment was 0.58 years), most patients had already been exposed to ≥1 immunomodulatory medication by enrolment, which could potentially influence the ANA result. Third, although we showed that demographic and clinical characteristics of the cohort subset with three available serum samples were largely similar to the remainder of the cohort, our sample included a larger proportion of Asian and fewer Hispanic participants. While our sample was racially and geographically diverse, it is not known if our findings are generalisable to other SLE cohorts. Fourth, the duration of follow-up, although relatively long at 5 years, does not capture potential seroconversions or measure assay performance later in the disease. Last, there are >10 different ANA immunoassays in use world-wide and our study used three. Regrettably, some manufacturers declined to participate in this study. Hence, generalisation to all ANA assays was not possible.[42 44]

In conclusion, we demonstrated that early in their disease course almost all adult SLE patients had highly positive ANAs. However, as the disease progressed, we observed increased frequency of ANA within the normal range and decreased ANA titres or AU by some assays likely related to differences in assay performance, medication exposure, decreased autoantibody responses over time and lower disease activity. Combining ANA assays resulted in fewer patients that tested within normal range and no patients who tested within the normal range over the 5 years with all three assays. A clinical implication of this study is that for patients who have a moderate-to-high suspicion of SLE, especially those early in the disease course but without an established diagnosis, screening on both ELISA and HEp-2 IFA is warranted if one or the other provides results in the normal range. Given the rather modest changes in ANA frequency (and/or titres or AU) observed in this longitudinal study of 5 years follow-up, it is difficult to perceive the actionable clinical value of ANA IFA or screening ELISA test results over this time period once the diagnosis of SLE has been established. Since there are differences in the performance characteristics of individual ANA assays, clinicians need to be aware of the performance characteristics of the ANA test that their laboratories use. Future studies testing the comparative performance of other ANA immunoassays over time in large populations will help inform approaches to an earlier and more accurate diagnosis and classification of SLE.

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TRANSLATIONAL SCIENCE

Auxilin is a novel susceptibility gene for congenital heart block which directly impacts fetal heart function

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ABSTRACT

Objective Neonatal lupus erythematosus (NLE) may develop after transplacental transfer of maternal autoantibodies with cardiac manifestations (congenital heart block, CHB) including atrioventricular block, atrial and ventricular arrhythmias, and cardiomyopathies. The association with anti-Ro/SSA antibodies is well established, but a recurrence rate of only 12%–16% despite persisting maternal autoantibodies suggests that additional factors are required for CHB development. Here, we identify fetal genetic variants conferring risk of CHB and elucidate their effects on cardiac function.

Methods A genome-wide association study was performed in families with at least one case of CHB. Gene expression was analysed by microarrays, RNA sequencing and PCR and protein expression by western blot, immunohistochemistry, immunofluorescence and flow cytometry. Calcium regulation and connectivity were analysed in primary cardiomyocytes and cells induced from pleuripotent stem cells. Fetal heart performance was analysed by Doppler/echocardiography.

Results We identified *DNAJC6* as a novel fetal susceptibility gene, with decreased cardiac expression of *DNAJC6* associated with the disease risk genotype. We further demonstrate that fetal cardiomyocytes deficient in auxilin, the protein encoded by *DNAJC6*, have abnormal connectivity and Ca^{2+} homoeostasis in culture, as well as decreased cell surface expression of the $Ca_v1.3$ calcium channel. Doppler echocardiography of auxilin-deficient fetal mice revealed cardiac NLE abnormalities in utero, including abnormal heart rhythm with atrial and ventricular ectopias, as well as a prolonged atrioventricular time intervals.

Conclusions Our study identifies auxilin as the first genetic susceptibility factor in NLE modulating cardiac function, opening new avenues for the development of screening and therapeutic strategies in CHB.

Key messages

What is already known about this subject?

⇒ Congenital heart block may develop after transplacental transfer of maternal autoantibodies.

⇒ A recurrence rate of only 12%–16% despite persisting maternal autoantibodies suggests that additional factors are required for congenital heart block (CHB) development.

What does this study add?

⇒ We here identify fetal genetic variants conferring risk of CHB and elucidate their effects on cardiac function.

How might this impact on clinical practice or future developments?

⇒ The findings open new avenues for the development of screening and therapeutic strategies in CHB.

INTRODUCTION

Neonatal lupus erythematosus (NLE) may develop in children of rheumatic women with autoantibodies to the Ro/SSA and La/SSB antigens.[1–4] The most common manifestations of NLE are skin rash and congenital heart block (CHB). While the former is most often benign and resolves as maternal autoantibodies are cleared from the child's circulation, the latter is characterised by an irreversible disruption of electric signal conduction at the atrioventricular (AV) node (third-degree AV block) and has a high mortality rate around 20% if left untreated,[5] with survivors often requiring pacemaker implants for the remainder of their life.[6 7]

CHB typically develops between weeks 18–24 of pregnancy and is often detected when the fetus presents with signs of bradycardia and complete AV block. The bradycardia is preceded and paralleled by other cardiac pathologies leading up to the end-stage third-degree AV block caused by fibrosis and calcification of the AV node.[8 9] Sinus node dysfunction, lower-degree AV block and a prolonged isovolumetric contraction time have thus been observed in early stages of CHB.[10–12] Up to 15%–20% of fetuses affected by CHB have also been shown to develop more diverse myocardial manifestations

before birth,[13 14] and signs of junctional ectopic tachycardia or ventricular tachycardia have been reported in nearly one third of fetuses with CHB.[15 16] CHB thus collectively refers to the spectrum of fetal cardiac manifestations occurring in neonatal lupus.

An association between CHB and the presence of maternal autoantibodies to the Ro/SSA autoantigen has long been established, and, when the diagnosis of fetal third-degree AV block without major malformations is established in utero, more than 95% of the mothers test positive for anti-Ro/SSA antibodies.[17] However, a recurrence rate of only approximately 12%–16% for second/third-degree AV block despite persisting maternal autoantibodies indicates that fetal susceptibility, governed by genetic factors, may contribute to disease development.[18–21] Fetal MHC alleles have been linked to the susceptibility, but no other genes thus far.[22–24] In this study, we, therefore, aimed at identifying genetic variants related to CHB by performing a genome-wide association study in families with at least one case of CHB, and sought to define the biological and functional relevance of identified candidate gene(s) for CHB.

PATIENTS AND METHODS

A detailed Patients and Methods section is available in online supplemental materials.

Study population and genotyping

The cohort of patients diagnosed with CHB (n=92) and their families has been previously described.[17 18] Briefly, AVB II–III in the index case and confirmed maternal Ro/SSA autoantibodies constituted inclusion criteria for a family, and families in which the index case had major cardiac structural abnormalities, postoperative or infection-induced block were excluded. Maternal diagnoses at the time of blood sampling were primary Sjögren's syndrome (n=14), SLE (n=12), SLE with secondary Sjögren's syndrome (n=18), rheumatoid arthritis (n=1), rheumatoid arthritis with secondary Sjögren's syndrome (n=1), while 39 mothers had no rheumatic diagnosis. Information was not available for two mothers. Anti-Ro52 autoantibodies were present in 96% of the mothers, anti-Ro60 in 61% and anti-La antibodies in 58%. Other analysed autoantibodies (anti-Histone, and-SmB, anti-SmD, anti-RNP, anti-Cenp-B and Ribosomal P) were present in less than 10% of the mothers (online supplemental table 1).

Genotyping was performed on the Illumina 660W-Quad Beadchip.

Statistical analysis

Genome-wide associations were analysed using PLINK. Statistica and SigmaPlot were used for analysing Doppler-recorded data. Graphpad Prism V.5 was used for all other statistical tests. The statistical tests used for analysis of data from individual experiments are stated in respective figure legend.

RESULTS

Identification of Auxilin/*DNAJC6* as a susceptibility gene for CHB

To identify genes that influence fetal susceptibility to CHB, we performed a genome-wide association study of >5 00 000 single-nucleotide polymorphisms (SNP) in a population-based cohort of families with children diagnosed with CHB. To segregate CHB-unique disease traits from potential inherited maternal traits reflecting the maternal rheumatic autoimmune status, we used a family-based study strategy and included SNP genotype data from index cases and their parents and unaffected siblings.

Analysing transmission of SNPs based on genotypes of index cases (n=92) and first-degree relatives (n=256) using the family-based association for disease trait (DFAM) method, we identified 32 polymorphisms associated with CHB at $p \leq 1 \times 10^{-4}$ (figure 1A, online supplemental figure 1 and online supplemental table 2). Subsequent validation analysis of these 32 CHB-associated polymorphisms in a population-based case-control (C-C) set-up confirmed the association of the locus on chromosome 1p31.3 at a higher level of significance (rs1570868, $P_{DFAM}=3\times10^{-5}$ and $P_{C\text{-}C}=6\times10^{-6}$), and verified suggestive associations in two other genomic regions 1q24.2 (rs7552323, $P_{DFAM}=3\times10^{-5}$, $P_{C\text{-}C}=2\times10^{-4}$) and 3p25.1 (rs1993331, $P_{DFAM}=5\times10^{-5}$ and $P_{C\text{-}C}=3\times10^{-4}$; rs2730335, $P_{DFAM}=5\times10^{-5}$ and $P_{C\text{-}C}=5\times10^{-4}$; and rs2730367, $P_{DFAM}=5\times10^{-5}$ and $P_{C\text{-}C}=2\times10^{-4}$) (figure 1B and online supplemental table 3). Parental transmission of the risk alleles to the affected individuals was 75% (95% CI 63.6% to 83.8%) for rs1570868, 80% (95% CI 60% to 71%) for rs7552323% and 78% (95% CI 64.4% to 87.3%) for rs1993331, rs2730335, and rs2730367, respectively (figure 1C and online supplemental table 2). ORs for the same SNPs in the validation analysis were 2.01 (95% CI 1.50 to 2.81) for rs1570868, 1.82 (95% CI 1.33 to 2.49) for rs7552323 and ranged between 1.82 and 1.90 (95% CI 1.30 to 2.67) for rs1993331, rs2730335 and rs2730367 (figure 1D and online supplemental table 3). Closer examination of the associated locus on chr 1p31.3 revealed the highest association with intronic variants in *DNAJC6* (figure 1E, online supplemental table 2).

Expression quantitative trait loci (eQTL) analysis in cardiac tissue of the genes present in the regions surrounding the top replicating SNPs (±500 kb) revealed a significant effect of rs1570868 on the expression of the *DNAJC6* gene, but not on the expression of other genes in the chromosomal interval (figure 2A). Interestingly, individuals carrying the risk allele at this position had a lower cardiac *DNAJC6* expression compared with carriers of the non-risk allele (figure 2B). Notably, *DNAJC6* expression in other tested tissues was not affected by the rs1570868 polymorphism (figure 2C and data not depicted).

DNAJC6 encodes the putative tyrosine-protein phosphatase auxilin, which is involved in clathrin-mediated endocytosis. Four protein-coding transcripts have been predicted for auxilin (figure 2D), and we could confirm expression of all four variants in cardiac tissue by qPCR (figure 2E, online supplemental figure 2). Auxilin-201 is the only transcript conserved between human and mouse, suggesting that it may be important functionally. Interestingly, analysis of transcript-specific auxilin expression according to the rs1570868 genotypes revealed that carriers of the CHB risk allele have a lower expression of auxilin-201 compared with carriers of the non-risk allele ($p<7\times10^{-4}$) (figure 2F). In contrast, cardiac expression levels of the three other transcript variants are not affected by the rs1570868 SNP.

Auxilin is highly expressed in the fetal heart and colocalised with clathrin in vesicular structures in primary cardiomyocytes

To address the functional basis for auxilin deficiency involvement in CHB, we first investigated whether auxilin is expressed in the heart during fetal development. We found that auxilin is indeed expressed in human fetal cardiac tissue both before and during the risk period for CHB development (figure 3A and online supplemental figure 3A-C). Interestingly, auxilin expression is remarkably higher in the fetal heart compared with the adult heart (figure 3A), as well as in comparison with other fetal tissues (figure 3B). Of note, cardiac tissue

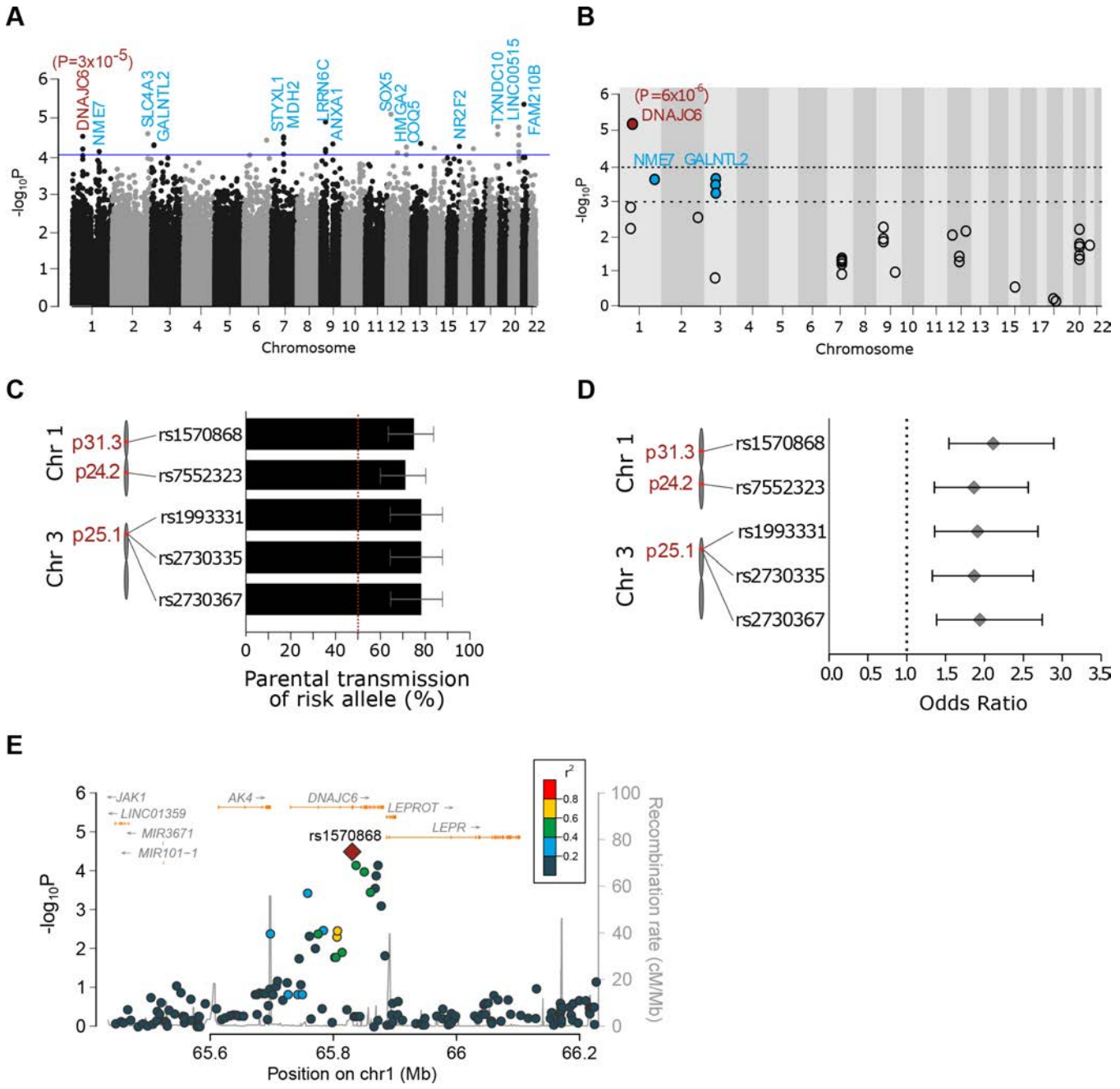

Figure 1 Identification of novel genomic loci associated with CHB. (A) Manhattan plot of genome-wide family-based association for disease trait (DFAM) transmission statistics based on SNP genotyping of CHB cases (n=92) and first-degree relatives (n=256). (B) Logistic regression analysis of association statistics for SNPs with $P_{DFAM} \leq 1\times10^{-4}$ comparing CHB cases (n=89) vs 1195 population-based out-of-study controls. Replicating polymorphisms with $p\leq1\times10^{-4}$ (red) and $p\leq1\times10^{-3}$ (blue). Approximate chromosomal positions are indicated. Dashed lines indicates $p=1\times10^{-4}$ and $p\leq1\times10^{-3}$ (A, B). (C) Percentage of parental transmission to CHB cases (95% CI) for replicating risk variants (DFAM analysis). Dashed line indicates 50% transmission. (D) OR (95% CI) for replicating risk variants (logistic regression). Dashed line indicates OR=1.0. (E) LocusZoom (http://locuszoom.org/) plot of the associated *DNAJC6* region on chromosome 1. CHB, congenital heart block; SNP, single-nucleotide polymorphisms;

expression profiling not only confirmed high expression of auxilin in fetal heart but also revealed that the homologous cyclin-G associated kinase (GAK), also denoted auxilin-2, is expressed only at low levels in the fetal heart (figure 3C). This relation is reversed in adult cardiac tissue, where auxilin is expressed at lower levels than GAK (figure 3D), suggesting that lack of auxilin may specifically affect the fetal rather than the adult heart. Auxilin expression was confirmed at RNA expression level (online supplemental figure 3D-F) and the protein level by immunoblotting of human fetal cardiac tissue and in cardiomyocytes (figure 3E–I). Ubiquitous expression of auxilin was observed throughout the fetal heart by immunohistochemistry (figure 3J), and these data were confirmed by similar auxilin expression levels in human fetal cardiac tissue surgically dissected from the apical myocardium and from the AV node (figure 3K and online supplemental figure 3G-I). Immunofluorescence staining of single cell preparations of human fetal cardiomyocytes demonstrated subcellular localisation of auxilin, which is present in the cytoplasm in a vesicular pattern and partly colocalises with clathrin (figure 3L).

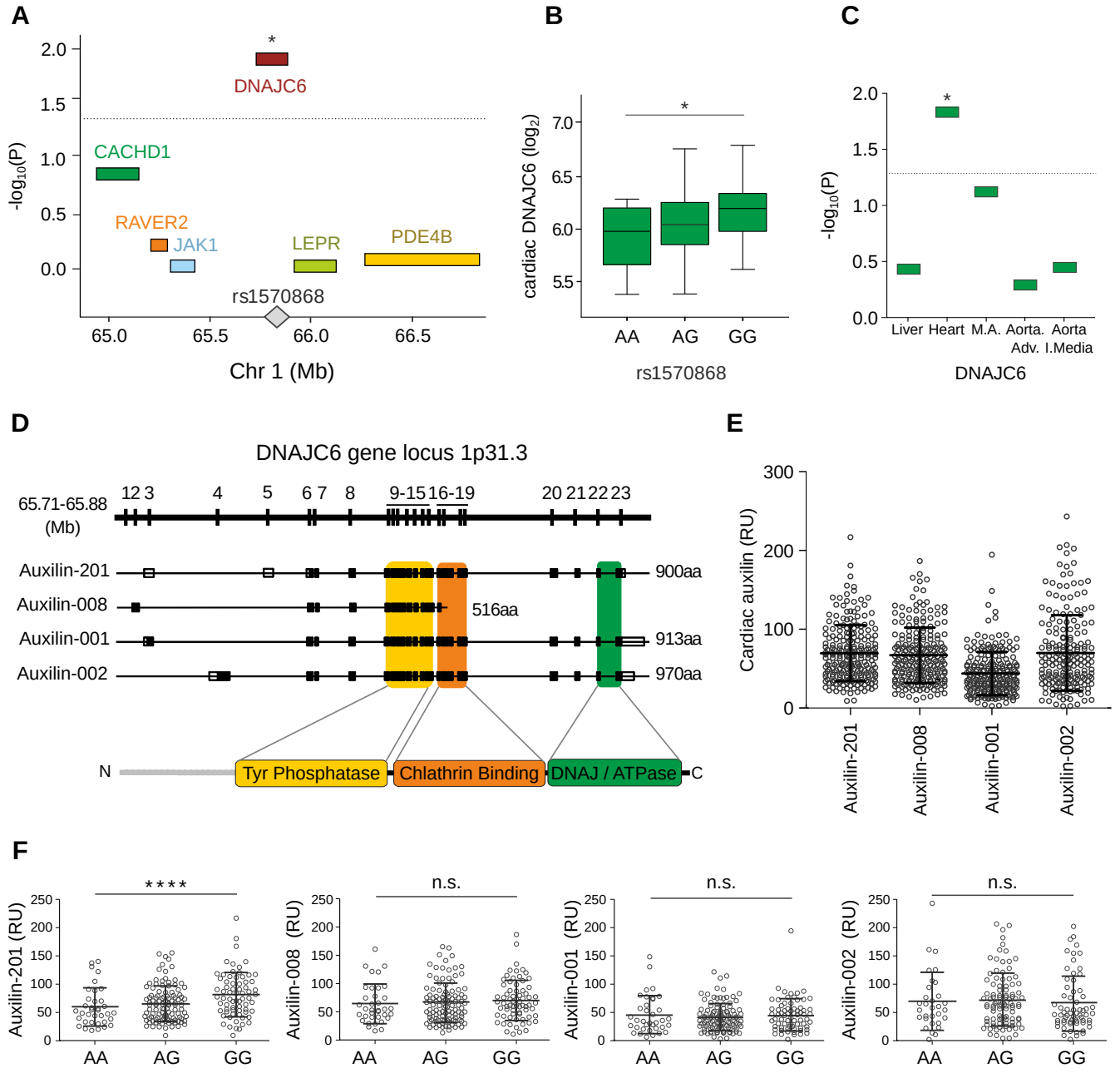

Figure 2 Identification of *DNAJC6* (*auxilin*) as a novel fetal genetic risk factor for CHB. (A) Effect of rs1570868 genotype on cardiac expression of genes located within a 1 Mb interval centred on rs1570868 (chromosomal position 1p31.3). (B) Cardiac *DNAJC6* expression stratified by rs1570868 genotypes. Allele frequency: 0.416 (A) and 0.584 (G) (n=101), β=0.066. (C) Effect of rs1570868 genotype on *DNAJC6* expression in different tissues. Liver (n=151), heart (n=101), m.a.; mammary artery (n=88), aorta AdV.; aorta adventia (n=90), aorta I. media; aorta intima media (n=104). An additive linear regression model was applied (A–C). Dashed line indicates p=0.05. (D) *DNAJC6* gene locus and predicted protein-coding auxilin transcript variants and functional domains. (E, F) Cardiac expression of auxilin transcript variants (E), stratified according to rs1570868 genotype (F), relative to cardiac β2-microglobulin expression (n=217). Results are shown as mean±SD. Linear regression analysis was applied. *P<0.05, ****p<0.0001. n.s, not significant.

Auxilin deficiency impairs cardiac cell connectivity and disturbs calcium homoeostasis

We next set out to investigate the role of auxilin in cardiac function using auxilin-deficient mice. We first confirmed that auxilin is indeed expressed in wild-type mouse neonatal heart (figure 4A), and also verified its described, high expression in mouse brain (figure 4B). In line with our findings of high fetal cardiac auxilin expression in human tissue, we observed that cardiac auxilin expression in the mouse also peaks in fetal heart tissue (figure 4C and online supplemental figure 3J,K), and verified that auxilin localises to vesicular structures partly colocalising with clathrin also in cultured neonatal mouse cardiomyocytes (figure 4D,E).

Ca^{2+} is one of the main regulators of cardiomyocyte function, and to evaluate the impact of auxilin deficiency on cardiomyocyte performance we analysed spontaneous $(Ca^{2+})_i$ oscillations in primary cultures of wild-type and auxilin knockout neonatal cardiomyocytes using the calcium sensitive fluorescent dye

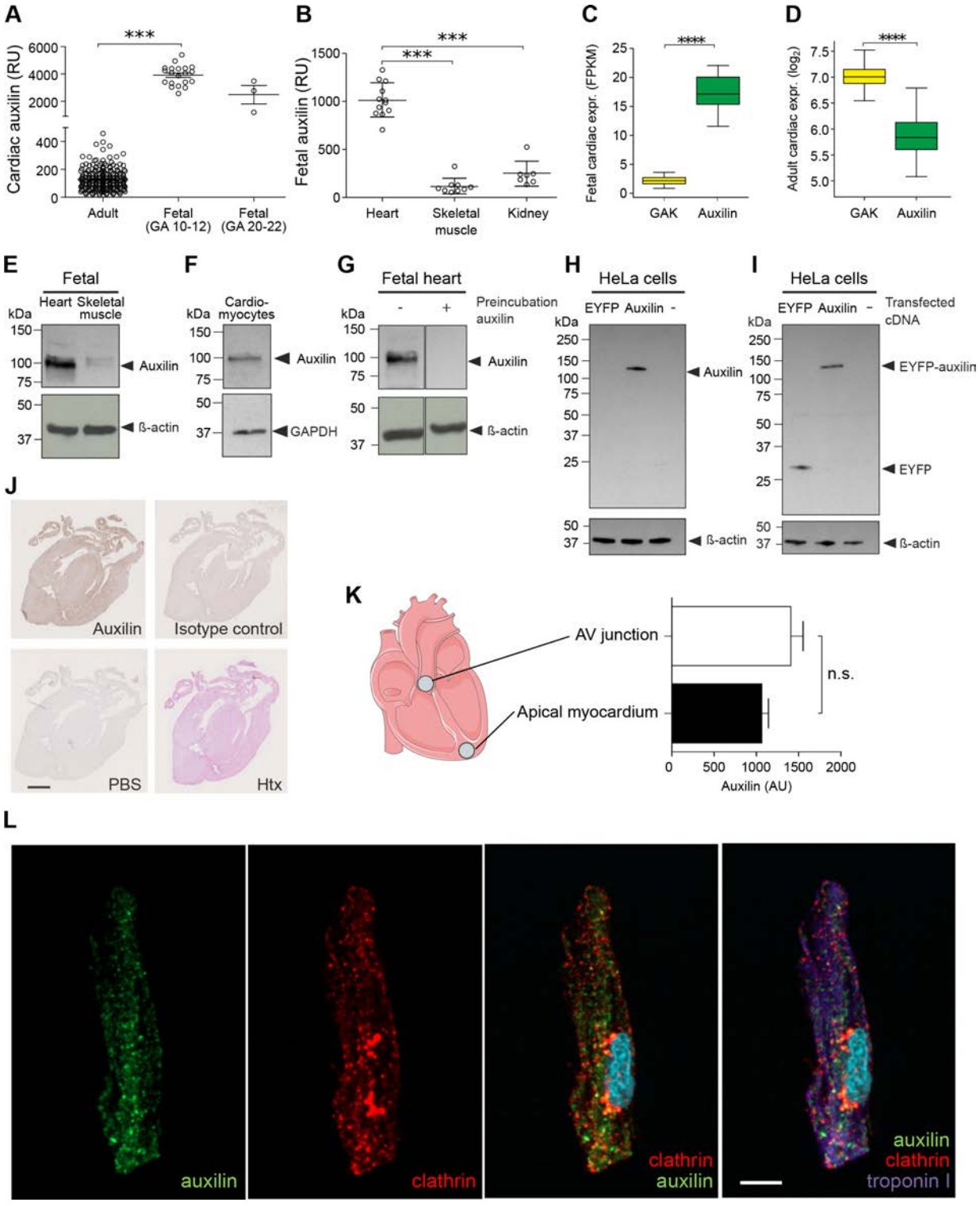

Figure 3 Auxilin is expressed in the human fetal heart and co-localises with clathrin in primary cardiomyocytes. (A) Auxilin cardiac expression in human adult (n=217) and fetal tissue, gestational age (GA) 10–12 weeks (n=20) and 20–22 weeks (n=3). (B) Auxilin expression in human fetal heart (n=12), skeletal muscle (n=9), and kidney (n=7), GA 10–12 weeks. auxilin expression relative to β2-microglobulin expression (A, B). (C) Human cardiac expression of GAK and auxilin in fetal tissue, GA 10–12 weeks (n=32). (D) Human cardiac expression of GAK and auxilin in adult tissue (n=127). (E) Auxilin protein expression in human fetal heart and skeletal muscle. (F) Auxilin protein expression in human cardiomyocytes differentiated from iPS cells. (G) Antibody specificity verified by preincubation with recombinant auxilin before Western blot of human fetal heart. (H, I) Immunoblotting with anti-auxilin (H) and anti-EYFP (I) of HeLa cell lysates transfected with recombinant EYFP (EYFP) or EYFP-auxilin (auxilin) or untransfected (-). (J) ubiquitous expression of auxilin detected by immunohistochemistry in sections of paraformaldehyde-fixed, paraffin-embedded human fetal cardiac tissue, GA 12 weeks. Scale bar represents 1 mm. (K) Auxilin mRNA expression within the apical myocardial and AV junctional tissue after microdissection of human fetal hearts (n=6; gestational age 20–22 weeks). (L) Subcellular localisation of auxilin and clathrin in cultured primary human fetal cardiomyocytes, GA 22 weeks. Counterstain by DAPI to visualise the nucleus (cyan). Scale bar represents 7.9 µm. Results are shown as mean±SE; Mann-Whitney U test. ***p<0.001, ****p<0.0001. AV, atrioventricular;

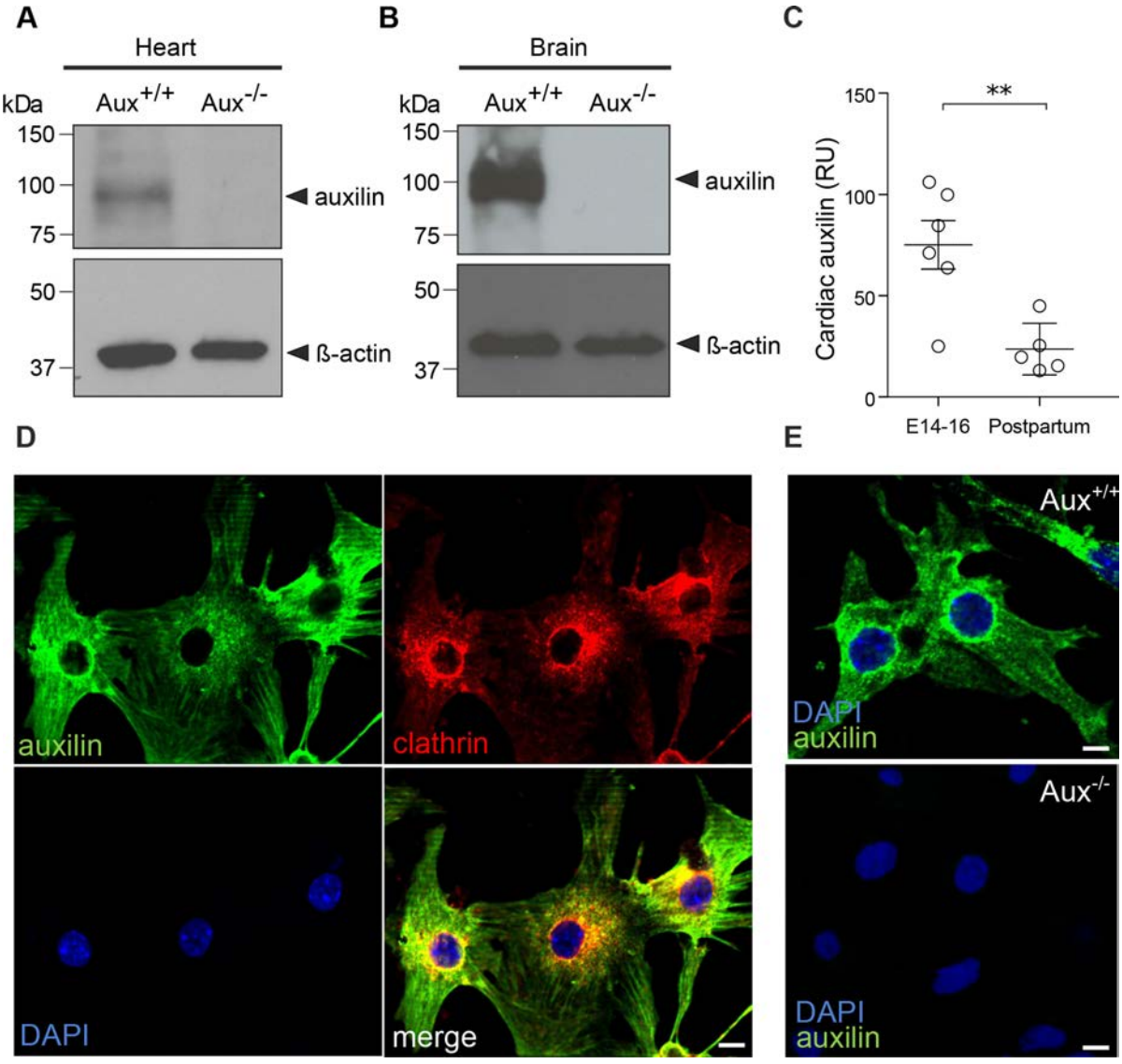

Figure 4 Auxilin is expressed in the mouse neonatal and fetal heart and colocalises with clathrin in primary cardiomyocytes. (A, B) Auxilin protein expression in the heart (A) and brain (B) of neonatal mice. Upper panel: auxilin; lower panel: β-actin. (C) Cardiac RNA expression of auxilin in mouse fetuses E14-E16 (n=6) and pups post partum (n=5). Expression levels are relative to TAF8 expression. (D) Subcellular localisation of auxilin and clathrin in cultured primary neonatal mouse cardiomyocytes. Scale bar represents 20 µm. (E) Immunofluorescence staining of cultured wild-type or auxilin knockout primary neonatal mouse cardiomyocytes using anti-auxilin antibody HPA031182, 1:200. Results are shown as mean±SE; two-tailed Student's t-test, **p<0.01.

(Fluo-4-AM) and time lapse imaging. Auxilin-deficient cardiomyocytes presented a pronounced irregular oscillation pattern (figure 5A,B and online supplemental movies 1 and 2), with reduced mean frequency and increased variability of $(Ca^{2+})_i$ oscillations compared with wild-type cells (figure 5C,D), though the total number or number of Ca-oscillating cells per area did not differ between wild-type and auxilin-deficient cultures (figure 5E). Strikingly, auxilin-deficient cells also appeared unable to organise into well-connected cellular networks in vitro, in contrast to wild-type cells (figure 5F). Cross-correlation analysis of cardiomyocyte $(Ca^{2+})_i$ activity demonstrated lower connectivity among auxilin-deficient cells (figure 5G), as well as a decreased mean path length reflecting an impaired capacity to communicate with cells at greater distances (figure 5H). Auxilin-deficient cells were, however, capable of interacting in so-called small-world networks (figure 5I).

Auxilin deficiency leads to decreased cell surface expression of the calcium channel $Ca_v1.3$ on cardiocytes

Given the described role of auxilin in the clathrin-mediated endocytic process and our finding that auxilin-deficient cardiomyocytes display a disturbed calcium homoeostasis, we hypothesised that absence of auxilin may impair the recycling of calcium channels to the plasma membrane of cardiomyocytes. Flow cytometry analysis of mouse neonatal $Sirpa^+$ cardiocytes revealed that the proportion of cells expressing the calcium channel $Ca_v1.3$ was comparable in auxilin-deficient and wild-type mice (figure 6A and B), but that $Ca_v1.3$ cell surface expression was significantly lower in $Sirpa^+Ca_v1.3^+$ auxilin-deficient cells compared with wild-type cells ($p<0.01$, figure 6C). Conversely, cardiac expression levels of $Ca_v1.3$ RNA transcripts were significantly higher in auxilin-deficient neonatal mice compared with wild-type mice (figure 6D), indicating that decreased $Ca_v1.3$ expression

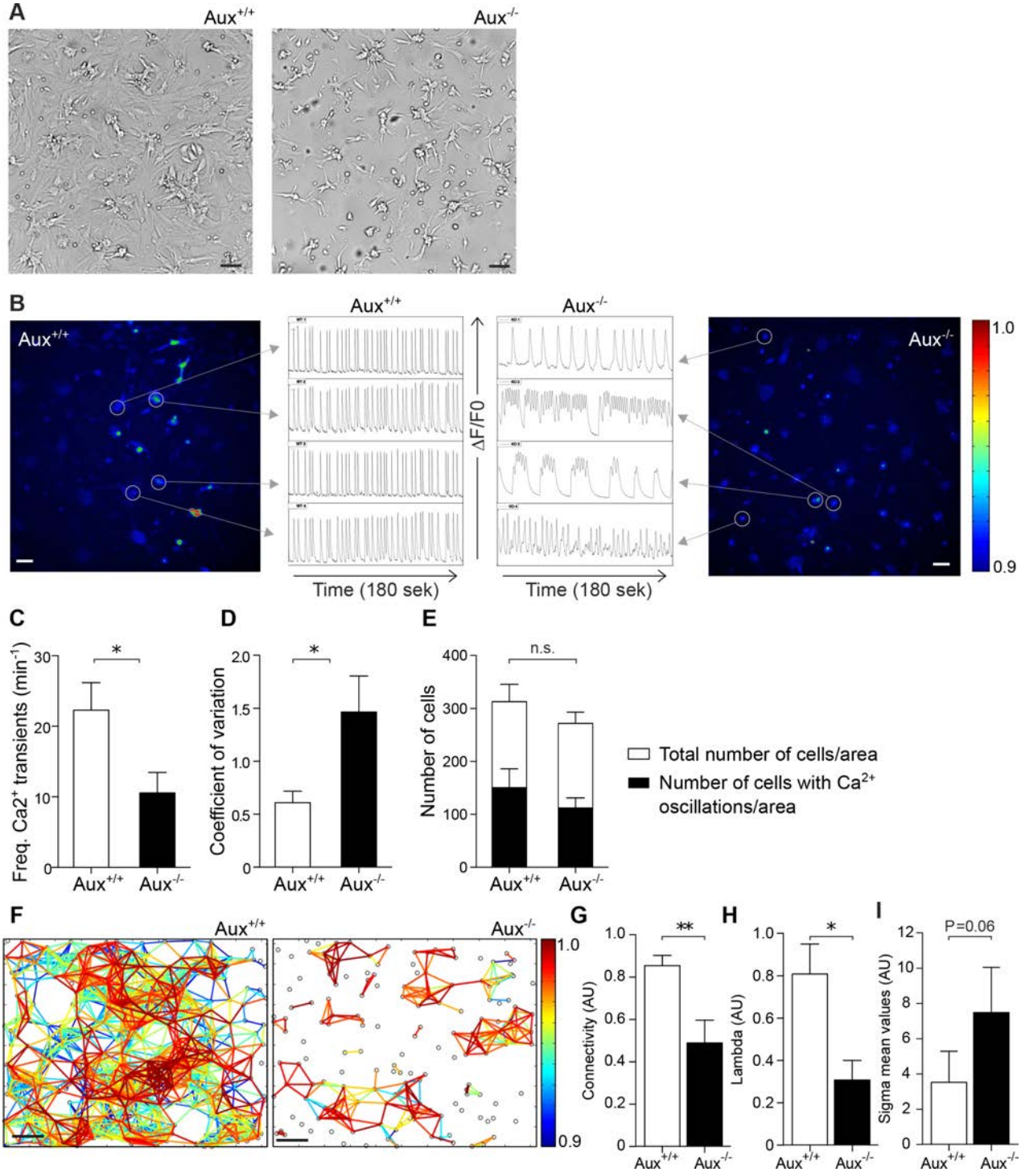

Figure 5 Auxilin deficiency causes impaired calcium homoeostasis and decreased intercellular connectivity in neonatal primary cardiomyocytes. (A) Phase-contrast images of primary neonatal cardiomyocytes of wild type and auxilin knockout mice in cultured monolayers. (B) Time lapse images of $(Ca^{2+})_i$ transients in spontaneously oscillating cardiomyocytes isolated from neonatal mice and loaded with Fluo4-AM. Examples of $(Ca^{2+})_i$ recordings from individual cardiomyocytes are shown. Videos of these cultures presented in online supplemental movie 2. (C, D) Frequency of $(Ca^{2+})_i$ oscillation transients and coefficient of variation in wild-type and knockout neonatal cardiomyocytes. Data are based on measurements from n=7 (wild-type) and n=6 (auxilin-knockout independent experiments with a mean of 165 cells analysed per experiment, each conducted with cells pooled from littermates (≥5 pups). (E) $(Ca^{2+})_i$ transients measurements in neonatal cardiomyocyte cultures showing the number of cells with $(Ca^{2+})_i$ transients per area and the total number of cells per area from wild-type vs auxilin knockout mouse pups. (F) Functional cell connection maps illustrating significantly correlated, thus connected, pairs of representative cultured cardiomyocytes. Multicoloured bar indicates correlation coefficient, with higher values representing a stronger correlation between the activities of the cells connected by the line. (G) Connectivity index in neonatal cardiomyocytes. (H) Lambda index representing the shortest mean path length in neonatal cardiomyocytes. (G–I) Representation of cardiomyocyte organisation into small-world networks in wild-type vs knockout cultures. Data are based on measurements from n=7 (wild-type) and n=6 (auxilin-knockout) independent experiments with a mean of 165 cell analysed per experiment, each conducted with cells pooled from littermates (≥5 pups). Au: arbitrary units. scale bars 100 µm. Results are shown as mean±SE; two-tailed Student's t-test, *p<0.05, **p<0.01.

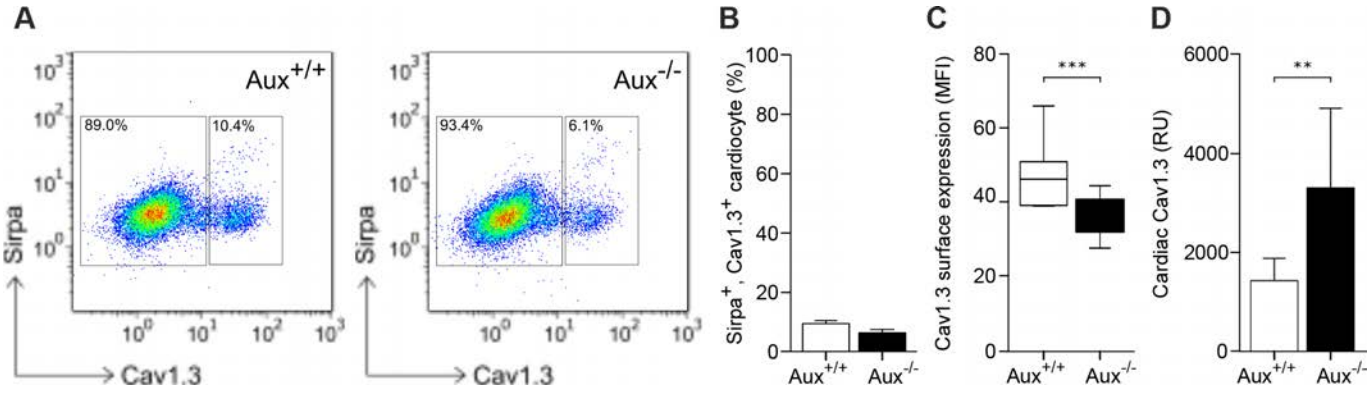

Figure 6 Auxilin deficiency leads to decreased cell surface expression of the $Ca_v1.3$ calcium channel in primary neonatal cardiomyocytes. (A, B) Flow cytometry analysis of wild-type and auxilin-deficient SIRPa and $Ca_v1.3$ double-stained primary mouse neonatal cardiomyocytes. (C) Mean fluorescence intensity (MFI) of $Ca_v1.3$ cell surface expression in $Sirpa^+Ca_v1.3^+$ primary neonatal cardiomyocytes from wild-type vs auxilin-deficient mice. Calculations are based on n=9 independent experiments per genotype with pooled cells from littermates (≥5 pups per experiment). (D) Cardiac RNA expression of $Ca_v1.3$ in primary neonatal cardiocytes from wild-type (n=5) and auxilin-deficient mice (n=5). Expression levels are relative to cardiac TAF8 expression. Results are shown as mean±SEM or minimal and maximal values; Mann-Whitney test, **p<0.01, ***p<0.001.

on the plasma membrane of auxilin-deficient cells was not due to a general decrease in expression, and further suggesting that auxilin-deficient cardiomyocytes upregulate the transcription of $Ca_v1.3$ to compensate for decreased protein levels on the cell surface.

Auxilin-deficient mice display Chb abnormalities during fetal development

To address whether the lack of auxilin affects fetal heart function in vivo, we monitored developing mice in utero by Doppler echocardiography. Notably, we observed several different CHB-related cardiac pathologies in auxilin-deficient mice at the fetal stage (figure 7A–F, online supplemental movies 3 and 4). Both the AV-time and isovolumetric contraction time were prolonged in auxilin knockout fetuses compared with wild-type fetuses (figure 7G,H). Furthermore, auxilin-deficient fetuses displayed abnormal heart rates and arrhythmias, including frequent ectopic beats generated in the atria and/or ventricles (figure 7I,J and online supplemental table 4). Interestingly, the effect was gene-dosage dependent as heterozygous animals showed an intermediate phenotype (figure 7G–J). Of note, the number of ectopic beat observations among auxilin knockout animals peaked at gestational day 13 (figure 7K), which corresponds to the window of disease onset for CHB in humans. Importantly, the cardiac abnormalities we observed in auxilin-deficient mice in utero are similar to those observed in human fetuses with CHB, as exemplified by one of our recorded human fetal case presenting with ectopic tachycardia at gestational age 21 weeks (figure 7L,M), and progressing to CHB at gestational age 24 weeks (figure 7N,O, online supplemental movies 5 and 6).

DISCUSSION

Given the low recurrence rate of CHB despite the persistence of autoantibodies in the mothers, fetal genetic factors have been suggested to contribute to disease development. Here, we identify auxilin/*DNAJC6* as a novel fetal susceptibility gene for CHB and report that decreased cardiac expression associates with the disease genotype. We further demonstrate that auxilin under normal circumstances is highly expressed in the fetal heart, and that auxilin deficiency impairs cardiomyocyte performance in vitro and leads to cardiac CHB abnormalities in vivo, thereby directly linking a novel susceptibility gene with disease mechanism and providing a functional basis for how decreased expression of auxilin may contribute to the development of CHB.

The majority of mothers of children with CHB carry autoantibodies to the Ro/SSA autoantigens and will thus have genetic traits reflecting their autoimmune status.[25 26] In order to segregate these potentially confounding maternal disease traits and identify genetic traits specific to CHB, we chose to perform a genome-wide association study using a family-based setup including individuals with CHB and their unaffected first-degree relatives. Interestingly, we found that the SNPs most significantly associated with CHB were located outside the human leucocyte antigen (HLA) region, in contrast to a previously published genome-wide association study in which the SNPs most significantly associated with CHB were found in the HLA region.[27] This discrepancy may be explained by the fact that the latter study[27] was based on a C-C set-up, and that its findings may therefore reflect inherited maternal traits linked to the autoimmune status of the mothers rather than CHB-specific disease traits.

Our family-based analysis strategy uncovered several CHB-specific polymorphisms across the whole genome, and additional validation of association combined with cardiac eQTL analysis identified auxilin/*DNAJC6* as the primary candidate for a susceptibility gene for CHB. This prompted us to further investigate the auxilin expression pattern. Indeed, auxilin expression and function had mainly been described in neuronal tissue,[28 29] and its potential involvement in heart function was unknown. Surprisingly, we found that auxilin was not only expressed in the heart, but that its levels were also markedly higher in fetal compared with adult cardiac tissue. In addition, we observed that the homologous protein GAK, which has been suggested to act as a functional substitute for auxilin,[30] was expressed only at low levels in the fetal heart, but at higher levels than auxilin in the adult heart. These findings, therefore, provide a rationale as to why a decreased expression of auxilin would be associated with a fetal cardiac phenotype.

Auxilin operates in the clathrin-mediated endocytic process,[31] and absence of auxilin may therefore impair the recycling of ion channels or other molecules important for cardiac function to the plasma membrane of cardiomyocytes. This in turn could explain the lower cellular connectivity and communication as well as the decreased and less well-coordinated Ca^{2+} oscillations we observed in auxilin-deficient cardiomyocytes.

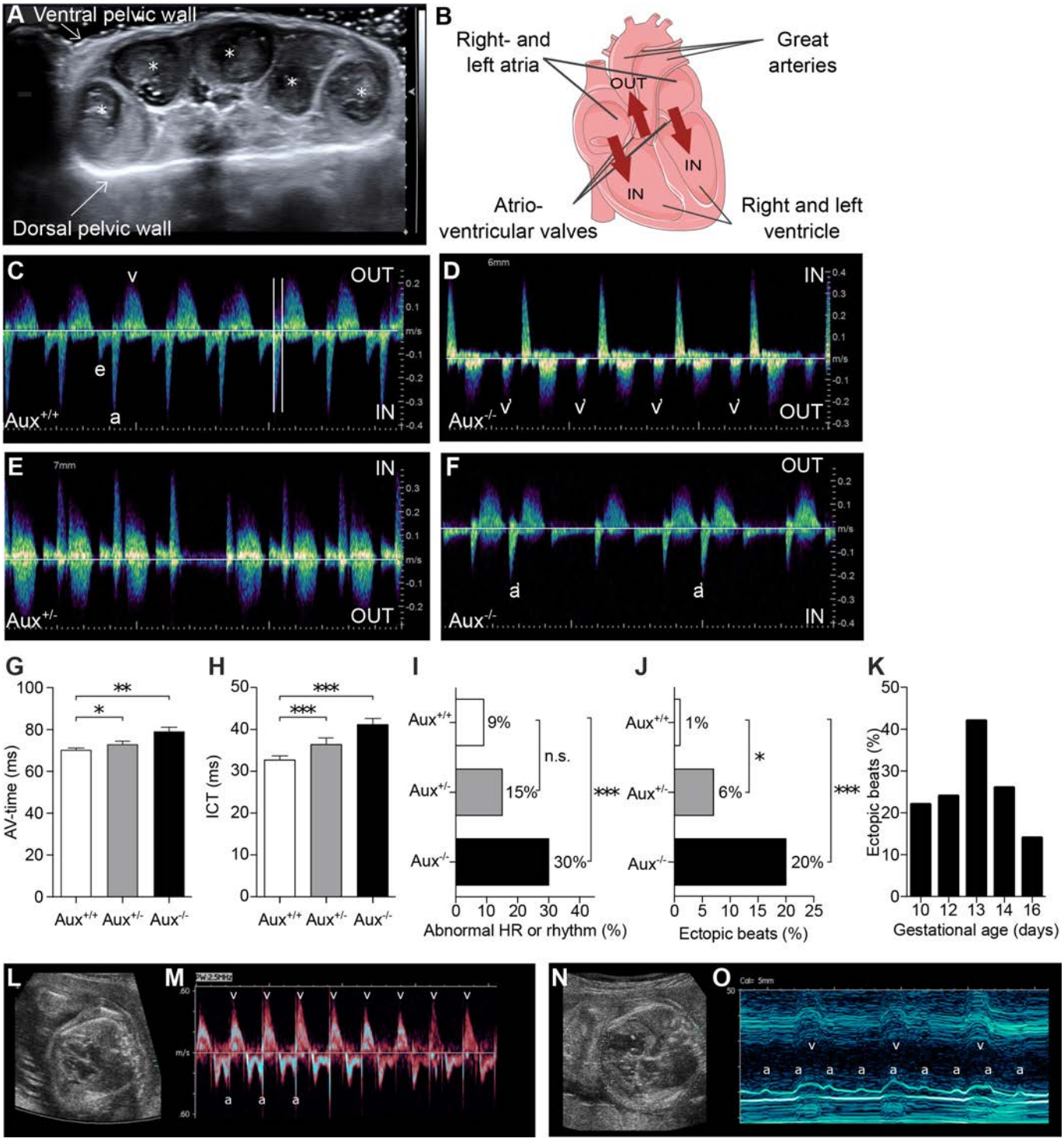

Figure 7 Auxilin deficiency causes cardiac abnormalities in vivo during fetal development. (A) Transsectional abdominal ultrasound view showing individual mouse fetuses (*) in utero. (B) Illustration of spatial and directional relationships between ventricular inflows and outflows registered in (C–F). (C–F) Echocardiographic Doppler flow velocity recordings from wild-type and auxilin-deficient fetuses, with cardiac inflow (IN) through atrioventricular valves and outflow (OUT) in the great arteries. (C) Normal recording showing two-peaked inflow with early passive e-wave (e), higher a-wave (a), and ventricular outflow (v). Vertical lines denote one AV-time interval. (D) Ventricular ectopic beats (VES) (v') in bigeminy. (E) Mobitz type II, second-degree AV-block. (F) Conducted premature ectopic supraventricular beats (SVES) (a'). (G, H) mechanical AV-time interval and isovolumetric contraction time (ICT), Kruskal-Wallis and Dunn's post hoc tests. Results are shown as mean±SE. (I, J) Proportion of fetuses with abnormal heart rate (HR) or rhythm (I), or with ectopic beats (SVES, VES) (J), χ^2 test. Auxilin$^{+/+}$ (n=147), auxilin$^{+/-}$ (n=89), and auxilin$^{-/-}$ (n=131) fetuses (G–J). (K) percentages of auxilin$^{-/-}$ fetuses with ectopic beats according to gestational age. Percentages are calculated based on the total number of auxilin$^{-/-}$ fetuses at each gestational age. l-o a human fetal case of junctional ectopic tachycardia progressing to CHB. (L, M) normal appearing heart with ectopic tachycardia at gestational age (GA) 21 weeks. (N, O) Complete CHB at GA 24 weeks with bradycardia and dilated echogenic heart. *P<0.05, **p<0.01, ***p<0.001.

In support of this hypothesis, we found that auxilin-deficient cardiac cells displayed lower levels of the calcium channel $Ca_v1.3$ on their plasma membrane compared with wild-type cells. Interestingly, $Ca_v1.3$-deficient mice have been reported to exhibit cardiac abnormalities such as sinus bradycardia and AV block at birth,[32 33] suggesting that decreased expression of $Ca_v1.3$ on the surface of auxilin-deficient cells may contribute in part to the cardiac abnormalities we observed in auxilin-deficient mice in utero. Indeed, we show here that auxilin-deficient mice develop cardiac abnormalities in utero similar to early CHB manifestations, such as ectopic beats, arrhythmias and prolongation of the AV time. AVBII/III was observed, but the occurrence did not reach statistical significance.

Calcium channels, including $Ca_v1.3$, have been described as potential targets of autoantibodies from mothers of children with CHB,[34–36] and maternal antibodies were reported to inhibit $Ca_v1.3$ calcium currents in exogenous expression systems.[34] A genetically determined lower cardiac auxilin expression, resulting in decreased calcium channel presence on the cell surface, may thus synergize with the inhibitory effect of maternal autoantibodies to further diminish $I_{Ca,L}$ current density and overall cardiomyocyte performance. Interestingly, fetal cardiomyocytes do not yet possess a fully developed sarcoplasmic reticulum, and the excitation-contraction coupling is thus largely dependent on cell surface calcium channels.[37 38] By contrast, adult cardiac cells rely mainly on sarcoplasmic calcium stores. Decreased expression of auxilin resulting in lower surface expression of calcium channels would therefore have a larger impact on cardiomyocyte function in the fetal heart than in the adult heart, rendering fetal cardiac cells particularly susceptible to the pathogenic effects of maternal antibodies while maternal cardiac cells are left relatively unaffected. This in turn might begin to explain why, despite the presence of circulating autoantibodies, cardiac manifestations similar to CHB are not detected in mothers of children with CHB.

Considering the role of auxilin in the clathrin-mediated endocytosis process, it is probable that auxilin deficiency may affect the presence of many different molecules to the plasma membrane of cardiac cells. Here, we limited our investigation to the $Ca_v1.3$ calcium channel as a proof of concept; however, it is likely that auxilin deficiency alters the surface expression also of other molecules, such as other ion channels or connexins involved in cardiac function, which in turn may contribute to CHB development. In addition, although we here focus on auxilin/*DNAJC6* as a susceptibility gene for CHB, it is likely that other genetic variants may contribute directly or indirectly to cardiomyocyte performance and hence also affect susceptibility to CHB. However, CHB is a rare disease in the general population, occurring in about 1 in 20 000 births,[21] and establishing large cohorts of patients that would enable the detection of many different risk variant combinations remains a challenge. The main limitations of this study are linked to this rarity of the studied condition, and includes the employed threshold at $p \leq 1 \times 10^{-4}$ for the family-based association for disease traits and the lack of a replication cohort of patients with CHB.

In all, we identify auxilin/DNAJC6 as a novel susceptibility gene for CHB and demonstrate a previously unreported role for auxilin in fetal cardiac function, revealing in particular that auxilin is necessary for cardiac cells to maintain normal calcium homoeostasis and establish functional networks. The disease-associated genetic variant, leading to decreased auxilin expression, may thus affect fetal myocardial function, both mechanically and electrically, as part of CHB. The involvement of maternal autoantibodies in CHB has long been recognised, especially regarding the establishment of inflammation and subsequent scaring of the AV node.[39 40] However, the mechanisms underlying the early phases and cardiac manifestations of NLE other than complete AV block remain unclear.[41] Importantly, we show here that auxilin-deficient mice develop cardiac abnormalities in utero similar to CHB manifestations such as prolonged AV time interval and isovolumetric contraction time, ectopic beats and arrhythmias, and provide a mechanistic basis as to how lack of auxilin may underlie such features at the molecular and cellular level. Identification of auxilin/DNAJC6 as a susceptibility gene for CHB that directly impacts cardiac function therefore begins to elucidate the tissue-dependent pathogenic mechanisms involved in CHB. This, in turn, shifts the focus from solely trying to prevent the pathogenic effects of maternal antibodies and instead taking into account intrinsic cardiac defects affecting fetal heart function, thus opening the road to conceiving new screening and therapeutic strategies for this often lethal condition.

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Contributors SM and MW-H designed the study with input from MH, AA, VKK, EH, AE, IK and S-ES. Members of TSCHBSG, AF-C, AH, TO, PE, JM and KG-D assisted in identification and characterisation of patients and controls. LG and RMH provided study material. SM, MH, VO, LB, LS, SR, LM, WN and S-ES performed the experiments. SM, MH, LF, GET, VO, DR, DF, LB, JM, EH, IK and S-ES analysed the data. SM, AA, GET and MW-H wrote the manuscript, and all authors participated in manuscript preparation until its final form. MW-H is the study guarantor.

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Patient consent for publication Not applicable.

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TRANSLATIONAL SCIENCE

CSF2-dependent monocyte education in the pathogenesis of ANCA-induced glomerulonephritis

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ABSTRACT

Objectives Myeloid cell activation by antineutrophil cytoplasmic antibody (ANCA) is pivotal for necrotising vasculitis, including necrotising crescentic glomerulonephritis (NCGN). In contrast to neutrophils, the contribution of classical monocyte (CM) and non-classical monocyte (NCM) remains poorly defined. We tested the hypothesis that CMs contribute to antineutrophil cytoplasmic antibody-associated vasculitis (AAV) and that colony-stimulating factor-2 (CSF2, granulocyte-macrophage colony-stimulating factor (GM-CSF)) is an important monocyte-directed disease modifier.

Methods Myeloperoxidase (MPO)-immunised $MPO^{-/-}$ mice were transplanted with haematopoietic cells from wild-type (WT) mice, C–C chemokine receptor 2 $(CCR2)^{-/-}$ mice to abrogate CM, or transcription factor CCAAT–enhancer-binding protein beta $(C/EBP\beta)^{-/-}$ mice to reduce NCM, respectively. Monocytes were stimulated with CSF2, and CSF2 receptor subunit beta (CSF2rb)-deficient mice were used. Urinary monocytes and CSF2 were quantified and kidney *Csf2* expression was analysed. CSF2-blocking antibody was used in the nephrotoxic nephritis (NTN) model.

Results Compared with WT mice, $CCR2^{-/-}$ chimeric mice showed reduced circulating CM and were protected from NCGN. $C/EBP\beta^{-/-}$ chimeric mice lacked NCM but developed NCGN similar to WT chimeric mice. Kidney and urinary CSF2 were upregulated in AAV mice. CSF2 increased the ability of ANCA-stimulated monocytes to generate interleukin-1β and to promote T_H17 effector cell polarisation. $CSF2rb^{-/-}$ chimeric mice harboured reduced numbers of kidney T_H17 cells and were protected from NCGN. CSF2 neutralisation reduced renal damage in the NTN model. Finally, patients with active AAV displayed increased urinary CM numbers, CSF2 levels and expression of GM-CSF in infiltrating renal cells.

Conclusions CMs but not NCMs are important for inducing kidney damage in AAV. CSF2 is a crucial pathological factor by modulating monocyte proinflammatory functions and thereby T_H17 cell polarisation.

Key messages

What is already known about this subject?

⇒ Antineutrophil cytoplasmic antibody-associated vasculitis is a myeloid cell-mediated vascular inflammation and glomerulonephritis. However, the detailed pathogenic contribution of distinct monocyte subsets is not clear.

What does this study add?

⇒ We identified C–C chemokine receptor 2 (CCR2)-positive classical monocytes as essential components for induction of antineutrophil cytoplasmic antibody-induced glomerulonephritis. Furthermore, colony-stimulating factor-2 (CSF2) and its receptor CSF2 receptor subunit beta are important for activation of monocytes and subsequent regulation of T cells.

How might this impact on clinical practice or future developments?

⇒ Direct targeting of monocytes (CCR2 antibodies) or CSF2 (granulocyte-macrophage colony-stimulating factor) could be a novel treatment strategy in human crescentic glomerulonephritis.

INTRODUCTION

Antineutrophil cytoplasmic antibody-associated vasculitis (AAV) is a systemic autoimmune disease featuring inflammation of small blood vessels and multiorgan damage, including necrotising crescentic glomerulonephritis (NCGN).[1 2] Antineutrophil cytoplasmic antibody (ANCA) recognises either myeloperoxidase (MPO) or proteinase 3[3] exclusively expressed by neutrophils and monocytes. ANCAs bind to their cell surface-presented antigens and activate both myeloid cell types.[4] The contribution of neutrophils to AAV is well documented, whereas the mechanistic role of monocytes remains incompletely understood.[5–7]

Murine monocytes are classified into distinct $Ly6C^{hi}$ inflammatory or classical monocytes (CMs) and $Ly6C^{lo}$ patrolling or non-classical monocytes (NCMs).[8] Corresponding human subsets are $CD14^+CD16^-$ and $CD14^{lo}CD16^+$ monocytes, respectively.[9] CMs are released from the bone marrow and give rise to circulating blood NCM.[10] Murine $Ly6C^{int}$ and human $CD14^+CD16^+$ intermediary monocytes (IMs) provide a third subset that possibly represents a maturation stage between CM and NCM. The CM to NCM conversion depends on the transcription factor CCAAT–enhancer-binding protein beta (C/EBPβ).[11] Similar to neutrophils, ANCAs induce monocyte activation resulting in reactive oxygen species (ROS) production[12]

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and release of proinflammatory cytokines, such as interleukin (IL)-6,[13] monocyte chemoattractant protein-1 (MCP-1)[14] and IL-1β, that are crucial for AAV induction in mice.[15 16] Monocyte exposure to MPO–ANCA promotes survival and macrophage differentiation.[17] Soluble CD163 (sCD163) shed from activated monocytes may provide a biomarker of renal flares in AAV.[18] Unselective monocyte depletion protected mice from anti-MPO-induced AAV.[7] Information on the mechanistic contribution of monocyte subsets in AAV is not available.

The colony stimulating factor 2 (CSF2), also known as granulocyte-macrophage colony-stimulating factor (GM-CSF), is produced by various cells, including myeloid, lymphoid and non-haematopoietic cells.[19] CSF2 binds to its receptor CSF2R (GM-CSFR) constituted by a ligand-binding α-chain and a signal-transducing β-chain (GM-CSF2Rβ or CSF2 receptor subunit beta (CSF2Rb)).[20 21] CSF2 was initially described as a haematopoietic growth factor for myeloid cell development. However, recent studies found a normal myelopoiesis in mice deficient either for CSF2 or for its receptor.[19] Instead, CSF2 was suggested as a modifier of myeloid cell activation in inflammatory conditions, including a murine experimental autoimmune encephalomyelitis (EAE) model where mice with a specific deletion of the GM-CSFRβ subunit ($Csf2rb^{-/-}$) in C–C chemokine receptor 2 $(CCR2)^{+}$ monocytes were protected.[22] Clinical studies indicated that CSF2 levels were strongly increased in patients with active AAV compared with patients on remission (Rem) and healthy controls (HCs).[23] We have previously shown that monocytes are important mediators of kidney damage in ANCA-induced vasculitis.[7] We now investigated the specific contribution of CM and NCM and explored whether CSF2 controls disease-mediating monocyte functions.

METHODS

Animal experiments

$MPO^{-/-}$ mice were immunised intraperitoneally with murine MPO in complete Freund's adjuvant, boosted intraperitoneally after 4 weeks with murine MPO in incomplete Freund's adjuvant, lethally irradiated and subsequently transplanted intravenously with bone marrow cells (1.5×10^{7}) from either C57BL/6 J wild type (WT) (The Jackson Laboratory), $CCR2^{-/-}$, $C/EBP\beta^{-/-}$ or $Csf2rb^{-/-}$ mice. Nephrotoxic nephritis (NTN) was induced by intraperitoneal injection of 10 µL/g BW NTS (Probetex, San Antonio, Texas, USA), and mice were sacrificed on day 6.

A detailed method section is provided in the online supplemental file 2.

RESULTS

CMs are essential for anti-MPO-induced glomerulonephritis

We first explored whether CMs mediate anti-MPO ANCA-induced glomerulonephritis in an MPO–ANCA mouse model. MPO-deficient mice were immunised with murine MPO, irradiated and transplanted with bone marrow (BM) cells from either WT or CCR2-deficient ($CCR2^{-/-}$) animals to generate $MPO^{-/-}$/WT mice and $MPO^{-/-}/CCR2^{-/-}$ chimeric mice, respectively (figure 1A). Mice were euthanised 7–8 weeks after transplantation, and blood, serum, urine, spleens and kidneys were analysed. Blood analysis was performed by flow cytometry with an adapted gating strategy to characterise neutrophils and both monocyte subsets (online supplemental figure 1A). The percentage of $CD11b^{+}Ly6G^{+}$ neutrophils was similar in both groups. $CD11b^{+}CD115^{+}Ly6C^{hi}$ CMs were almost absent in $CCR2^{-/-}$ chimeric mice (online supplemental figure 1B). Consequently, a non-significant marginal reduction of $CD11b^{+}CD115^{+}Ly6C^{lo}$ NCMs was observed. Renal histology revealed protection from kidney injury in $CCR2^{-/-}$ chimeric mice with a significantly reduced percentage of crescentic and necrotic glomeruli (figure 1B). Correspondingly, erythrocyturia and albuminuria were also significantly decreased (figure 1C,D). Urinary neutrophil gelatinase-associated lipocalin (NGAL) was similar in both groups (figure 1E), suggesting tubular injury. We analysed renal infiltrating inflammatory cells by flow cytometry to identify neutrophils and monocyte subsets (online supplemental figure 2). We observed strongly reduced numbers of kidney-infiltrating $CD11b^{+}Ly6C^{hi}$ CM, whereas both $CD11b^{+}Ly6C^{-}MHCII^{-}CD11c^{mid/hi}$ NCM and $CD11b^{+}Ly6G^{+}$ neutrophil influx were not altered (figure 1F,G and online supplemental figure 3). These experiments firmly establish the central role of CM in mediating anti-MPO-induced NCGN.

NCMs are dispensable for anti-MPO-induced glomerulonephritis

Next, we studied whether NCM contribute to disease induction as previous data suggested that NCM are essential in mediating glomerular neutrophil influx.[24] We took advantage of mice deficient in the transcription factor C/EBPβ, which is essential for NCM $Ly6C^{-}$ monocyte development and survival.[11] MPO-deficient mice were immunised with murine MPO, irradiated and transplanted with BM cells from either C/EBPβ-positive ($C/EBP\beta^{+/+}$) or C/EBPβ-deficient ($C/EBP\beta^{-/-}$) animals to generate $MPO^{-/-}$ $C/EBP\beta^{+/+}$ mice (WT) and $MPO^{-/-}$ $C/EBP\beta^{-/-}$ chimeric (NCM-deficient) mice, respectively (figure 2A). Both chimeric mice had similar numbers of circulating neutrophils (online supplemental figure 1A,C). As expected, $C/EBP\beta^{-/-}$ chimeric mice displayed an almost total absence of circulating NCM together with a marginal, non-significant reduction of blood CM. Renal histology showed no differences in glomerular crescents and necrosis between WT and NCM-deficient mice (figure 2B). Urinalysis did not show differences by dipstick analysis, albuminuria and urinary NGAL concentration (figure 2C–E). We observed a profound reduction in NCM influx, whereas CM and neutrophil influx were not affected (figure 2). These data establish that NCMs are dispensable for anti-MPO induced NCGN.

CSF2 is increased in kidneys and urine of AAV mice, and CSF2rb deletion in haematopoietic cells protects from anti-MPO induced glomerulonephritis

We next investigated CSF2 as a monocyte-targeting disease modifier in AAV. Using immunohistochemistry, we found that CSF2 protein was expressed in kidney sections from AAV but not HC mice (figure 3A). We observed strong CSF2 expression in tubular cells and in some glomerulus-infiltrating leucocytes. We confirmed these results on CSF2 protein assessing kidney lysates by ELISA. High CSF2 protein was detectable in AAV but not in control mice (figure 3B). Moreover, urinary CSF2 was strongly upregulated in AAV mice but undetectable in urine from healthy mice (figure 3C). In parallel, kidney *Csf2* mRNA expression was strongly upregulated, confirming that CSF2 is locally expressed (figure 3D). Finally, we found a positive correlation between CSF2 protein level and the percentage of renal crescents (figure 3E). These results indicate that CSF2 is strongly upregulated in kidneys of AAV mice. We next explored the hypothesis that CSF2 is a critical mediator of AAV.

To study the mechanistic role of CSF2 in AAV, MPO-deficient mice were immunised with murine MPO, irradiated and transplanted with BM cells from either C57BL/6J WT (WT) or CSF2rb-deficient ($CSF2rb^{-/-}$) animals to generate $MPO^{-/-}$ $CSF2rb^{+/+}$ mice (WT)

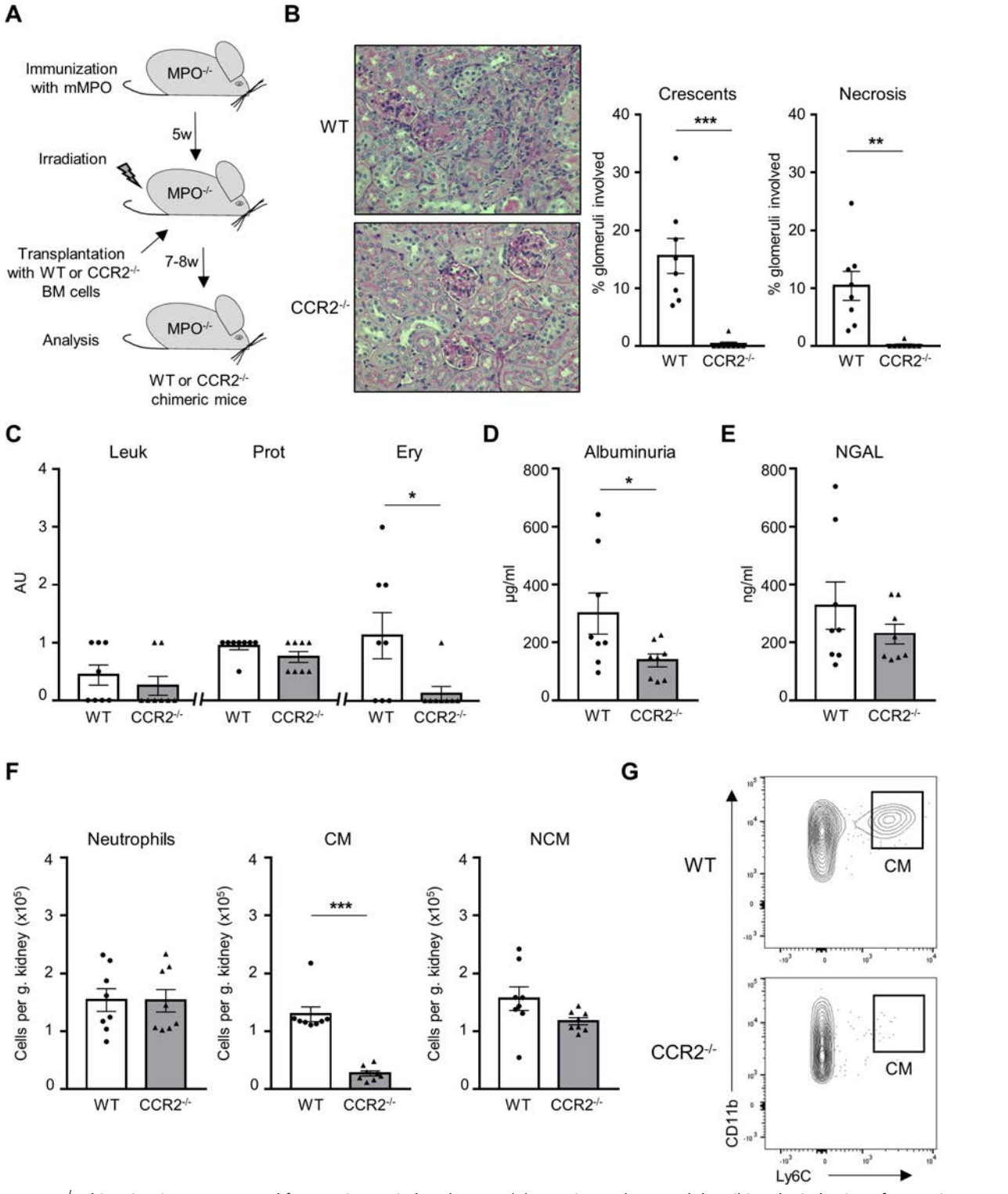

Figure 1 CCR2$^{-/-}$ chimeric mice are protected from anti-MPO-induced NCGN. (A) Experimental protocol describing the induction of NCGN in MPO$^{-/-}$. Chimeric mice were analysed 7–8 weeks following BM transplantation. (B) CCR2$^{-/-}$ chimeric mice showed reduced renal damage compared with WT chimeric mice with significant reduction of crescentic and necrotic glomeruli. For each group, a representative image of a kidney section stained with PAS at high magnification (×40) is shown. (C) CCR2$^{-/-}$ chimeric mice displayed reduced Ery by dipstick. Leu and Prot were similar in both groups. (D) Reduction in albuminuria by ELISA in CCR2$^{-/-}$ chimeric mice. (E) Urinary NGAL levels by ELISA were similar in CCR2$^{-/-}$ and WT chimeric mice. (F) Renal CD11b^{+}Ly6G^{+} neutrophils, CD11b^{+}Ly6G^{-}Ly6C^{+} CMs and CD11b^{+}Ly6G^{-}Ly6C^{-}MHC-II^{-}CD11c$^{mid/+}$ NCMs were analysed by flow cytometry. Infiltration of CM was strongly reduced in CCR2$^{-/-}$ chimeric mice, whereas infiltration of neutrophils and NCMs was similar in both groups. The number of immune cells is expressed per gram kidney and calculated using counting beads. (G) Representative flow cytometry plot showing that the renal infiltration of CM is strongly reduced in CCR2$^{-/-}$ chimeric mice. *P<0.05, **P<0.01, ***P<0.001. CCR2, C–C chemokine receptor 2; AU, arbitrary unit; CM, classical monocyte; Ery, erythrocyturia; Leu, leukocyturia; mMPO, murine MPO; MPO, myeloperoxidase; NCGN, necrotising crescentic glomerulonephritis; NCM, non-classical monocyte; NGAL, neutrophil gelatinase-associated lipocalin; PAS, periodic acid-Schiff; Prot, proteinuria; WT, wild type.

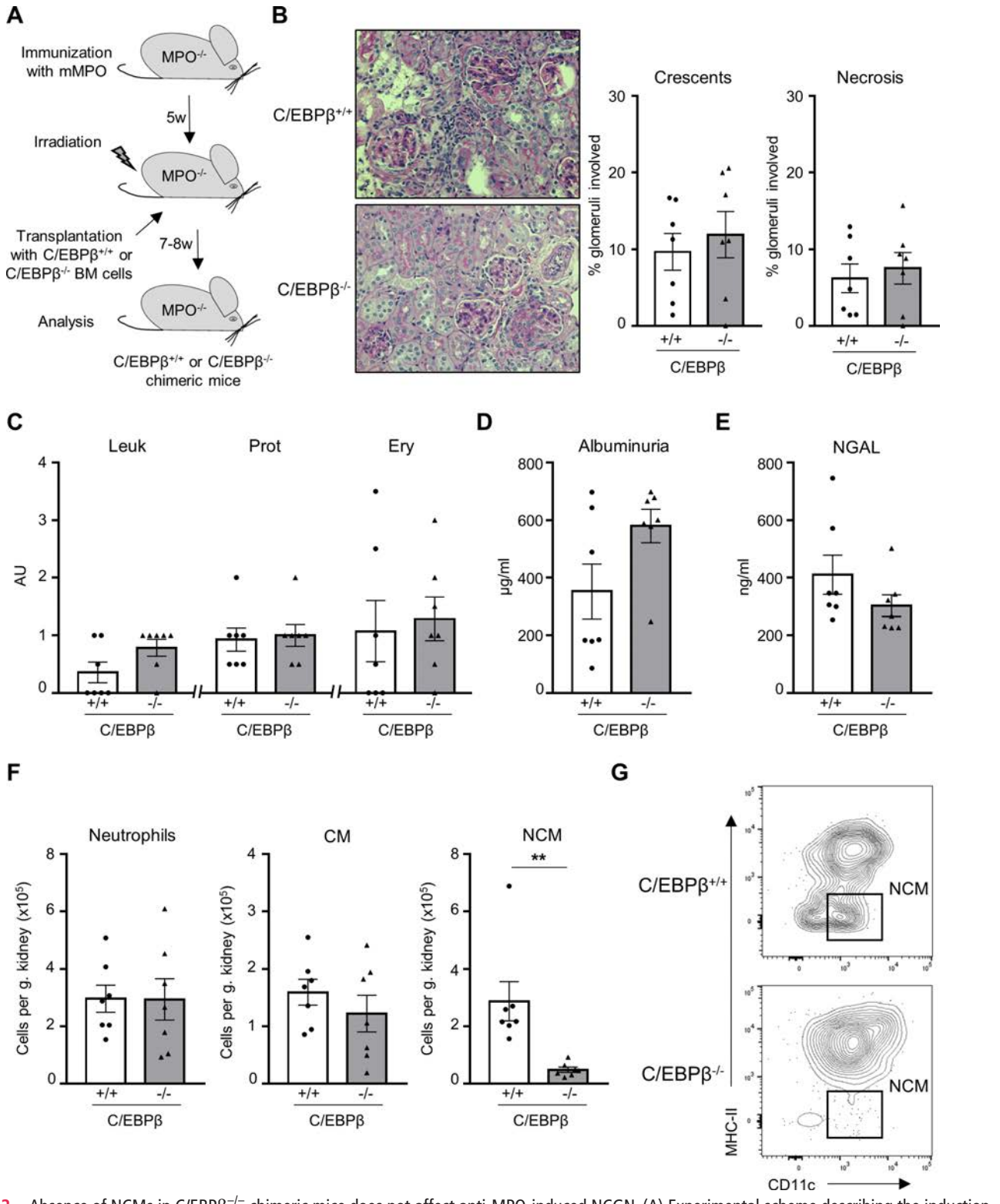

Figure 2 Absence of NCMs in C/EBPβ$^{-/-}$ chimeric mice does not affect anti-MPO-induced NCGN. (A) Experimental scheme describing the induction of NCGN in MPO$^{-/-}$ mice. (B) No difference in histological renal damage between C/EBPβ$^{-/-}$ and WT chimeric mice. For each group, a representative image of a kidney section stained with PAS at high magnification (×40) is shown. (C) C/EBPβ$^{-/-}$ chimeric mice showed no difference in Leu, Prot and Ery by dipstick compared with C/EBPβ$^{+/+}$ chimeric mice. (D) Albuminuria by ELISA was similar in both groups. (E) Urinary NGAL levels by ELISA were similar in both groups. (F) Renal CD11b^{+}Ly6G^{+} neutrophils, CD11b^{+}Ly6G^{-}Ly6C^{+} CMs and CD11b^{+}Ly6G^{-}Ly6C^{-}MHC-II^{-}CD11c$^{mid/+}$ NCMs were analysed by flow cytometry. Infiltration of NCM was strongly reduced in C/EBPβ$^{-/-}$ chimeric mice, whereas infiltration of neutrophils and CM was similar in both groups. The number of immune cells is expressed per gram kidney and calculated using counting beads. (G) Representative flow cytometry plot showing that the renal infiltration of NCM is strongly reduced in C/EBPβ$^{-/-}$ chimeric mice. **P<0.01. AU, arbitrary unit; C/EPBβ, CCAAT–enhancer-binding protein beta; CM, classical monocyte; Ery, erythrocyturia; Leu, leukocyturia; MPO, myeloperoxidase; mMPO, murine MPO; NCGN, necrotising crescentic glomerulonephritis; NCM, non-classical monocyte; NGAL, neutrophil gelatinase-associated lipocalin; PAS, periodic acid-Schiff; Prot, proteinuria; WT, wild type.

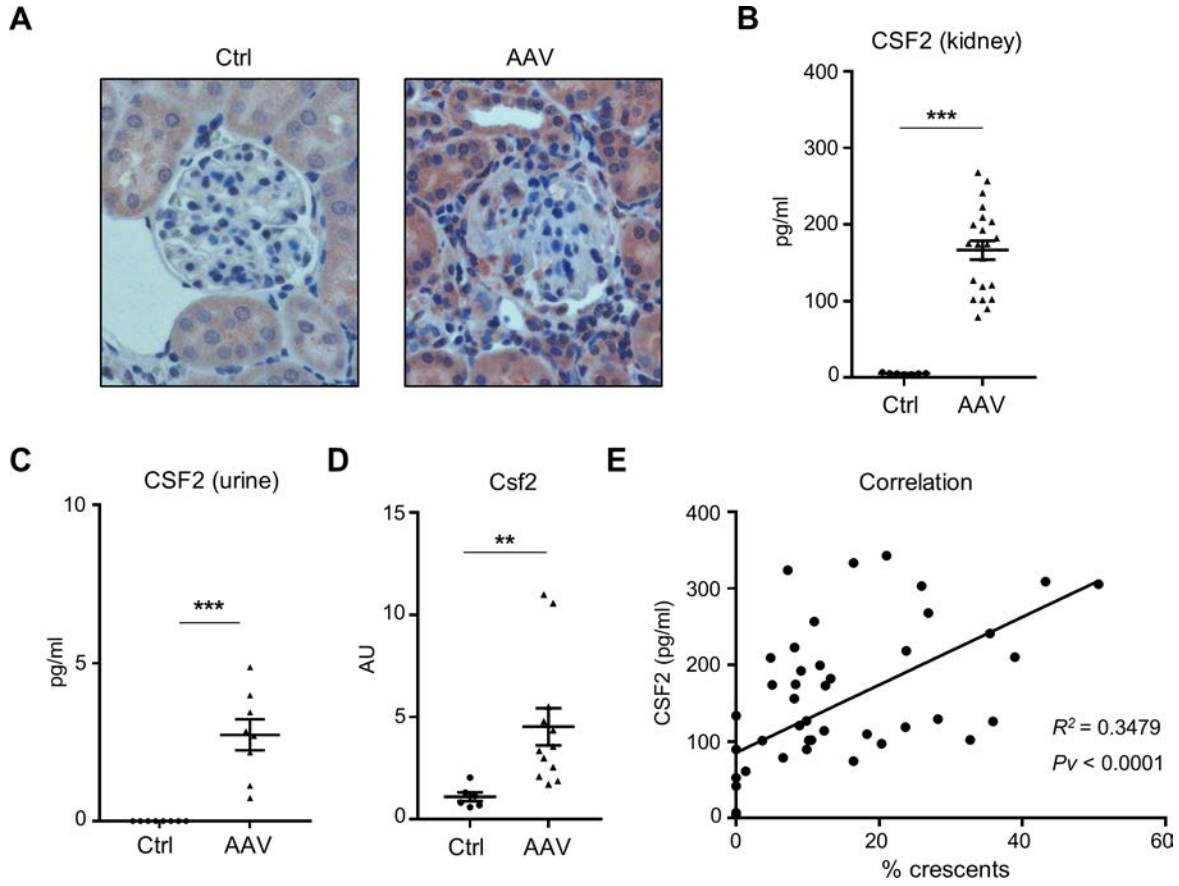

Figure 3 CSF2 expression is increased in kidneys from mice with anti-MPO-induced NCGN. (A) Immunofluorescence images show strong CSF2 expression in kidney sections from mice with AAV compared with Ctrl mice. A representative image at magnification ×40 is shown for each group. 4',6-Diamidin-2-phenylindol (DAPI) was used to stain nuclei (blue). (B) CSF2 level in urine by ELISA is increased in AAV mice compared with Ctrl mice. (C) CSF2 level by ELISA in renal lysates is increased in AAV mice compared with Ctrl mice. (D) *Csf2* mRNA by RT-PCR expression in kidney lysates is increased in AAV mice compared with Ctrl mice. (E) Correlation between the percentage of crescents and the amount of renal CSF2. **P<0.01, ***P<0.001. AAV, antineutrophil cytoplasmic antibody-associated vasculitis; AU, arbitrary unit; CSF2, colony-stimulating factor-2; Ctrl, control; MPO, myeloperoxidase; NCGN, necrotising crescentic glomerulonephritis.

and MPO$^{-/-}$ CSF2rb$^{-/-}$ chimeric (CSF2rb$^{-/-}$) mice, respectively (figure 4A). Anti-MPO titres and blood cell counts were similar in both animal groups (online supplemental figure 4). Renal histology revealed strongly reduced crescent formation in CSF2rb$^{-/-}$ chimeric mice compared with the WT group (figure 4B). Urine analysis did not show group differences by dipstick analysis, albumin ELISA or NGAL ELISA (figure 4C–E). Remarkably, quantification of kidney-infiltrating leucocytes revealed no difference in either neutrophil, CM or in NCM influx (figure 4F and online supplemental figure 3). These data demonstrate that CSF2 and its interaction with its CSF2rb receptor on myeloid cells are essential for crescent formation and that the effect is not mediated by reduced myeloid cell influx. Thus, we next explored CSF2 effects on monocyte functions in the presence of MPO–ANCA.

CSF2 modulates ANCA-stimulated monocytes

Monocytes were isolated from human blood and murine BM. First, we analysed the effect of CSF2 priming on anti-MPO IgG-induced IL-1β generation. We confirmed that anti-MPO IgG-stimulated human and murine monocytes released IL-1β (figure 5A,B).[15 16] Importantly, CSF2 priming significantly increased the IL-1β release by MPO-ANCA. We next tested the capacity of supernatants from ANCA-stimulated murine monocytes to promote the polarisation of CD4$^+$ T cells into IL-17A-producing T-helper cells (T_H17), which are known to be an important contributor to kidney injury in AAV. Monocyte supernatants were added to CD4$^+$ T cells from murine spleens. After 5 days, T_H17 cells were detected by flow cytometry. Supernatants from anti-MPO IgG-stimulated monocytes did not induce T_H17 polarisation, whereas supernatants from anti-MPO IgG-stimulated monocytes that were primed with CSF2 strongly induced T_H17 polarisation in vitro (figure 5B,C). Importantly, we found a reduction in numbers of kidney-infiltrating T_H17 cell in MPO$^{-/-}$ CSF2rb$^{-/-}$ chimeric mice compared with MPO$^{-/-}$ WT mice, suggesting that this polarisation effect occurs also in vivo (figure 5D). Together, these data establish that CSF2 increases proinflammatory monocyte functions and promote the subsequent polarisation of CD4$^+$ T cells. Importantly, human monocyte-derived macrophages lost MPO protein expression during differentiation with either GM-CSF or macrophage colony-stimulating factor (M-CSF) (online supplemental figure 5A,B) and could not be activated by anti-MPO ANCA, thereby excluding a direct role of macrophages in this context (online supplemental figure 5C,D).

Patients with ANCA-induced NCGN display elevated renal CSF2 level and urinary myeloid cells

Next, we analysed CSF2 expression by in situ hybridisation in kidney biopsies from patients with active AAV and controls (figure 6A). We observed strong upregulation of CSF2 expression in glomerulus-infiltrating cells and detected increased CSF2 in urine from patients with active AAV (figure 6B). Finally, we

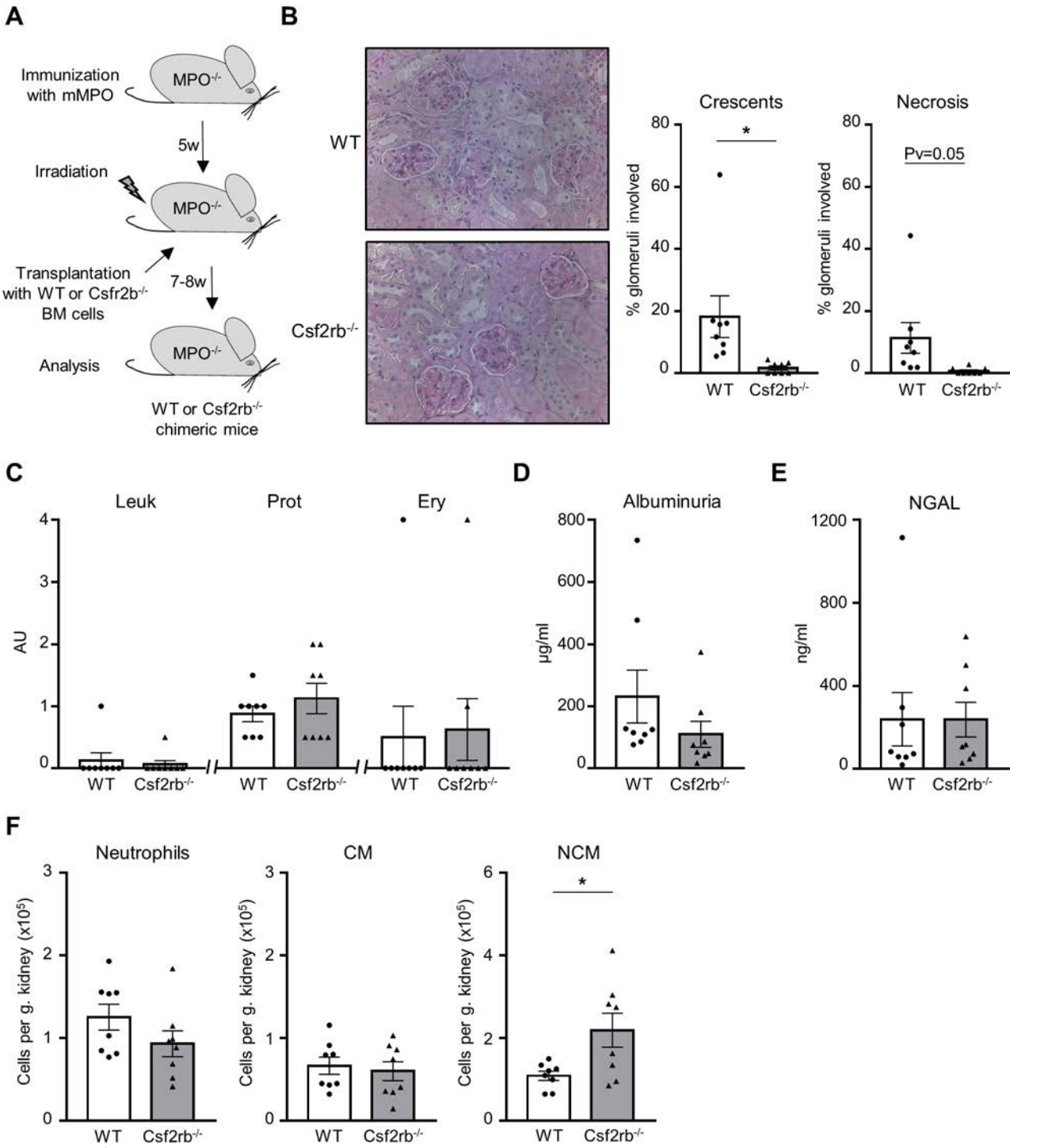

Figure 4 Csf2rb$^{-/-}$ chimeric mice are protected from anti-MPO-induced NCGN. (A) Experimental settings describing the induction of NCGN. (B) Csf2rb$^{-/-}$ chimeric mice developed less renal damage compared with WT chimeric mice with a reduction of crescentic and necrotic glomeruli. A representative image of a kidney section stained with PAS at high magnification (×40) is shown for each group. (C) Leu, Prot and Ery by urine dipstick are similar in both groups. (D) Albuminuria and (E) NGAL urine levels by ELISA were similar in both groups. (F) Renal infiltration of immune cells was analysed by flow cytometry. Infiltration of CD11b^{+}Ly6G^{+} neutrophils and CD11b^{+}Ly6G^{-}Ly6C^{+} CMs was similar in both groups, whereas CD11b^{+}Ly6G^{-}Ly6C^{-}MHC-II^{-}CD11c$^{mid/+}$ NCM infiltration was slightly increased in Csf2rb$^{-/-}$ chimeric mice compared with WT chimeric mice. The number of immune cells is expressed per gram kidney and calculated using counting beads. *P<0.05. AU, arbitrary unit; CM, classical monocyte; Csf2rb, CSF2 receptor subunit beta; Ery, erythrocyturia; Leu, leukocyturia; MPO, myeloperoxidase; NCGN, necrotising crescentic glomerulonephritis; NCM, non-classical monocyte; NGAL, neutrophil gelatinase-associated lipocalin; PAS, periodic acid-Schiff; Prot, proteinuria; WT, wild type.

analysed urinary myeloid cells by flow cytometry in 59 subjects, 31 with active antineutrophil cytoplasmic antibody-associated vasculitis kidney manifestation (kAAV), 20 patients in stable AAV Rem but previous AAV kidney manifestation and 8 HCs. Patient characteristics are given in the online supplemental table 1. The gating strategy for the distinct myeloid cell populations is shown in online supplemental figure 6. We found significantly increased urinary neutrophil and monocyte cell counts in kAAV

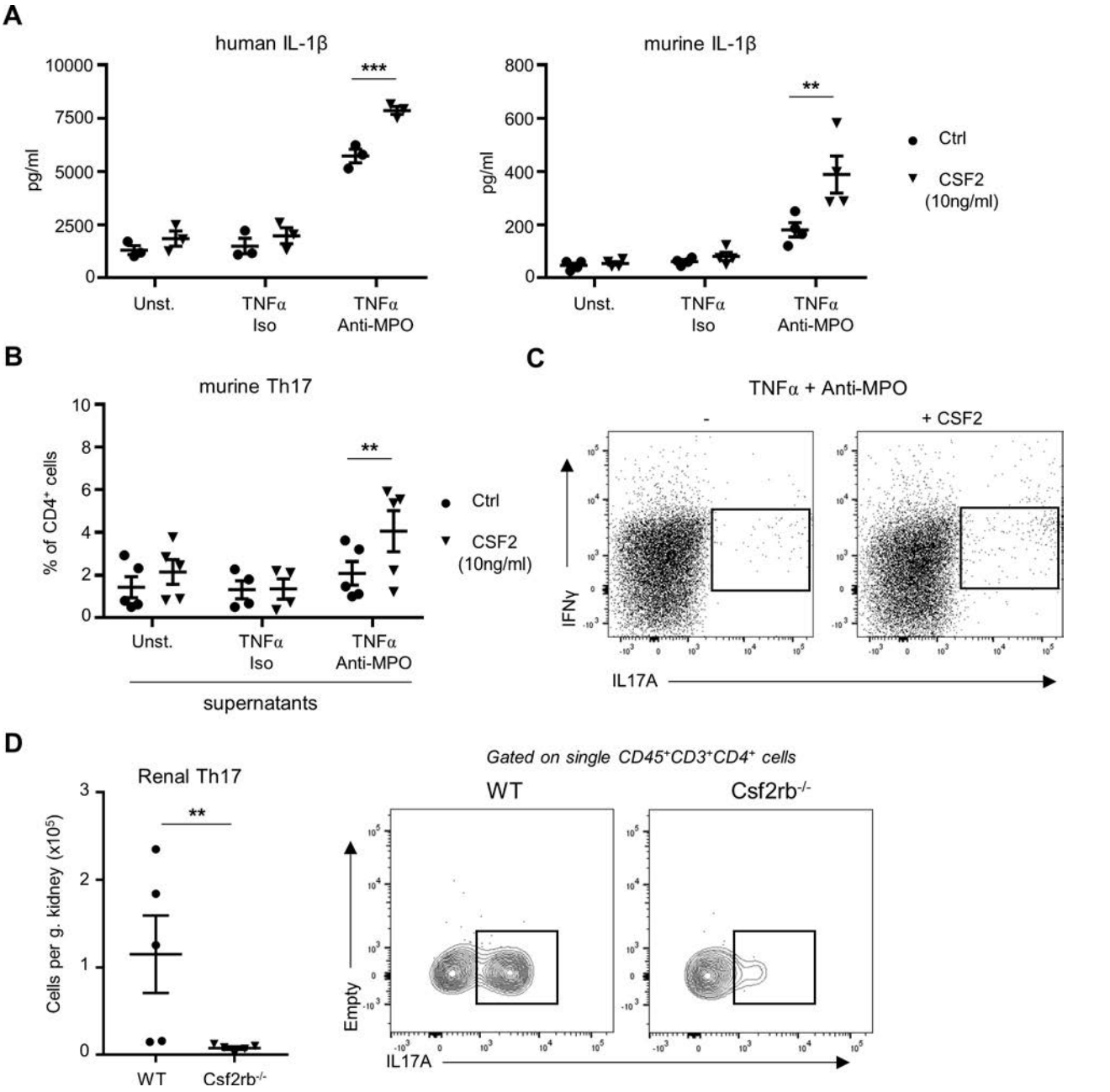

Figure 5 CSF2 increases the capacity of anti-MPO stimulated monocytes to release IL-1β and polarise T_H17 $CD4^+$ T cells. (A) TNFα-primed human or murine neutrophils were isolated and stimulated with either isotype IgG or anti-MPO IgG. Unstimulated cells were used as negative controls. Addition of recombinant murine or human CSF2 (10 ng/mL) increases IL-1β generation by TNFα-primed-monocytes stimulated with anti-MPO as measured in culture medium by ELISA. (B, C) $CD4^+$ T cells were sorted from murine spleen and cultured in medium containing anti-IFN-γ, anti-IL-4, anti-IL-2 antibodies, together with supernatants from TN-α-primed monocytes stimulated with either isotype IgG or anti-MPO IgG. Supernatants from unstimulated monocytes were used as negative control. Addition of recombinant murine CSF2 (10 ng/mL) increases the capacity of TNFα-primed anti-MPO stimulated monocytes to induce T_H17 polarisation. A representative flow cytometry plot is shown. (D) Renal T_H17 $CD4^+$ T cells were analysed by flow cytometry in WT and $Csf2rb^{-/-}$ chimeric mice. **P<0.01, ***P<0.001. CSF2, colony-stimulating factor-2; Csf2rb, CSF2 receptor subunit beta; Ctrl, control; IFN-γ, interferon gamma; IL, interleukin; MPO, myeloperoxidase; TNFα, tumour necrosis factor alpha; WT, wild type.

(median: 17599, IQR 5459–72 194) in comparison to all other groups (Rem: 1116, IQR 638–2376; HC: 1263, IQR 406–2064) (figure 6C). Analysis of distinct monocyte subpopulations by surface staining showed that classical, intermediate and NCMs were strongly increased in patients with kAAV compared with HCs (figure 6D). Moreover, urinary CMs were increased in patients with kAAV compared with patients in Rem, while NCMs were similar (figure 6D,E).

CSF2 blockade reduces kidney damage in crescentic glomerulonephritis

Finally, we performed an animal experiment to address the potential of CSF2 neutralisation as therapeutic option for crescentic glomerulonephritis. To this purpose, we used the nephrotoxic nephritis model (figure 7A). One group of mice received daily injection of anti-CSF2 antibody, whereas control mice received the same amount of isotype IgG. Although mice from

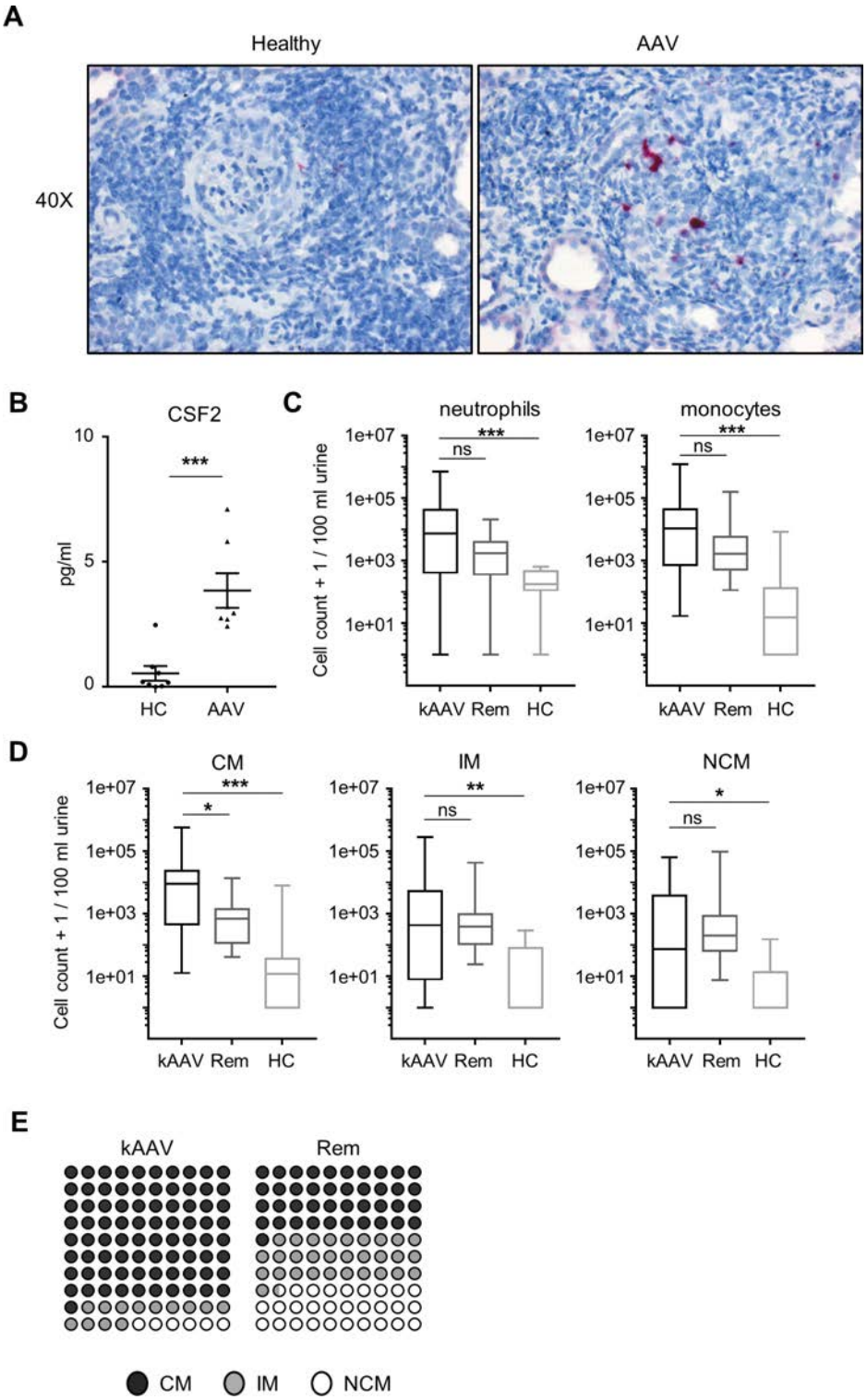

Figure 6 CSF2 and monocyte subsets in patients with AAV and HCs. (A) Human kidney biopsies from patients with AAV were analysed for in situ expression of Csf2 usingRNAscope Probe-Hs-CSF2 mRNA and appropriate positive and negative controls. Counterstaining was done with haematoxylin. A representative picture at ×40 magnification is shown for both groups (left: negative control, right: RNAscope for Csf2). (B) CSF2 protein in human urines was measured by ELISA. CSF2 was increased in urine from patients with active AAV compared with HCs. (C,D) Myeloid cells in urines from patients with active AAV with kidney involvement (kAAV), patients in Rem and HCs were analysed by flow cytometry. Neutrophils, monocytes and the three different monocyte subsets were quantified and expressed as cell number per 100 mL urine. Total neutrophil and monocyte numbers are significantly higher in patients with kAVV compared with HCs. $CD14^{+}CD16^{-}$ CMs, $CD14^{+}CD16^{+}$ IMs and $CD14^{-}CD16^{+}$ NCMs are significantly increased in urine from patients with kAAV compared with HC. In addition, CMs are increased in urine from patients with kAAV compared with patients with AAV in Rem. (E) Dot plots representing the distribution of the different monocyte subsets in urine from patients with kAAV and Rem. *P<0.05, **P<0.01, ***P<0.001. AAV, antineutrophil cytoplasmic antibody-associated vasculitis; CM, classical monocyte; CSF2, colony-stimulating factor-2; HC, healthy control; IM, intermediary monocyte; kAAV, antineutrophil cytoplasmic antibody-associated vasculitis kidney manifestation; NCM, non-classical monocyte; ns, not significant; Rem, remission.

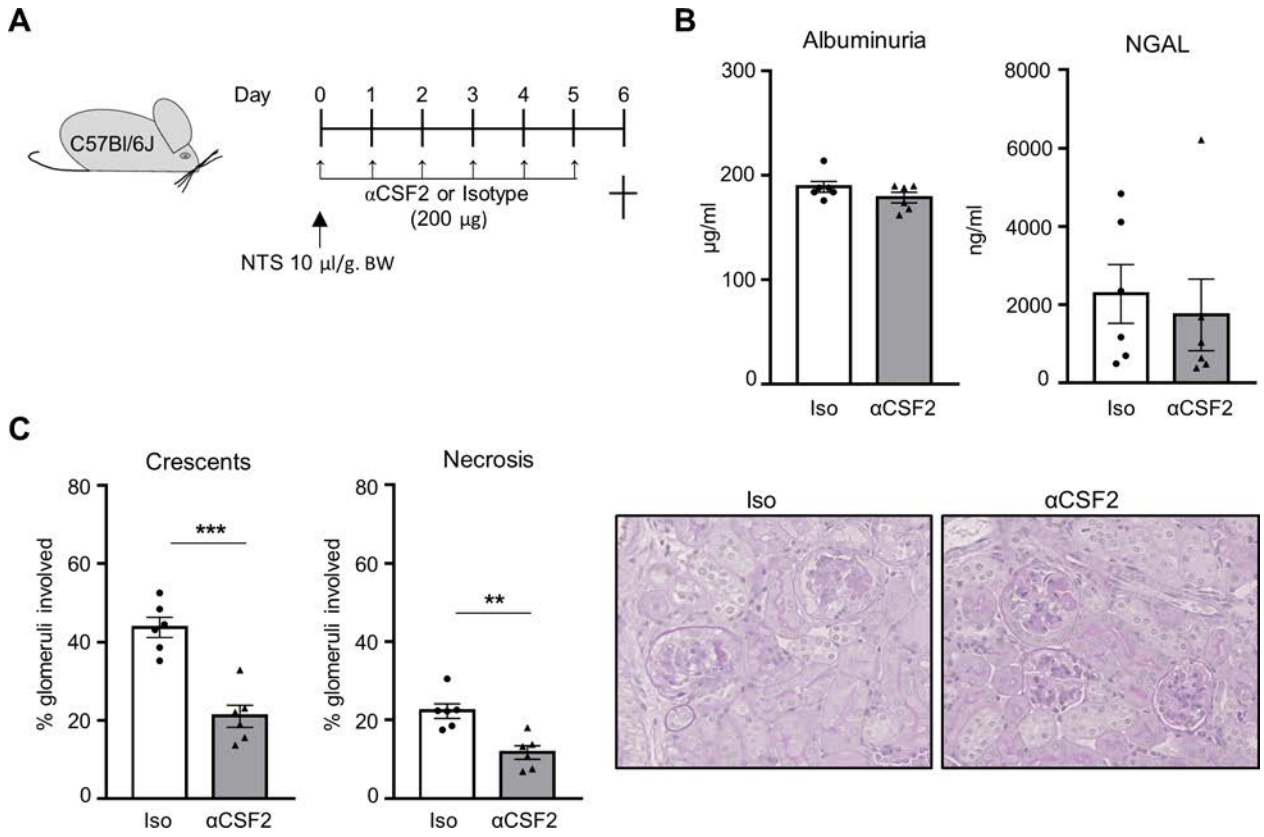

Figure 7 CSF2 neutralisation attenuates crescentic glomerulonephritis. (A) Experimental protocol describing the induction of cGN in C57Bl/6J mice. Animals were sacrificed 6 days after the induction of cGN with NTS. Anti-CSF2 antibodies were injected daily. Control mice received the same amount of isotype. (B) Albuminuria and urine NGAL were determined by ELISA. (C) Mice treated with anti-CSF2 antibodies showed reduced renal damage compared with control mice with significant reduction of crescentic and necrotic glomeruli. For each group, a representative image of a kidney section stained with PAS at high magnification (×40) is shown. **P<0.01, ***P<0.001. cGN, crescentic glomerulonephritis; CSF2, colony-stimulating factor-2; NGAL, neutrophil gelatinase-associated lipocalin; NTS, nephrotoxic serum; PAS, periodic acid-Schiff.

both groups developed high albuminuria and urine NGAL levels (figure 7B), CSF2 blockade attenuated the development of renal damage as the amount of crescentic and necrotic glomeruli was decreased in treated mice compared with control mice injected with isotype IgG (figure 7C).

DISCUSSION

We studied the mechanistic role of monocytes and their subsets in inducing ANCA vasculitis. First, our study shows that CMs play an important non-redundant role in AAV, whereas NCMs are dispensable. Second, we demonstrate the importance of renal CSF2 as a monocyte-targeting disease modifier. Renal CSF2 was increased in mice and patients with AAV and accelerated ANCA-induced monocyte activation, IL-1β release and monocyte-mediated T_H17 polarisation. CSF2rb deficiency reduced the kidney-infiltrating T_H17 cells and protected from NCGN. Third, we show that patients with active AAV with NCGN feature key findings characterised in the mechanistic murine studies, namely, increased renal and urine CSF2, and increased urinary CM numbers. Fourth, we could demonstrate that CSF2 blockade attenuated the development of renal damage in the NTN model of crescentic glomerulonephritis.

The central role of neutrophils in the pathogenesis of AAV is supported by a multitude of in vitro and in vivo studies. For example, Xiao *et al* showed that neutrophil depletion with the NIMP-R14 monoclonal antibody protected mice. However, as this antibody targets the Gr-1 antigen, also known as Ly-6G/Ly6-C, that is expressed by both neutrophils and monocytes, the contribution of monocytes could not be completely ruled out.[25] Conceivably, both monocytes and neutrophils are essential for NCGN induction with distinct time-specific roles. Our results do not exclude a role of neutrophil in the development of AAV but rather suggest that either monocytes modulate neutrophil functions or both cell subsets work in concert to promote vasculitis and renal damage. Our data now firmly establish that CMs are important contributors to MPO–ANCA-induced vasculitis, whereas NCMs are dispensable. Although we cannot completely exclude that minimal residual NCM in C/EBPβ$^{-/-}$ chimeric mice played a minor role, the almost unaffected circulating NCMs in CCR2$^{-/-}$ chimeric mice and the even increased infiltration of NCM in kidneys of CSF2rb$^{-/-}$ chimeric mice that displayed strongly reduced renal damage further support our conclusion derived from C/EBPβ$^{-/-}$ mice. Using ANCA unrelated animal models, NCMs were described to perform patrolling functions, thereby controlling both CM and neutrophil influx. Using imaging studies, Finsterbusch *et al* demonstrated in a murine NTN model that NCMs continuously traffic along the glomerular endothelium and that CX3CR1 expressed by NCM is crucial for this process.[24] Turner-Stokes *et al* showed in a rat NTN model that monocytes infiltrate the inflamed glomerulus in a two-wave recruitment process.[26] The investigators showed that NCMs enter the glomerulus initially, whereas CMs follow at later time points. However, NTN does not feature characteristics of human ANCA-induced NCGN, and our murine and human data support the notion that CMs rather than NCMs are important in inducing ANCA-mediated pauci-immune NCGN.

Importantly, although NCMs have been described to play an important role in the earlier phase of disease induction in the NTN model, our overall injury data at later time points clearly demonstrate that CMs are the major inducer of cumulative kidney damage in an anti-MPO IgG-induced NCGN. Conceivably, with single-cell studies, the distinction of monocytes into three subsets may become obsolete. These techniques will provide a better tool to characterise additional monocyte and monocyte-derived cell populations in physiological and pathological conditions.[27–30]

Our data provide a mechanistic explanation how monocytes contribute to vasculitis and the subsequent kidney damage. We previously showed that ANCA-stimulated monocytes released significantly more IL-1β compared with neutrophils and that blocking the IL-1β receptor protected mice from AAV.[15 31] Now, we show that increased CSF2 augmented the capacity of ANCA-stimulated monocytes to release IL-1β and subsequently to induce T_H17 polarisation in vitro. T_H17 cells play an important role in the pathogenesis of NCGN,[32] including NCGN induced by ANCA.[33] Conceivably, additional monocyte-mediated disease mechanisms are at work. For example, monocytes release extracellular traps in response to ANCA that can directly induce endothelial cell damage (personal data). In addition, the absence of CM likely reduces the amount of circulating IM. The contribution of IM in AAV was suggested but remains controversial.[16 34] Moreover, the local renal environment of monocyte-derived macrophages and dendritic cells is likely altered by the absence of CM. In summary, we believe that CMs predominantly mediate their pathogenic roles by indirect effects by modulating $CD4^+$ T-cell polarisation rather than by directly damaging effects.

CSF2 has evolved as a key proinflammatory cytokine involved in the pathogenesis of several inflammatory and autoimmune disorders, including experimental autoimmune encephalomyelitis and arthritis.[35] CSF2 was produced by resident renal cells in an NTN model,[36] and CSF2 released by renal and immune cells contributed to renal injury in a murine anti-GBM glomerulonephritis model.[37 38] Interestingly, CSF2 induced a pathogenic signature in $Ly6C^{hi}$ $CCR2^+$ monocytes and monocyte-derived dendritic cells that was characterised by an increased IL-1β generation.[22] Our data now establish a mechanistic role of CSF2 in regulating monocyte activation in AAV. We demonstrated that CSF2 is not only increased in kidney and urine of mice with AAV but also augments the capacity of ANCA-activated monocytes to release IL-1β and to induce T_H17 polarisation. We confirmed the mechanistic CSF2 role in vivo by showing that mice lacking CSF2rb in their haematopoietic cells were protected from the development of ANCA-induced NCGN and had reduced numbers of kidney-infiltrating T_H17 cells. Importantly, we found increased CSF2 expression in kidney biopsies and increased CSF2 protein in the urine from active AAV patients with NCGN. Importantly, CSF2 neutralisation attenuated kidney damage in the NTN model of crescentic glomerulonephritis. Together with the finding that urinary CMs were increased in active, but not patients with AAV on Rem, we confirmed key elements of the mechanistic CSF2-CSF2rb-CM concept in the human AAV disease. Of note, other cytokines (such as tumour necrosis factor alpha or interferon gamma among others) besides CSF2 can potentially activate, modulate or even dampen monocyte functions and thereby can also be involved in the regulation of renal injury in AAV. Our results may encourage future studies exploring CSF2 and its receptor interaction as a potential therapeutic target in AAV, particularly since compounds targeting either CSF2 or its receptor are currently tested in clinical trials (reviewed in Ingelfinger *et al*[35]).

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Patient and public involvement Patients and/or the public were not involved in the design, conduct, reporting or dissemination plans of this research.

Patient consent for publication Not applicable.

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TRANSLATIONAL SCIENCE

Low-ratio somatic NLRC4 mutation causes late-onset autoinflammatory disease

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ABSTRACT

Objectives We aim to investigate the genetic basis of a case of late-onset autoinflammatory disease characterised by arthritis, recurrent fever and skin rashes.

Methods We performed whole-exome/genome sequencing and digital droplet PCR (ddPCR) to identify the pathogenic somatic mutation. We used single-cell RNA sequencing (scRNA-seq), intracellular cytokine staining, quantitative PCR, immunohistochemistry and western blotting to define inflammatory signatures and to explore the pathogenic mechanism.

Results We identified a somatic mutation in *NLRC4* (p.His443Gln) with the highest mosaicism ratio in the patient's monocytes (5.69%). The somatic mutation resulted in constitutive NLRC4 activation, spontaneous apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) aggregation, caspase-1 hyperactivation and increased production of interleukin (IL)-1β and IL-18. Moreover, we demonstrated effective suppression of inflammatory cytokine production by targeting gasdermin D, an approach that could be considered as a novel treatment strategy for patients with *NLRC4*-associated autoinflammatory syndrome.

Conclusions We reported a case of a late-onset autoinflammatory disease caused by a somatic *NLRC4* mutation in a small subset of leucocytes. We systemically analysed this condition at a single-cell transcriptomic level and revealed specific enhancement of inflammatory response in myeloid cells.

Key messages

What is already known about this subject?

⇒ Germline gain-of-function mutations in *NLRC4* have been linked to autoinflammation with infantile enterocolitis/recurrent macrophage activation syndrome or familial cold autoinflammatory syndrome.

What does this study add?

⇒ Our study reported a case of a late-onset autoinflammatory disease caused by a low-ratio somatic NLRC4 mutation, with enhanced inflammatory signalling specifically in myeloid cells. The study highlights the importance of in-depth genetic testing for adult-onset autoinflammatory diseases.

⇒ Mechanistic studies indicated that H443Q somatic mutation resulted in stronger NLRC4 activation and more interleukin (IL)-1β and IL-18 production than other germline pathogenic mutations.

How might this impact on clinical practice or future developments?

⇒ Treatment with IL-6 inhibitor is effective in inhibiting inflammation for the patient with somatic *NLRC4* mutation. And blocking cytokine secretion by targeting gasdermin D may provide a novel therapeutic strategy for inflammasomopathies.

INTRODUCTION

Inflammasomopathies are autoinflammatory diseases typically caused by gain-of-function mutations that enhance the activation of various inflammasomes including NLRP1, NLRP3, pyrin and NLRC4.[1] NLRC4 functions as a cytosolic innate immune receptor for sensing bacterial flagellin and restriction of bacterial replication.[2] On stimulation or activation by gain-of-function mutations, NLRC4 can bind the inflammasome adaptor ASC to promote caspase-1 activation.[2] Active caspase-1 functions to cleave and release IL-1β and IL-18 and also cleaves gasdermin D to trigger pyroptosis.[3–5] Germline gain-of-function mutations in *NLRC4* have been linked to autoinflammation with infantile enterocolitis/recurrent macrophage activation syndrome (AIFEC; OMIM 616050) or familial cold autoinflammatory syndrome (FCAS4; OMIM 616115).[4–7]

Somatic mutations, which are acquired postfertilisation, have been reported as causal variants in several autoinflammatory diseases, such as somatic *NLRP3* in cryopyrin-associated periodic syndrome (CAPS)[8 9] and somatic *UBA1* in vacuoles, E1 enzyme, X-linked, autoinflammatory, somatic (VEXAS) syndrome.[10] To date, *NLRC4* somatic mosaicism has only been reported in two patients presented with neonatal-onset autoinflammatory disease.[11 12] However, late-onset autoinflammatory disease associated with *NLRC4* somatic mutation has not been reported yet.

PATIENTS AND METHODS

Patient (somatic H443Q) and patient control (T177N/WT) were evaluated under protocols approved by the institutional review board of Second Affiliated Hospital and Yuying Children's Hospital, respectively. Both patients provided written informed consent. We performed genetic analysis and functional studies with the use of samples including serum, peripheral blood

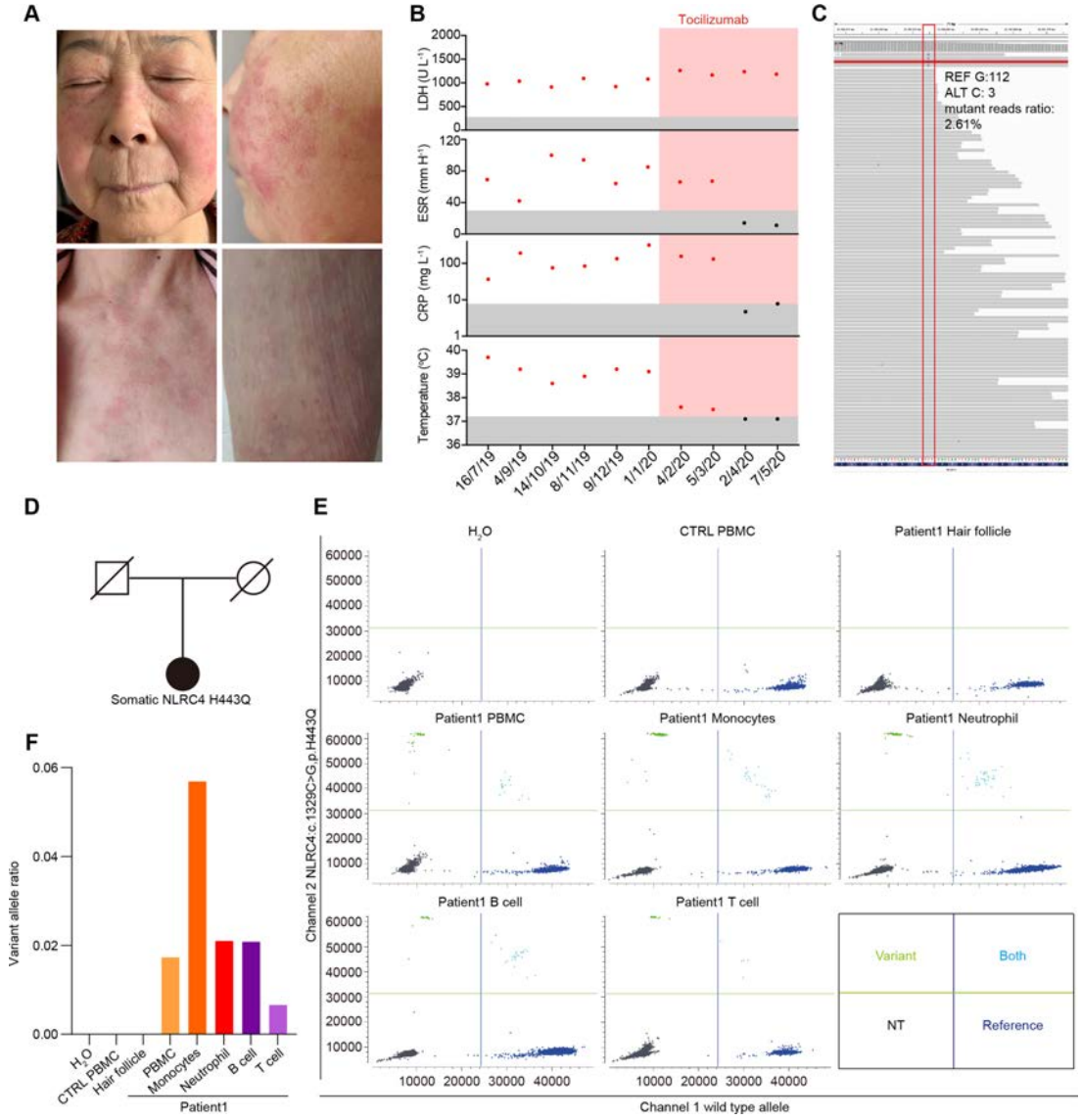

Figure 1 Somatic mutation *NLRC4* H443Q in myeloid cells associated with late-onset autoinflammatory disease. (A) Images of skin rashes in face (upper), chest (lower left) and arm (lower right) from patient 1 (P1). (B) Laboratory parameters from P1 including lactate dehydrogenase (LDH), erythrocyte sedimentation rate (ESR) and C reactive protein (CRP), and daily maximum body temperature reading before and after tocilizumab treatment over 10 months. (C) Exome sequencing reads covering the somatic H443Q variant (NM_001199138: c.1329C>G) in P1 displayed by the integrative genomics viewer. (D) Family pedigree of P1 with the somatic variant in *NLRC4*. (E–F) Quantification of variant allele ratio by ddPCR in H_2O and DNA from control (CTRL) PBMCs and P1's hair follicles, PBMCs, and sorted monocyte, neutrophil, B-cell, T-cell populations. Two-dimensional fluorescence amplitude plots (E) show droplets with *NLRC4* variant p.H443Q (upper left), NLRC4 wild type (WT) (lower right), both templates (upper right) or no *NLRC4* template (lower left) as indicated, each dot represents a droplet. (F) Quantification of variant allele ratio based on droplet counts.

mononuclear cells (PBMCs) and muscle tissue from patients and control or transfected HEK293T cell lysates. The materials and methods are available in the online supplemental information file.

RESULTS

A patient with late-onset autoinflammatory disease due to a *NLRC4* somatic mutation

A 69-year-old Chinese woman (patient 1, P1) presented with recurrent rash and long-lasting fever episodes in her early 60s (figure 1A,B). Skin biopsy suggested cutaneous vasculitis. Laboratory studies consistently showed elevated erythrocyte sedimentation rate (42–100 mm/hour), C reactive protein (36–190 mg/L) and lactic dehydrogenase (LDH; 912–1800 U/L).

To investigate the pathogenicity of the disease, we performed whole-exome sequencing (WES) and whole-genome sequencing but the results did not reveal any causal variants. Considering the late disease onset, we reanalysed WES data for somatic mutation and identified a somatic missense variant c.1329C>G (p.His443Gln) in *NLRC4*, with the mosaicism ratio of 2.61% (figure 1C,D). Allele-specific PCR and Sanger sequencing of PCR products also confirmed the presence of somatic mutation (online supplemental figure 1A,B).

The mosaicism ratio determined by ddPCR was about 1.73% in PBMCs and was highest in monocytes (5.69%), followed by neutrophil (2.10%), B cells (2.08%) and lowest in T cells (0.66%). The mutant allele was not detected in the patient's hair follicles (0%) or in PBMCs from a healthy control (figure 1E,F).

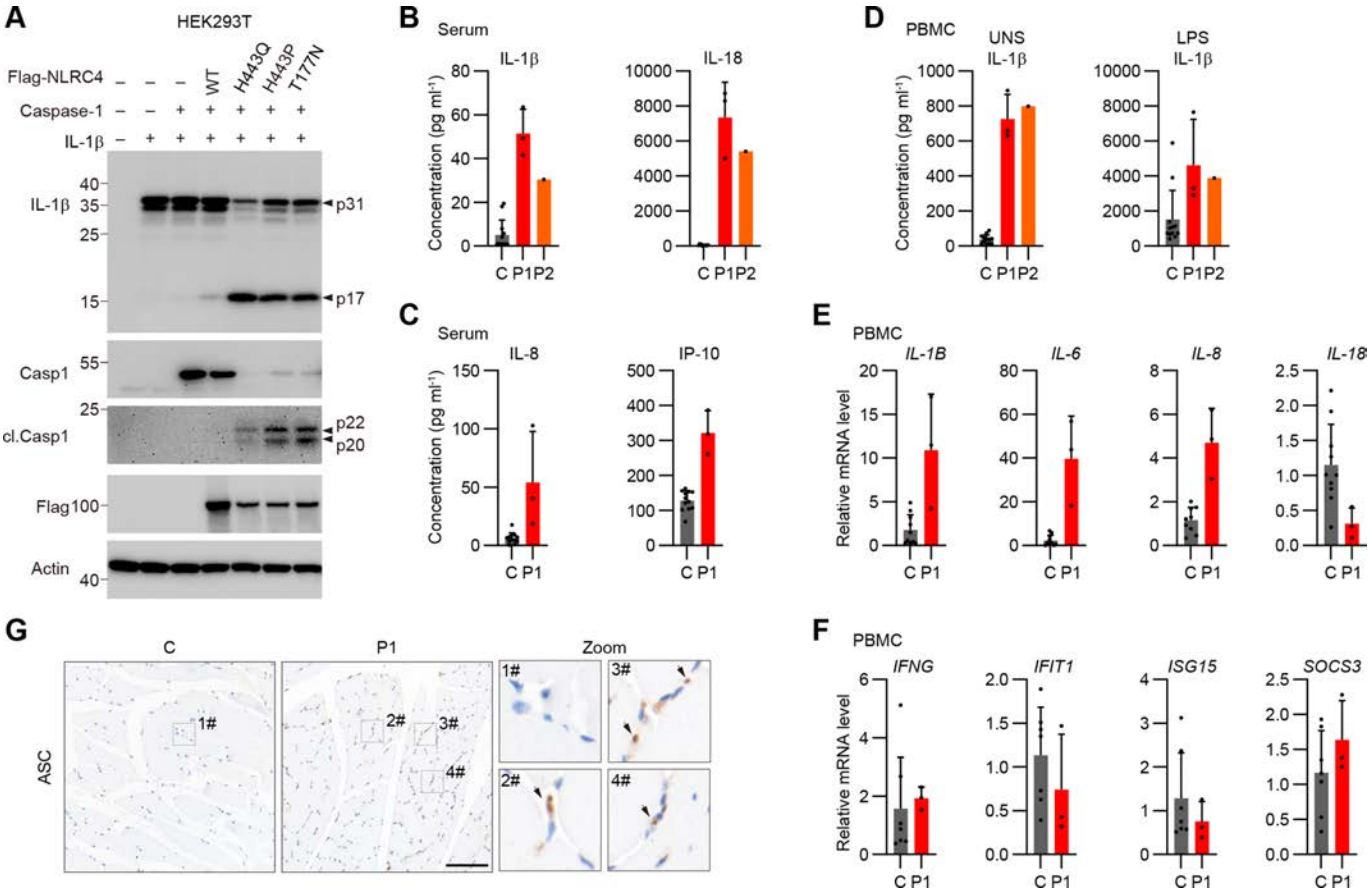

Figure 2 Somatic *NLRC4* H443Q mutation leads to constitutive activation of inflammasome signalling. (A) In vitro validation of NLRC4 H443Q gain of function. Flag-tagged wild-type or mutant NLRC4, caspase-1, interleukin (IL)-1β were coexpressed in HEK293T as indicated for 20 hours. Whole-cell lysates were immunoblotted with indicated antibodies.(B–D) Serum or supernatant cytokine levels of patient 1 (P1) (n=3 samples), patient 2 (P2, germline NLRC4 T177N/wild type (WT) carrier; n=1 sample) and 12 healthy controls. IL-1β and IL-18 levels in the serum were determined by ELISA (B). IL-8 and IFN-gamma-inducible protein 10 (IP-10) were determined using the cytometric bead array (CBA) assay (C). IL-1β in the supernatant of cultured PBMCs incubated for 9 hours with or without lipopolysaccharide (LPS) were determined by ELISA (D). (E–F) Quantification of cytokine mRNA levels in PBMCs isolated from three P1 samples and 10 healthy controls (E) and expression of interferon-inducible genes in P1 samples and seven controls (F) Dots in (B–F) represent different time points for P1 and P2, and different individuals in the control group. Graphs show mean±SD. (G) ASC staining of muscle tissue sections from P1's skin lesion and a control patient with myasthenia without evidence of inflammatory signature. Arrows, ASC aggregates. Scale bar, 200 µm.

Therefore, the low ratio of somatic *NLRC4* mutation likely occurred as a late-onset event and clonally expanded mainly in the myeloid cells.

H443Q promotes constitutive inflammasome activation

The H443Q substitution in *NLRC4* is a novel mutation which was not present in gnomAD or in Kaviar database. It was also absent in Chinese cohorts in ChinaMAP and HUABIAO. This novel p.H443Q variant was predicted to be damaging by multiple software and with combined annotation-dependent depletion (CADD) score of 16.54 (online supplemental figure 1C). Germline pathogenic variant p.His443Pro has been reported in a family with FCAS.[6] The His443 residue is located in the winged-helix domain and forms hydrogen bonds with ADP, which is critical for NLRC4 autoinhibition.[13] The H443Q mutation likely disrupts ADP-mediated autoinhibition and potentially causes constitutive NLRC4 activation (online supplemental figure 1D). Taken together, the H443Q is a likely pathogenic variant according to ACMG variant interpretation guideline.

Next, we examined the effect of mutant NLRC4 on the activation of caspase-1 and the processing of IL-1β in vitro.[13] Germline gain-of-function mutations including the reported mutation p.His443Pro and p.Thr177Asn, a new de novo mutation identified in a 9-year-old affected girl (P2) (online supplemental figure 1E,F) could activate the cleavage of procaspase-1 and pro-IL-1β. Interestingly, the H443Q mutation showed greater ability to process pro-IL-1β than the other two pathogenic mutations (figure 2A), which suggests that H443Q mutant has a more substantial effect on inflammasome activation.

In addition, proinflammatory cytokines including IL-1β, IL-18, IL-8 and IP-10 were all significantly upregulated in the patient P1's serum during disease flares (figure 2B,C). Although IL-1β and IL-18 decreased during remission, the levels remained elevated (online supplemental figure 2A,B). Compared with healthy controls, patients with either somatic mutation or germline mutation possessed higher IL-1β and IL-18 levels in the serum (figure 2B), and their PBMCs also secreted more IL-1β in the supernatants, with or without lipopolysaccharide priming (figure 2D). The mRNA expression of *IL-1B*, *IL-6*, *IL-8*, but not *IL-18* were all elevated at the basal level in patient P1's PBMCs during flares (figure 2E) and at baseline (online supplemental figure 2C), supportive of a strong proinflammatory signature triggered by NLRC4 activation in the patient. Furthermore, we detected increased LDH and adenylate kinase release by PBMCs from both patients, indicating

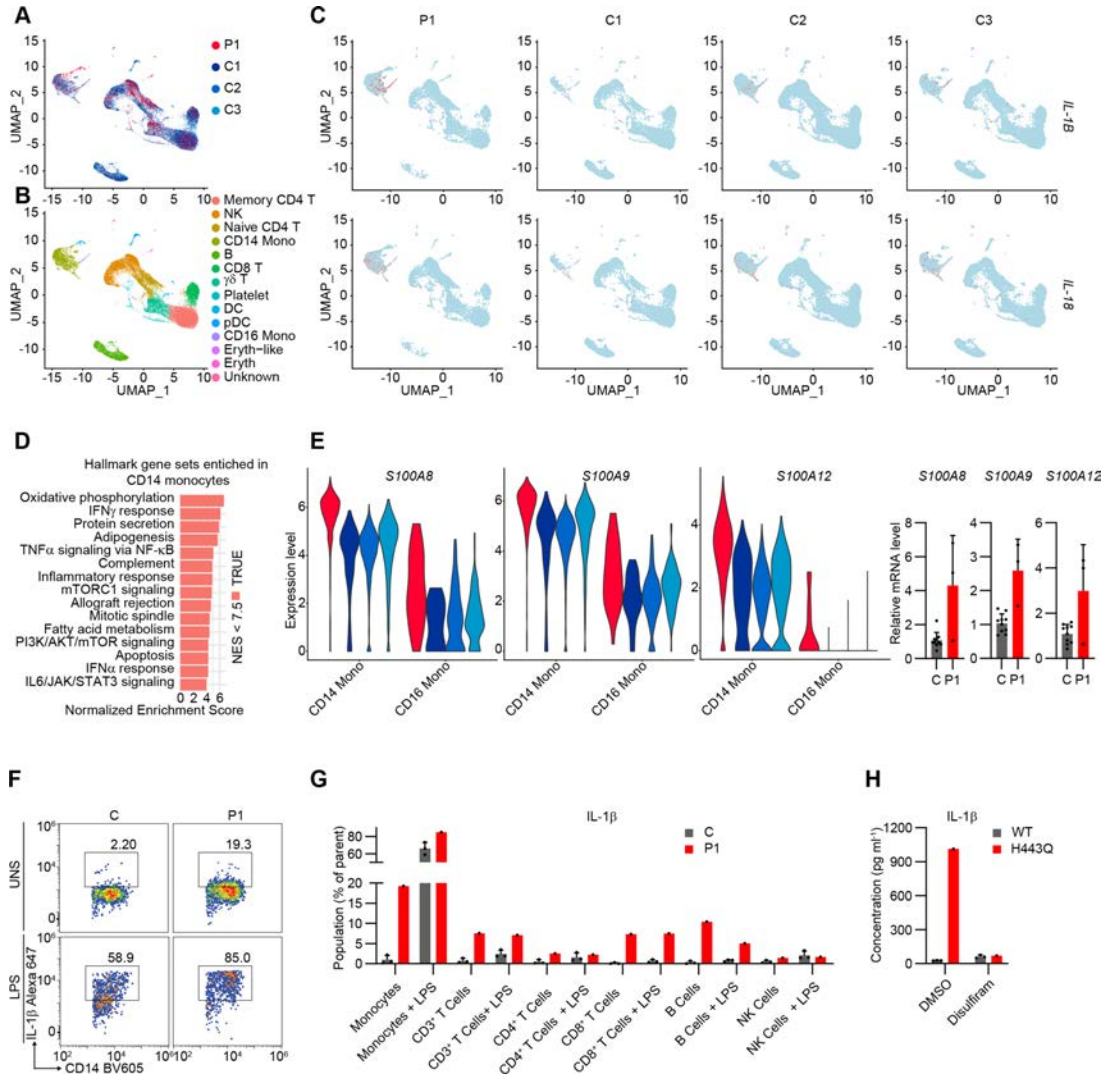

Figure 3 sc-RNA sequencing analysis of patient 1 (P1's) PBMCs. (A) Uniform manifold approximation and projection (UMAP) of 44 687 cells, split between P1 and three healthy controls (C1–C3) after alignment. (B) UMAP visualisation and marker-based annotation of 14 cell subtypes, coloured by cluster identity. (C) Visualisation of expression of interleukin (*IL)-1B and IL-18* (coloured single cells) on UMAP plot. (D) Gene set enrichment analysis (GSEA) analysis of CD14+ monocytes showed enriched pathways from hallmark gene sets. NES, normalised enrichment score. (E) Violin plots (left) shows the expression of *S100A8, S100A9*, *S100A12* in monocytes from P1 or controls, bar graph (right) shows relative mRNA levels in PBMCs from three P1 samples and 10 healthy controls quantified by quantitative PCR. Dots represent individual time points for P1 or different individuals in the control group. (F,G) Intracellular cytokine analysis of IL-1β in whole blood from P1 and three healthy controls treated with or without 1 mg/mL lipopolysaccharide (LPS) for 6 hours. Representative fluorescence-activated cell sorting (FACS) plot of IL-1β staining in CD14+ monocytes (F) and aggregated results (G) are presented. UNS for unstimulated. (H) PBMCs isolated from P1 and three healthy controls were treated with 40 µM disulfiram or dimethyl sulfoxide (DMSO) for 9 hours, IL-1β in supernatant were determined with ELISA. Dots in G–H represent each individual and all graphs show mean±SD.

that *NLRC4* somatic or germline mutations may also promote cell death (online supplemental figure 2D).

In addition to recurrent fever episodes, the patient also presented with skin rashes and muscle pain, which may be caused by inflammatory infiltrates in the dermis or muscle. In the patient's muscle biopsy, we observed a higher level of ASC specks in endomysium compared with control muscle tissue by immunohistochemistry staining, which provides evidence for infiltration of immune cells with activated inflammasomes in vivo (figure 2G).

scRNA-seq reveals enhanced inflammatory signalling in myeloid cells specifically

scRNA-seq was performed for further investigation of the effect of somatic mutation on different cell types at the transcriptome level (figure 3A,B). The mRNA levels of *IL-1B*, but not *IL-18*, were increased in the monocytes of patient than healthy controls (figures 2E and 3C).

Although activation of nuclear factor kappa B (NF-κB) and type-I interferon (IFN) pathways was undetectable in patient's PBMCs by quantitative PCR (qPCR) (figure 2F; online supplemental figure 3A), scRNA-seq shows that genes involved in IFN, NF-κB signalling and inflammatory response were enriched and upregulated in monocytes (figure 3D; online supplemental figure 3B, online supplemental figure 4). The calgranulin genes *S100A8*, *S100A9* and *S100A12*, which function as damage-associated molecular patterns (DAMPs) to induce inflammatory response,[5] were highly expressed specifically in myeloid cells, and their upregulation was also detectable in PBMCs by qPCR (figure 3E).

Intracellular cytokine staining confirmed IL-1β overproduction in T cells, B cells and especially in monocytes at baseline in the patient compared with healthy controls (figure 3F,G). These results demonstrated that the inflammatory response associated with *NLRC4* H443Q was mainly driven by monocytes. Given that non-immune cell populations were not thoroughly examined in this study, it remains a possibility that non-immune cells also harbour the mutation and contribute to disease.

Blocking cytokine secretion by targeting gasdermin D

IL-1β and IL-18 blockade have both been reported as effective treatment options for patients carrying germline NLRC4 mutations.[5 14] These choices may also be effective for our patient with NLRC4 somatic mutation, but these agents are currently unavailable in our region. Treatment with the IL-6 inhibitor tocilizumab (320 mg/month) effectively suppressed fever, skin rash and acute phase reactants (figure 1B), although LDH levels remain elevated and muscle pain is not alleviated in the patient.

Disulfiram, an Food and Drug Administration-approved alcohol deterrent, was reported to covalently target gasdermin D protein to block pyroptosis and inflammasome-mediated cytokine release.[15] Indeed, disulfiram effectively inhibited basal IL-1β release by patient P1's PBMCs (figure 3H), illustrating the possibility of using disulfiram to treat patients with NLRC4-associated inflammasomopathies.

DISCUSSION

Here we report a case of late-onset autoinflammatory syndrome associated with a novel somatic mutation H443Q in *NLRC4*. Germline H443P mutation was associated with the familial cold autoinflammatory syndrome,[6] and the low-temperature induced hyperactivation of caspase-1 by H443P might be due to the loss of inhibition by HSC70.[16] However, the patient with somatic H443Q mutation does not show cold-induced urticaria. Macrophage activation syndrome, enterocolitis, hepatomegaly, hepatitis, monocytopaenia, hyperferritinaemia and hypertriglyceridaemia, which are typical clinical features of patients with NLRC4 germline mutations,[4 5] were also not present in this patient. The H443P mutation was demonstrated to constitutively activate caspase-8-dependent cell death signalling,[17] but cell death due to NLRC4 somatic mutation was significantly lower than that caused by germline mutation (online supplemental figure 2D). Both H443P and H443Q are gain-of-function mutations, but H443Q displayed stronger NLRC4 activation. These findings suggest that even though the mutations occurred on the same amino acid residue His443, the effects are distinct and the pathological mechanisms may be different.

We also reported the efficacy of tocilizumab in ameliorating the fever and elevated acute phase reactants for the patient with somatic *NLRC4* mutation. Moreover, we provided proof of concept for blocking cytokine secretion by targeting gasdermin D with disulfiram. The efficacy and safety of disulfiram or other gasdermin inhibitors need to be addressed with future investigation. Specific NLRC4 inhibitors may be another choice of treatment in the future.[18]

The variant allele fraction (VAF) in this patient is relatively low compared with VEXAS.[10] However, it is comparable to the VAF detected in patients with somatic mutations in *NLRP3*.[19 20] Perhaps a VAF of 5%–15% is sufficient to cause pathology in inflammasomopathies, because a low ratio of mutant-activated inflammasome is sufficient to produce IL-1β and IL-18 and trigger inflammation, while further clonal expansion might be limited by pyroptosis.

Somatic mutation may account for many autoimmune and autoinflammatory diseases with undetermined genetic explanation due to difficulties in detecting low levels of a mutant allele. The discovery of somatic mutation in *NLRC4* with a low allelic frequency in this study together with a contemporaneous study which reports a distinct somatic *NLRC4* mutation[21] highlights the necessity to search for somatic mutations in adult-onset autoinflammatory diseases, which may be otherwise missed by standard diagnostic testing.

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Contributors QZ, TJ designed the study, directed and supervised the research. TJ, JW, XY and RF performed experiments and analysed the data. QY, WZ, FL, YS, ZD and PYL enrolled the patients, collected and interpreted clinical information. QZ, TJ, JW and PYL wrote the manuscript with input from others. All authors contributed to the review and approval of the manuscript. QZ is the author acting as guarantor.

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Competing interests ZD is the employee of MyGenostics.

Patient and public involvement Patients and/or the public were not involved in the design, conduct, reporting or dissemination plans of this research.

Patient consent for publication Obtained.

Ethics approval This study involves human participants and was approved by the institutional review boards of the Second Affiliated Hospital of Jiaxing University (JXEY-2021ZXFQ016) and the Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University (2021-K-327-02). Participants gave informed consent to participate in the study before taking part.

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TRANSLATIONAL SCIENCE

Krüppel-like factor-4 and Krüppel-like factor-2 are important regulators of joint tissue cells and protect against tissue destruction and inflammation in osteoarthritis

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ABSTRACT

Objectives Analysing expression patterns of Krüppel-like factor (KLF) transcription factors in normal and osteoarthritis (OA) human cartilage, and determining functions and mechanisms of KLF4 and KLF2 in joint homoeostasis and OA pathogenesis.

Methods Experimental approaches included human joint tissues cells, transgenic mice and mouse OA model with viral KLF4 gene delivery to demonstrate therapeutic benefit in structure and pain improvement. Mechanistic studies applied global gene expression analysis and chromatin immunoprecipitation sequencing (ChIP-seq).

Results Several KLF genes were significantly decreased in OA cartilage. Among them, KLF4 and KLF2 were strong inducers of cartilage collagen genes and Proteoglycan-4. Cartilage-specific deletion of *Klf2* in mature mice aggravated severity of experimental OA. Transduction of human chondrocytes with Adenovirus (Ad) expressing KLF4 or KLF2 enhanced expression of major cartilage extracellular matrix (ECM) genes and SRY-box transcription factor-9, and suppressed mediators of inflammation and ECM-degrading enzymes. Ad-KLF4 and Ad-KLF2 enhanced similar protective functions in meniscus cells and synoviocytes, and promoted chondrocytic differentiation of human mesenchymal stem cells. Viral KLF4 delivery into mouse knees reduced severity of OA-associated changes in cartilage, meniscus and synovium, and improved pain behaviours. ChIP-seq analysis suggested that KLF4 directly bound cartilage signature genes. Ras-related protein-1 signalling was the most enriched pathway in KLF4-transduced cells, and its signalling axis was involved in upregulating cartilage ECM genes by KLF4 and KLF2.

Conclusions KLF4 and KLF2 may be central transcription factors that increase protective and regenerative functions in joint tissue cells, suggesting that KLF gene transfer or molecules upregulating KLFs are therapeutic candidates for OA.

Key messages

What is already known about this subject?

⇒ We previously performed RNA-seq analysis of normal and osteoarthritis (OA) human knee cartilage and found that several members in the Krüppel-like factor (KLF) family of transcription factors are suppressed in OA.

What does this study add?

⇒ The suppression of KLF4 and KLF2 seen in OA does have functional consequences for the phenotype of cells in joint tissues, leading to increased OA severity.

⇒ KLF4 and KLF2 upregulate major cartilage extracellular matrix (ECM) and chondrogenic genes, and they also suppress mediators of inflammation and ECM-degrading enzymes.

⇒ Delivery of KLF4 gene into mouse knee joints reduces severity of experimental OA-associated changes in cartilage, meniscus and synovium and improves pain behaviours.

How might this impact on clinical practice or future developments?

⇒ KLF4 and KLF2 are central transcription factors regulating protective functions in joints, and are promising therapeutic targets for joint diseases such as OA.

INTRODUCTION

Osteoarthritis (OA) is the most common joint disease.[1] Despite substantial progress in identifying mechanisms of OA pathogenesis and molecular targets for intervention,[2] there have thus far not been any successful clinical trials and there are no approved pharmacological treatments to prevent the disease onset or progression. A potential explanation is that more important molecular mechanisms than those previously targeted are involved in OA.

Degradation and loss of articular cartilage is a major factor of OA pathogenesis.[2] Cartilage extracellular matrix (ECM) molecules, including type-2 and type-11 collagen (COL2A1 and COL11A2), aggrecan (ACAN) and cartilage oligomeric matrix protein (COMP), are regulated by SRY-box transcription factor-9 (SOX9) cooperating with SOX5 and SOX6.[3–7] Proteoglycan-4 (PRG4) also known as lubricin is dominantly expressed in the superficial zone of articular cartilage, and is essential for homoeostasis of articular joints to prevent damage to the articular surface.[8] While several transcription

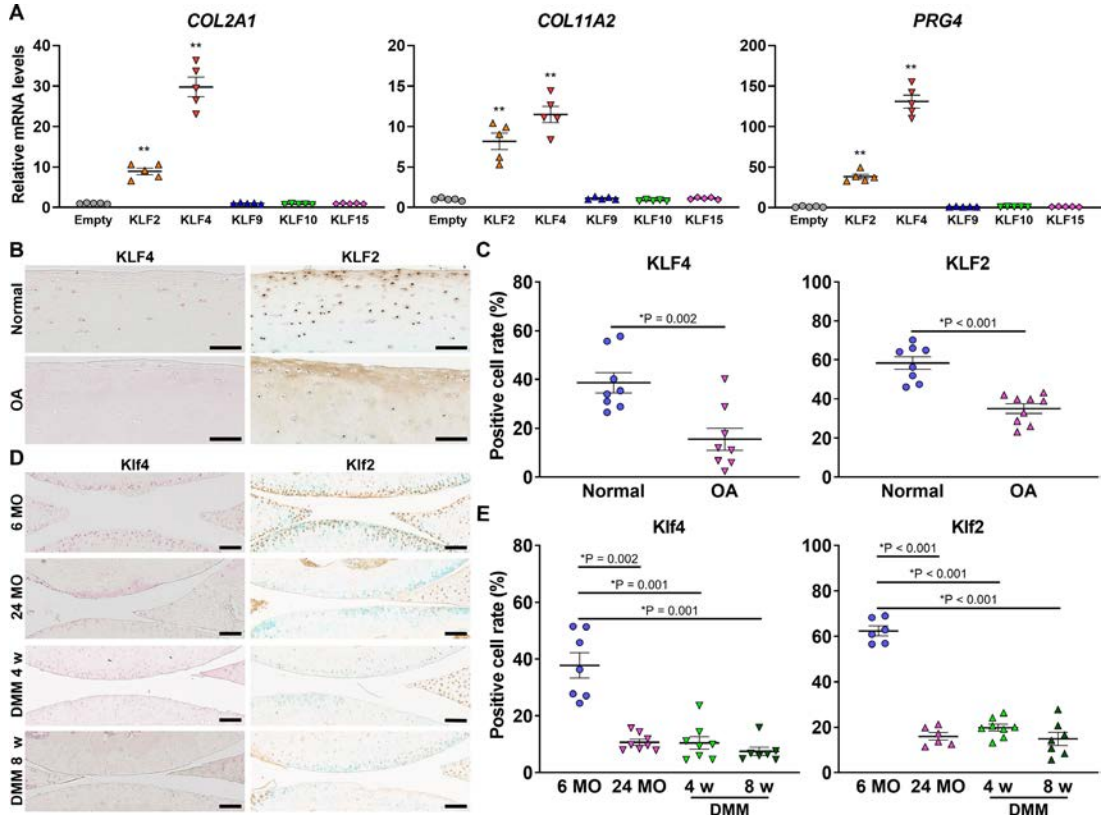

Figure 1 Regulation of cartilage collagen genes and *PRG4* by KLFs, and expression of KLF4 and KLF2 in human and mouse cartilage tissues. (A) TC28a2 cells were transfected with an empty vector, or expression vectors for either KLF2, KLF4, KLF9, KLF10 or KLF15, and RNA was collected 48 hours after transfection (n=5 from five independent experiments). mRNA levels relative to empty vector are shown. *P<0.05, **P<0.01 vs empty vector, Dunnett's test. (B, C) Immunohistochemistry (IHC) for KLF4 and KLF2 in human knee cartilage. For KLF4, normal cartilage samples were from eight donors (5 males and 3 females; age 22–48, mean 33±3), while osteoarthritis (OA)-affected cartilage samples were from eight donors (4 males and 4 females; age 29–90, mean 65±7). For KLF2, normal cartilage samples were from eight donors (5 males and 3 females; age 18–53, mean 38±4), while OA-affected cartilages were from nine donors (3 males and 6 females; age 51–90, mean 70±5). *P<0.05, Welch's t-test in (C). (D, E) IHC for KLF4 and KLF2 in mouse knee cartilage (n=6–8 per condition). While 6 MO samples were from knees of 6-month-old mice, 24 MO indicates samples from knees of 24-month-old mice. DMM 4 w and DMM 8 w samples were from knees of 6-month-old mice 4 and 8 weeks after destabilisation of the medial meniscus (DMM) surgery. *P<0.05 vs 6 MO, Dunnett's T3 test in (E). All quantitative data are expressed as means±SE, and results of omnibus tests for multiple comparisons are shown in online supplemental table 2. Scale bars, 100 µm.

factors are reported to regulate PRG4,[9–11] PRG4 is not subject to regulation by SOX9, which is a different regulatory mechanism compared with other cartilage ECM genes described earlier.[5 12] A transcription factor that upregulates all these cartilage signature genes would be a promising therapeutic for cartilage engineering and in treatment of OA.

Activation of catabolic and inflammatory events is another key mechanism in OA.[2 13 14] Therefore, suppressing catabolic and inflammatory genes, including a disintegrin and metalloproteinase with thrombospondin motifs-5 (ADAMTS5), matrix metalloproteinase-3 (MMP3), MMP13, interleukin-6 (IL6), prostaglandin-endoperoxide synthase-2 (PTGS2) and nitric oxide synthase-2 (NOS2), will also be important for therapeutic intervention in OA.

We previously performed RNA-seq analysis of normal and OA human knee cartilage and found that expression of several members in the Krüppel-like factor (KLF) family of transcription factors was suppressed in OA.[15] The KLF family includes 17 zinc-finger transcription factors that are typically categorised into several groups based on similarities in structure and transcriptional activity: (1) KLF-3, KLF-8 and KLF-12 serving as transcriptional repressors; (2) KLF-1, KLF-2 to KLF-4, KLF-5 to KLF-6 and KLF-7 functioning predominantly as transcriptional activators; (3) KLF-9, KLF-10 to KLF-11, KLF-13 to KLF-14 and KLF-16 having repressor activity; KLF-15 and KLF-17 have not been classified because their interaction motifs have yet to be determined.[16] While the zinc-finger domains are highly conserved among the KLFs, the non-DNA-binding regions are highly divergent, and modular activation and repression domains have been suggested to regulate transcriptional activity.[16] KLFs are involved in various biological and pathological mechanisms, including differentiation, apoptosis and tumuorigenesis.[16] However, there is limited knowledge about potential roles of KLFs in cartilage and OA pathogenesis.

Here, we analysed functions of KLF4 and KLF2 in cells from human joint tissues and OA models in vivo and determined mechanisms by which KLF4 and KLF2 protected joint tissues from OA-associated damage.

RESULTS

Expression of KLF family genes in cartilage and regulation of cartilage ECM genes by KLFs

We used our previous RNA-seq dataset of human knee cartilage tissues,[15] to determine which KLF genes were highly expressed in normal cartilage and differentially expressed between normal

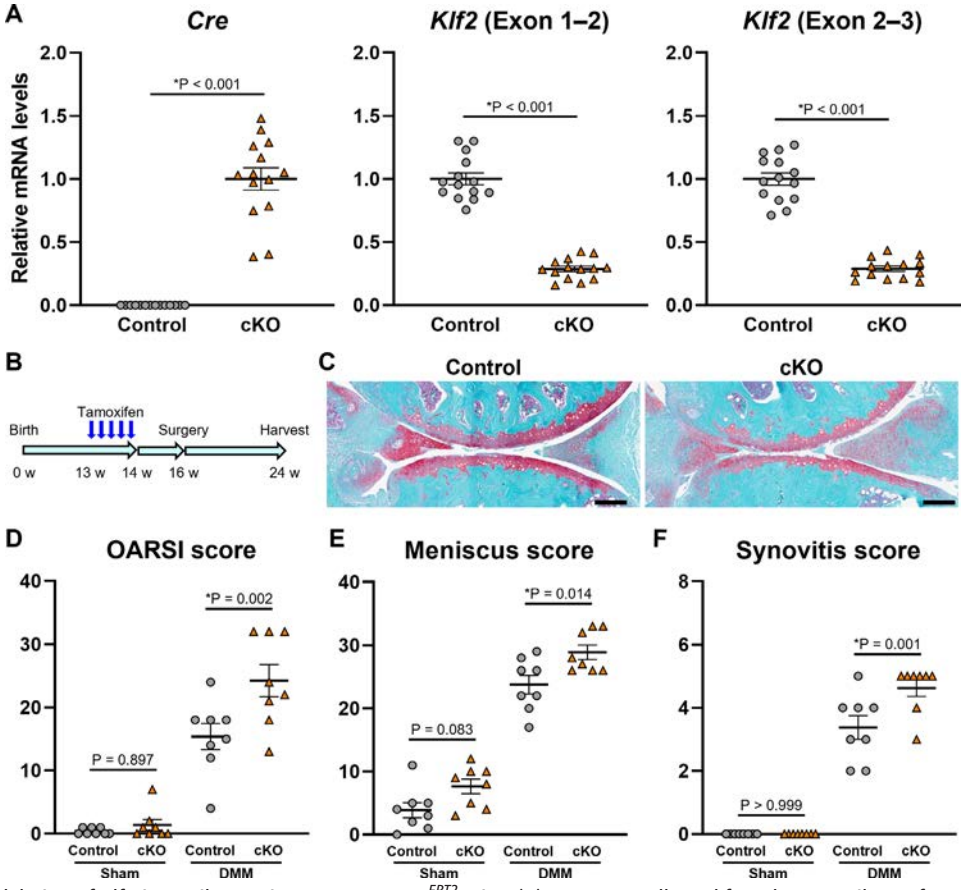

Figure 2 Postnatal deletion of Klf2 in cartilage using *Aggrecan-Cre^{ERT2}* mice. (A) RNA was collected from knee cartilage of 12-week-old *Aggrecan-Cre^{ERT2};Klf2$^{flox/flox}$* (cKO) and littermate control mice 2 weeks after tamoxifen injection (n=14 per group). mRNA levels are relative to cKO for *Cre*, and relative to control for *Klf2*. *P<0.05, unpaired Welch's t-test. (B) Sixteen-week-old cKO and littermate control mice underwent DMM or sham surgery 2 weeks after tamoxifen injection. The knees were harvested at 8 weeks postoperatively for histological analysis (n=8 per group). (C) Representative Safranin-O staining images of control and cKO mice with DMM surgery. Scale bars, 200 µm. (D) Summed Osteoarthritis Research Society International (OARSI) scores for the medial femoral condyle and tibial plateau. (E) Meniscus histopathological scores. (F) Synovitis scores. For (D–F), *P<0.05, Sidak's multiple comparison test. All quantitative data are expressed as means±SE, and results of two-way analysis of variance (ANOVA) test are shown in online supplemental table 2.

and OA cartilage (online supplemental figure 1; online supplemental table 1). *KLF1*, *KLF14* and *KLF17* were excluded from further analyses because of their low expression levels. KLF genes with log2(counts per million reads mapped (CPM))>5 in normal cartilage were considered to be expressed. Among those KLFs, *KLF2*, *KLF4*, *KLF9*, *KLF10* and *KLF15* were significantly decreased in OA cartilage, which suggested that these five KLFs might be associated with the compromised maintenance of cartilage homoeostasis in OA.

We transduced TC28a2 human chondrocyte cells[17] with the five KLFs (figure 1A; online supplemental figure 2). Overexpression of KLF2 and KLF4 upregulated expression of *COL2A1* and *COL11A2*. Notably, these two KLFs also increased expression of *PRG4*. Based on these findings, we focused subsequent studies on KLF4 and KLF2.

Decreased expression of KLF4 and KLF2 in cartilage with OA and ageing

Among human primary joint cells and bone marrow-derived mesenchymal stem cells (BMSCs), chondrocytes had the highest expression levels of *KLF4* and *KLF2* (online supplemental figure 3). Then, we examined whether there were zone-specific expression patterns of *KLF4* and *KLF2* in articular cartilage, utilising our DNA microarray dataset for three zones (superficial, middle and deep) of human knee cartilage tissues.[18] Expression levels of *KLF4* and *KLF2* were not significantly different among the zones (online supplemental figure 4). Six-month-old mouse knee joints were analysed with immunohistochemistry (IHC), and there were no differences in positive cell rates of Klf4 and Klf2 between articular cartilage and subchondral bone, and between weight-bearing and non-weight-bearing regions of articular cartilage (online supplemental figure 5).

Next, we examined how expression of KLF4 and KLF2 would change with OA and ageing in human and mouse cartilage. Positive cell rates of both KLF4 and KLF2 were decreased in human OA cartilage compared with those in normal cartilage (figure 1B,C). IHC of Klf4 and Klf2 was also performed in knee joints from 6-month-old and 24-month-old mice, and mice 4 or 8 weeks after OA induction by surgical destabilisation of the medial meniscus (DMM)[19] (figure 1D,E). Positive cell rates of both Klf4 and Klf2 in 24-month-old mice and in mice after DMM surgery were decreased when compared with 6-month-old control mice. *KLF4* and *KLF2* in human normal chondrocytes were downregulated by proinflammatory cytokine IL-1β (online supplemental figure 6), which is compatible with previous studies.[20 21] Meanwhile, treatment of human OA chondrocytes with TGF-β3 upregulated *KLF4* and *KLF2* (online supplemental figure 7). Collectively, these findings indicated

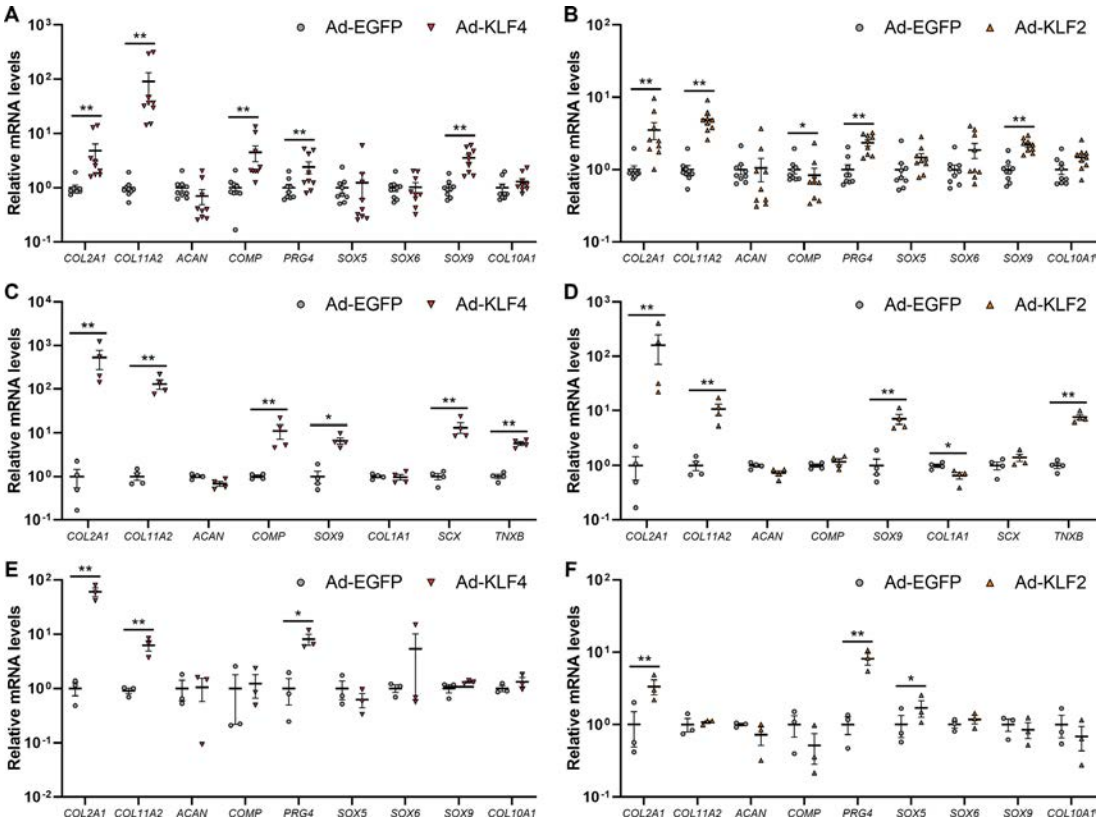

Figure 3 Regulation of chondrogenic and anabolic genes by KLF4 and KLF2 in human OA chondrocytes, meniscal cells and BMSC pellets. (A, B) Human OA chondrocytes were transfected with adenovirus (Ad-KLF4 or Ad-EGFP for (A), and Ad-KLF2 or Ad-EGFP for (B)), and RNA was collected 48 hours after transfection (n=9 donors). (C, D) Human meniscal cells were transfected with adenovirus (Ad-KLF4 or Ad-EGFP for (C), and Ad-KLF2 or Ad-EGFP for (D)), and RNA was collected 48 hours after transfection (n=4 donors). (E, F) Human bone marrow-derived mesenchymal stem cells (BMSCs) were transfected with adenovirus (Ad-KLF4 or Ad-EGFP for (E), and Ad-KLF2 or Ad-EGFP for (F)), and were cultured in pellets. RNA was collected 2 weeks after pellet culture (n=3 donors). mRNA levels are expressed as means±SE, relative to Ad-EGFP. *P<0.05, **P<0.01, paired t-test.

OA- and ageing-associated suppression of KLF4 and KLF2 in cartilage, and that mediators of joint tissue catabolism and inflammation were potential mechanisms of this suppression.

Klf2 functions in joint homoeostasis and OA pathogenesis

When KLF2 and KLF4 were knocked down using small interfering RNAs (siRNAs) in human normal chondrocytes, expression levels of *COL2A1*, *COL11A2*, *PRG4* and *SOX9* were suppressed, while *IL6*, *MMP3*, *PTGS2*, *ADAMTS5* and *MMP13* were upregulated (online supplemental figure 8).

We obtained *Klf2*$^{flox/flox}$ mice[22] and *Aggrecan-Cre*^{ERT2} knock-in mice,[23] and created *Aggrecan-Cre*^{ERT2}*;Klf2*$^{flox/flox}$ (conditional knock-out, cKO) mice to analyse roles of Klf2 in maintenance of mature articular cartilage and in OA pathogenesis. *Klf2* deletion in articular cartilage of skeletally mature mice was confirmed using knee cartilage of 12-week-old cKO mice after tamoxifen injection (figure 2A). We performed DMM surgery to induce OA in cKO and littermate control mice and harvested knee joints 8 weeks after surgery for histological analysis (figure 2B). Severity of OA in cKO mice was significantly higher than in control mice, as shown by the Osteoarthritis Research Society International (OARSI) scores[24] (figure 2C,D). In cKO mice, meniscus histopathological scores,[25] synovitis scores[26] and bone scores[9] were also higher than in control mice (figure 2E,F; online supplemental figure 9). These results demonstrated that Klf2 deletion in articular cartilage increased severity of OA.

KLF4 and KLF2 regulation of cartilage signature genes

To investigate functions of KLF4 and KLF2 in regulation of joint tissue homoeostasis, we used adenovirus (Ad) to overexpress KLF4 and KLF2 in several different cell types. In human OA chondrocytes, Ad-KLF4 increased expression of *COL2A1*, *COL11A2*, *COMP*, *PRG4* and *SOX9* (figure 3A; online supplemental figure 10A), and Ad-KLF2 upregulated *COL2A1*, *COL11A2*, *PRG4* and *SOX9* (figure 3B; online supplemental figure 10B). Human meniscal cells transfected with Ad-KLF4 showed higher mRNA levels of *COL2A1*, *COL11A2*, *COMP* and *SOX9* (figure 3C; online supplemental figure 10C). Transduction of KLF4 also upregulated scleraxis (*SCX*) and tenascin-B (*TNXB*), which are reported to be highly expressed in the meniscus.[27] In Ad-KLF2-transduced meniscal cells, expression of *COL2A1*, *COL11A2*, *SOX9* and *TNXB* was increased (figure 3D; online supplemental figure 10D).

We further studied KLF4 and KLF2 in chondrogenesis of human BMSCs. In BMSCs under monolayer culture, Ad-KLF4 increased *COL2A1*, *COL11A2*, *ACAN*, *COMP*, *PRG4*, *SOX5* and *SOX9* (online supplemental figure 11), while Ad-KLF2 upregulated expression of *COL2A1*, *COL11A2* and *PRG4* (online supplemental figure 12). During 2-week pellet culture of BMSCs, KLF4 upregulated *COL2A1*, *COL11A2* and *PRG4* (figure 3E; online supplemental figure 13A), and transduction of KLF2 increased *COL2A1*, *PRG4* and *SOX5* (figure 3F; online supplemental figure 13B). Collectively, KLF4 and KLF2 upregulated various chondrogenic and anabolic genes in human chondrocytes, meniscal cells and BMSCs.

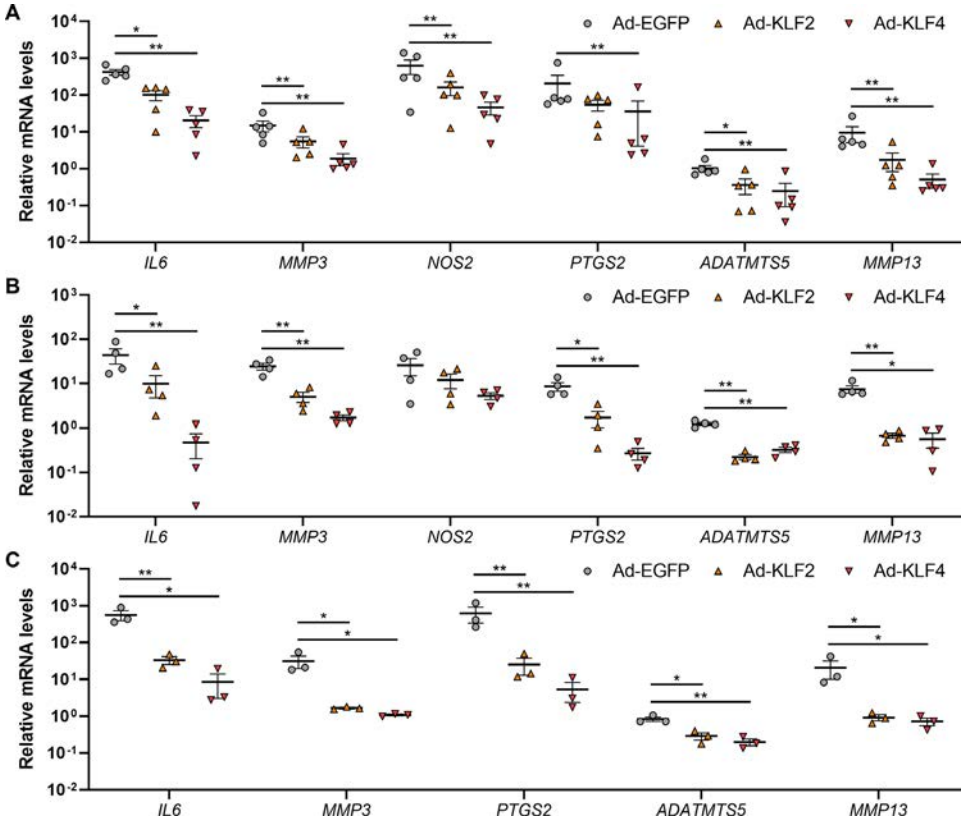

Figure 4 Regulation of inflammatory and catabolic genes by KLF4 and KLF2 in human OA chondrocytes, meniscal cells and synoviocytes on IL-1β stimulation. Cells were transfected with Ad-EGFP, Ad-KLF2 or Ad-KLF4, and RNA was collected 48 hours after adenoviral transfection and 6 hours after treatment with 10 ng/mL of interleukin-1β (IL-1β). (A) n=5 donors were used for human OA chondrocytes. (B) n=4 donors were used for human meniscal cells. (C) n=3 donors were used for human synoviocytes. mRNA levels are expressed as means±SE, relative to Ad-EGFP without IL-1β as controls. *P<0.05, **P<0.01 vs Ad-EGFP, Dunnett's test. Results of one-way mixed-effects ANOVA test are shown in online supplemental table 2.

Suppression of inflammatory and catabolic genes by KLF4 and KLF2

We examined regulation of genes related to inflammation and ECM degradation by the KLFs. When OA chondrocytes were treated with IL-1β, Ad-KLF4 downregulated *IL6*, *MMP3*, *NOS2*, *PTGS2*, *ADAMTS5* and *MMP13*, and Ad-KLF2 decreased expression levels of *IL6*, *MMP3*, *NOS2*, *ADAMTS5* and *MMP13* (figure 4A; online supplemental figure 14). In IL-1β stimulated meniscal cells, *IL6*, *MMP3*, *PTGS2*, *ADAMTS5* and *MMP13* were downregulated by KLF4 or KLF2 transduction (figure 4B; online supplemental figure 15). Similarly in synoviocytes, Ad-KLF4 and Ad-KLF2 suppressed expression levels of *IL6*, *MMP3*, *PTGS2*, *ADAMTS5* and *MMP13* under IL-1β stimulation (figure 4C; online supplemental figure 16). These findings suggested that KLF4 and KLF2 suppressed catabolic and inflammatory genes in joint tissue cells.

Therapeutic effects of KLF4 gene delivery using adeno-associated virus

To directly confirm therapeutic effects against OA, mice with DMM or sham surgery received intraarticular injection of adeno-associated virus (AAV) expressing KLF4 (figure 5A; online supplemental figure 17A), AAV-EGFP (online supplemental figure 17B) or phosphate-buffered saline (PBS) at 2 and 4 weeks after surgery. Knee joints were harvested at 8 weeks postoperatively, as illustrated in figure 5B. We performed von Frey test[28 29] preoperatively, and at 4 and 8 weeks after surgery to evaluate mechanical allodynia (figure 5C; online supplemental figures 18 and 19). AAV-KLF4 injected mice with DMM surgery showed significantly decreased numbers of paw withdrawals at 4 and 8 weeks. Histological analyses revealed that AAV-KLF4 injection significantly improved the OARSI scores, meniscus histopathological scores, synovitis scores and bone scores (figure 5D–G; online supplemental figure 20). These results clearly demonstrated that KLF4 reduced OA-associated joint damage and mechanical allodynia.

Global analysis of KLF4-regulated genes

To study genes regulated by KLF4 comprehensively and to elucidate regulatory mechanisms of KLF4, we performed global expression profiling by digital RNA with pertUrbation of Genes (DRUG-seq) analysis[30] of Ad-KLF4-transduced, Ad-EGFP-transduced and non-transduced TC28a2 cells with and without IL-1β stimulation (online supplemental figure 21). For differential expression analysis, we compared cells that were treated with various combinations of IL-1β, Ad-EGFP and Ad-KLF4. Lists of all significantly upregulated genes (URGs) and downregulated genes (DRGs) in each comparison are shown in online supplemental tables 3–10, where genes with a false discovery rate (FDR) of <0.05 and a |log2(fold change (FC))|>1 were considered to be significantly differentially expressed genes.

Ad-KLF4 significantly upregulated cartilage ECM genes such as *COL2A1*, *COL11A2*, *ACAN*, *COMP* and *PRG4* (figure 6A and online supplemental table 7). Notably, a number of genes which are anabolic or protective against OA were also significantly upregulated, including tissue inhibitor of metalloproteinases-1 (*TIMP1*), *TIMP2*, *TIMP3*, cluster of

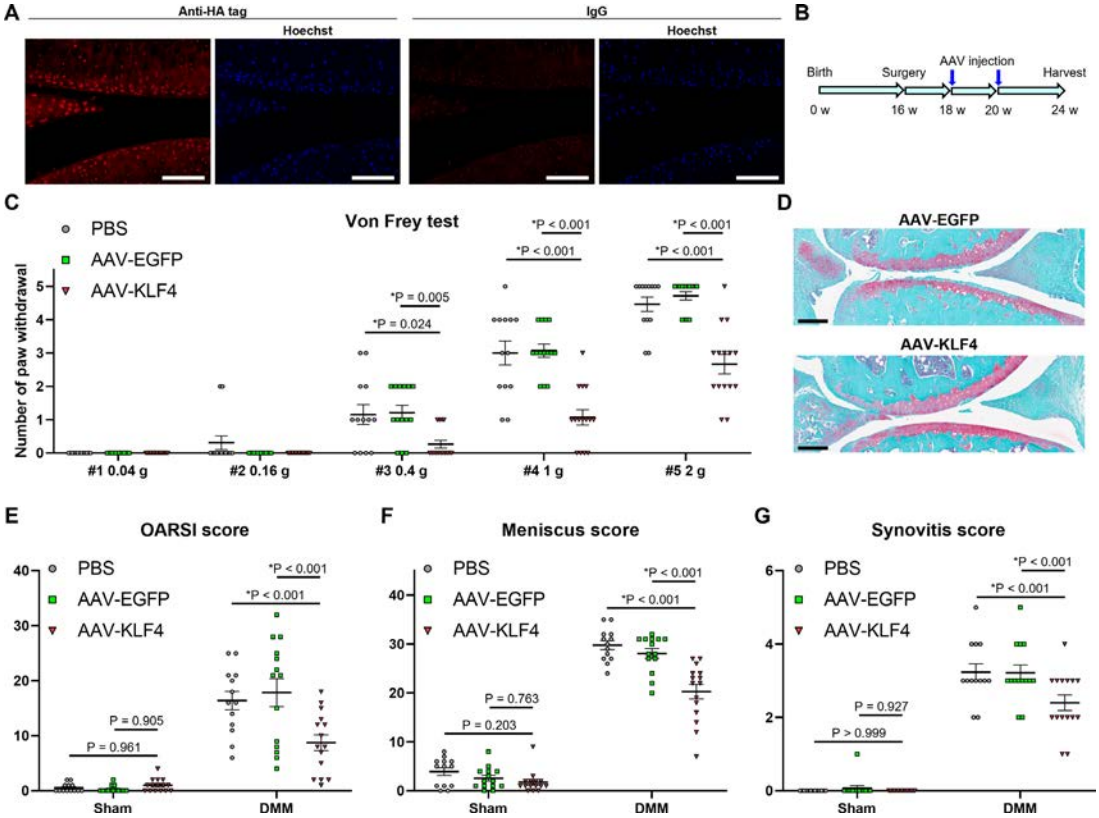

Figure 5 Therapeutic effects of OA by KLF4 gene delivery. (A) Immunofluorescence of HA tag with nuclear staining by Hoechst 33342 in 12-week-old mouse knee joints 1 week after injection of adeno-associated virus (AAV) expressing KLF4 and HA tag. Representative images from n=6 are shown. Scale bars, 100 µm. (B) Sixteen-week-old mice underwent DMM or sham surgery, and AAV-KLF4 (n=15), AAV-EGFP (n=14) or PBS (n=13) was injected into knee joints at 2 and 4 weeks after surgery. Knees were harvested at 8 weeks postoperatively for histological analysis. (C) von Frey test in mice at 8 weeks after DMM surgery. Numbers of paw withdrawals from five stimuli per filament per mouse are shown. *P<0.05 vs AAV-KLF4, Dunn's test. (D) Representative Safranin-O staining images of AAV-EGFP-injected and AAV-KLF4-injected mice with DMM surgery. Scale bars, 200 µm. (E) Summed OARSI scores for the medial femoral condyle and tibial plateau. (F) Meniscus histopathological scores. (G) Synovitis scores. For (E–G), *P<0.05 vs AAV-KLF4, Dunnett's test. All quantitative data are expressed as means±SE, and results of omnibus tests for multiple comparisons are shown in online supplemental table 2. PBS, phosphate-buffered saline.

differentiation-24 (*CD24*), *COL6A1*, *COL6A2*, *COL9A2*, *COL9A3*, nuclear factor of activated T cells-1 (*NFATC1*), *NFATC2*, forkhead box O1 (*FOXO1*), *FOXO3* and fibroblast growth factor-18 (*FGF18*)[9 31–38] (online supplemental table 7).

In the presence of IL-1β, Ad-KLF4 significantly suppressed *IL6*, MMP3, PTGS2, ADAMTS5, MMP13 and also other genes associated with OA pathogenesis, including fibronectin-1 (*FN1*), periostin (*POSTN*), cellular communication network factor-2 (*CCN2*), leukaemia inhibitory factor (*LIF*), gremlin-1 (*GREM1*), *SOX4*, IL-1β receptor type 1 (*IL1R1*), *IL1B, IL11*, *IL33*, *ADAMTS1*, *ADAMTS12* and *MMP1*[14 39–49] (figure 6B; online supplemental table 10). These findings suggested that KLF4 suppressed key mediators of OA pathogenesis.

Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analyses[50] were performed using URGs and DRGs. Significantly enriched pathways are shown in online supplemental tables 11–16. In the URGs by KLF4, 'ECM-receptor interaction', where cartilage ECM genes such as COL2A1, *COL11A2* and *COMP* are annotated, was significantly enriched (figure 6C; online supplemental table 13). 'Rap1 signalling pathway' was the top pathway, and several signalling pathways including 'Calcium signalling pathway' and 'MAPK signalling pathway' were highly ranked, suggesting their regulation by KLF4. Among the DRGs by KLF4 on IL-1β stimulation, 'TNF signalling pathway' was the second most enriched pathway (figure 6D; online supplemental table 16).

Genome-wide analysis of KLF4-DNA association in KLF4-transduced cells

Chromatin immunoprecipitation sequencing (ChIP-seq) was performed using TC28a2 cells transfected with a vector expressing KLF4 with HA tag (HA-KLF4) or an empty vector. Non-specific peaks were removed, and only specific peaks were used for subsequent analyses (online supplemental figure 22). In gene ontology analysis, terms related to chondrocyte differentiation were significantly enriched, such as 'growth plate cartilage chondrocyte differentiation', and 'regulation of chondrocyte development' (online supplemental table 18). Next, de novo motif analysis was performed for the obtained ChIP-seq peaks. A KLF motif (figure 7A) was top-ranked among the significantly recovered motifs (online supplemental figure 23), and this motif was highly enriched at the predicted centres of the peaks (figure 7B). These findings supported direct binding of KLF4 to DNA as well as integrity of the ChIP-seq dataset. Then, we examined peak signals of the ChIP-seq dataset around cartilage signature genes, as well as the top four URGs by KLF4 in the DRUG-seq analysis (online supplemental table 7). Large numbers of peaks

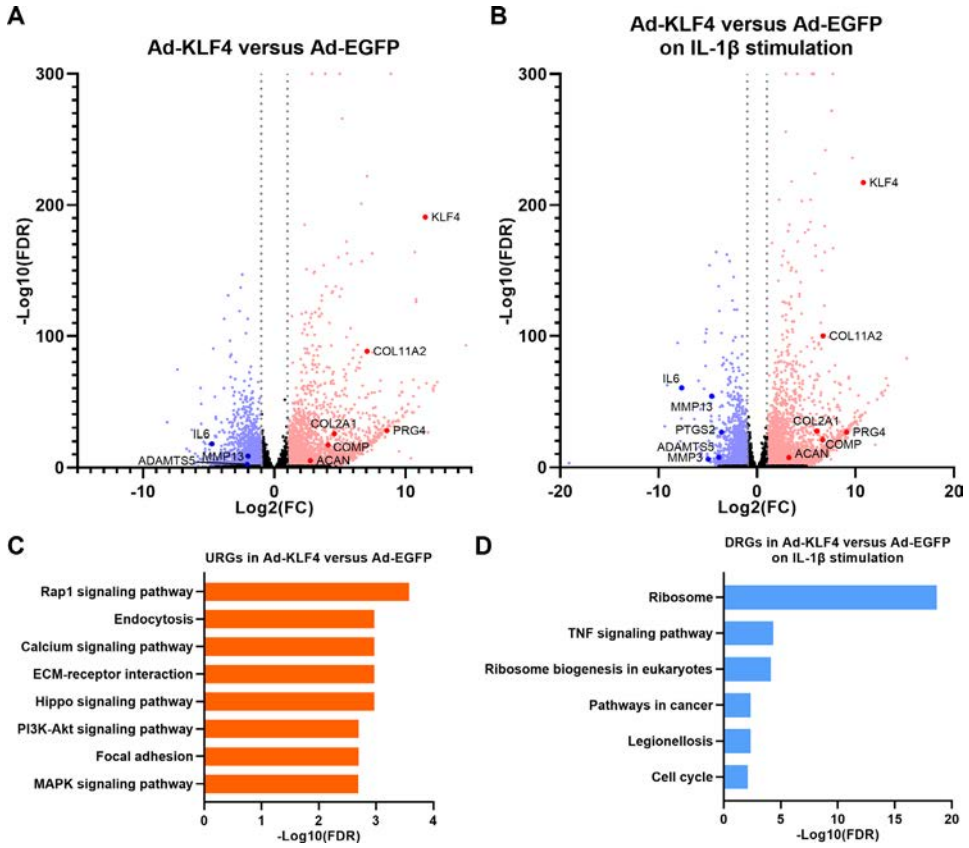

Figure 6 Digital RNA with pertUrbation of Genes (DRUG-seq) analysis of Ad-EGFP or Ad-KLF4 transfected TC28a2 cells. In samples with IL-1β stimulation, cells were treated with 10 ng/mL of IL-1β for 6 hours before RNA collection. n=7 per condition from seven independent experiments were analysed. (A) Volcano plot analysis to identify differentially expressed genes (DEGs) in Ad-KLF4 vs Ad-EGFP transfected samples. (B) Volcano plot analysis to identify DEGs in Ad-KLF4 vs Ad-EGFP transfected samples on IL-1β stimulation. For (A, B), grey dotted lines indicate |log2(fold change (FC))|=1. Significantly upregulated genes (URGs) are shown as red dots, while significantly downregulated genes (DRGs) are indicated as blue dots; black dots represent non-significant DEGs. (C) Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis for URGs in Ad-KLF4 vs Ad-EGFP transfected samples. The top eight enriched pathways are shown. (D) KEGG pathway analysis for DRGs in Ad-KLF4 vs Ad-EGFP transfected samples on IL-1β stimulation. All significantly enriched pathways are shown. FDR, false discovery rate.

were detected in the putative regulatory regions of *SOX9*, *COL2A1*, *COL11A2*, *PRG4*, *ACAN* and *COMP*, and most of these peak regions contained the recovered KLF motif, while similar findings were found for the top URGs (figure 7C,D; online supplemental figures 24–31). These data suggested that KLF4 directly bound to DNA around major chondrogenic genes, indicating that KLF4 was an important regulator of chondrogenesis.

Regulation of chondrogenesis by KLFs via PKA-RAP1-MEK-CREB signalling axis

In addition to the function of KLF4 as a transcription factor to directly upregulate the cartilage signature genes, we focused on relation between Ras-related protein-1 (RAP1) signalling and KLFs, since 'Rap1 signalling pathway' was the top-ranked enriched pathway in the KEGG pathway analysis (figure 6C; online supplemental tables 13 and 15). It is reported that activation of RAP1 promotes chondrogenesis.[51] While KLF4 and KLF2 did not increase expression levels of *RAP1A* and *RAP1B* in OA chondrocytes (online supplemental figure 32) or in TC28a2 cells (online supplemental table 19), KLF4 directly interacts with RAP1 protein.[52] Our results showed that treatment of KLF4- and KLF2-transfected TC28a2 cells with Rap1 inhibitor GGTI-298 diminished or eliminated upregulation of cartilage ECM genes, *COL2A1*, *COL11A2*, *ACAN*, *COMP* and *PRG4* (figure 8A; online supplemental figure 33).

RAP1 is a small GTPase[53] and a member of the PKA (protein kinase A)-RAP1-MEK (mitogen-activated protein kinase kinase)-CREB (cyclic-AMP (cAMP) response element-binding protein) signalling axis.[54–56] Fluid flow shear stress induces expression of Prg4 in articular cartilage via activation of this signalling pathway,[11] and treatment of TC28a2 cells with the cAMP inducer forskolin increased expression levels of cartilage ECM genes (online supplemental figure 34). We thus hypothesised that KLF4 and KLF2 might upregulate cartilage ECM genes via the PKA-RAP1-MEK-CREB signalling axis. To test this hypothesis, we treated KLF4- and KLF2-transfected TC28a2 cells with inhibitors of several members in the signalling axis; PKA inhibitor H89, MEK inhibitor U0126, and CREB inhibitor 666–15. Upregulation of the cartilage ECM genes was attenuated or cancelled by each of these inhibitors (figure 8B–D; online supplemental figure 35). Collectively, these findings demonstrated that KLF4 and KLF2 upregulated major cartilage ECM genes, not only by direct regulation as a transcription factor but also via the PKA-RAP1-MEK-CREB signalling axis (online supplemental figure 36).

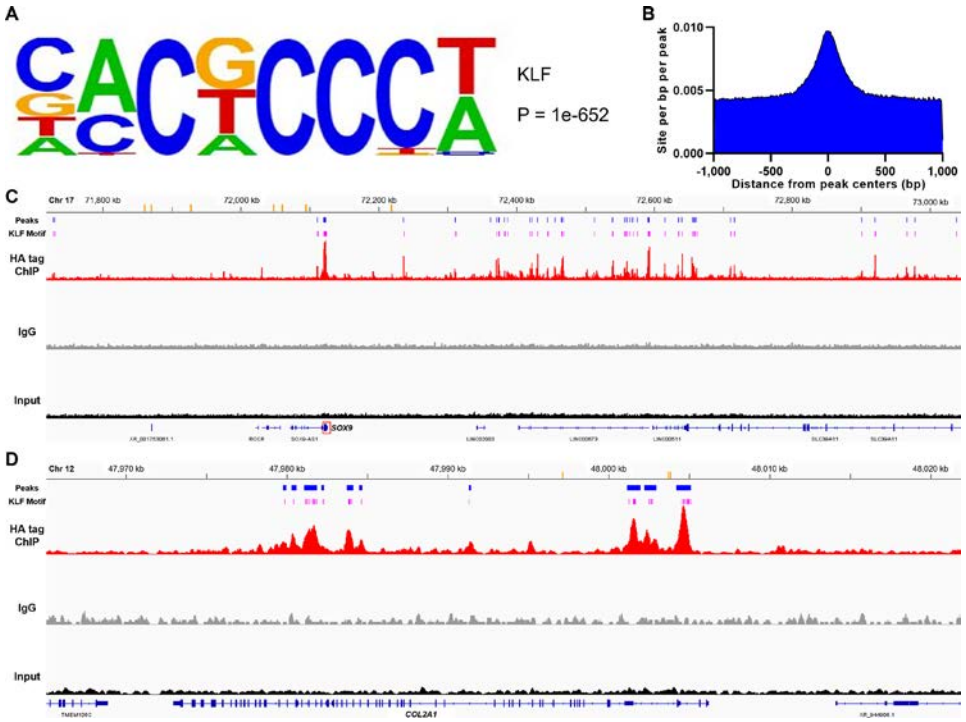

Figure 7 Chromatin immunoprecipitation sequencing (ChIP-seq) for genome-wide analysis of KLF4-DNA association in KLF4 overexpressed TC28a2 cells. (A) A KLF motif recovered from ChIP-seq peaks. The motif logo displays nucleotide frequencies (scaled relative to the information content) at each position. (B) Distribution of the KLF motif within a 1000 base pair (bp) window from the peak centres with a 10 bp step size. (C, D) Visualisation of aligned reads for HA tag ChIP-seq, IgG and input samples around the *SOX9* gene (C) and around the *COL2A1* gene (D). Orange bars indicate previously identified enhancers for each gene. Blue bars indicate peak regions that were putative regulatory domains of *SOX9* or *COL2A1* and common between the replicates of ChIP samples. Pink bars show the predicted sites of the recovered KLF motif which were located within the peak regions. Chr, chromosome. kb, kilobase.

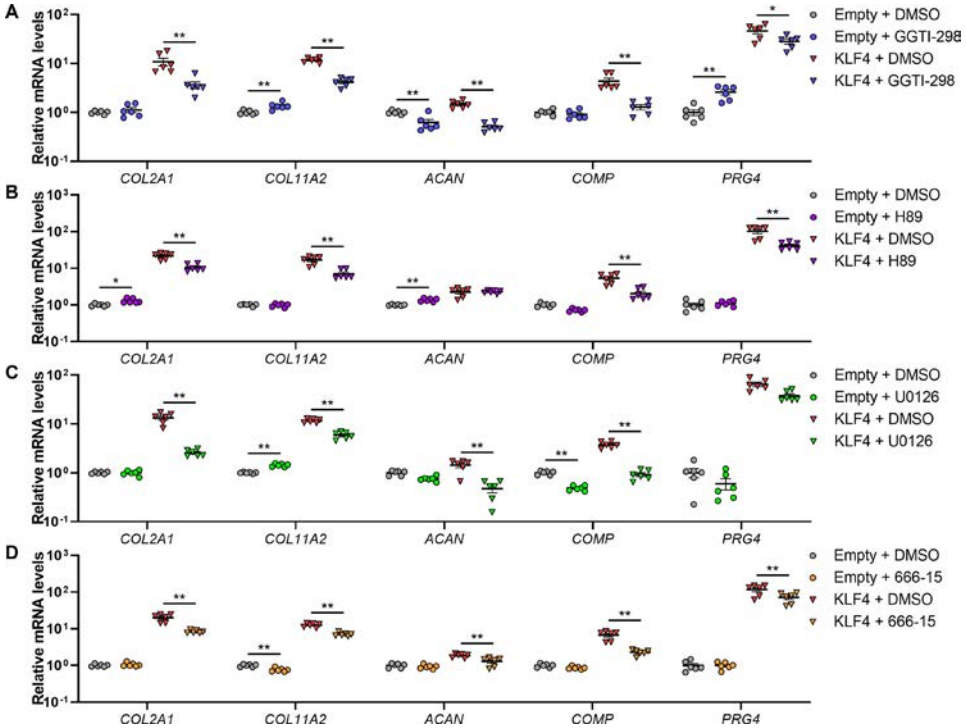

Figure 8 KLF4 regulation of cartilage extracellular matrix genes through the RAP1-related signalling axis. TC28a2 cells were transfected with an expression vector encoding KLF4 or an empty vector. Then, cells were treated with GGTI-298 (A), H89 (B), U0126 (C) or 666-15 (D), or dimethyl sulfoxide (DMSO). RNA was collected 48 hours after transfection (n=6 from six independent experiments). mRNA levels are expressed as means±SE, relative to empty vector-transfected samples with DMSO as controls. *P<0.05, **P<0.01, Sidak's multiple comparison test. Results of two-way mixed-effects ANOVA test are shown in online supplemental table 2.

DISCUSSION

This is the first detailed analysis of KLFs in the context of cartilage biology, joint homoeostasis and OA pathogenesis. We focused on KLF4 and KLF2 as they were the strongest inducers of cartilage collagen genes and *PRG4*, and as their expression was suppressed in OA cartilage. KLF4 and KLF2 are closely related members within the KLF family.[16 57]

KLF4 and KLF2 downregulated mediators of inflammation and ECM-degrading enzymes in human joint tissue cells, which are compatible with previous studies showing that they suppressed NF-κB activity.[58 59] Previous reports show KLF4 binding to the promoter proximal region of *COL2A1* gene[60] and KLF4 colocalisation and interaction with SOX9 in chondrocytes.[61] This study suggested that KLF4 and KLF2 were important regulators of chondrogenesis in joint tissue cells because they upregulated cartilage signature genes with different regulatory mechanisms; *SOX9*, a master regulator of chondrogenesis, SOX9-regulated genes such as *COL2A1*, *COL11A2*, *ACAN* and *COMP*,[3–7] and *PRG4* which is not subject to regulation by SOX9.[5 12] ChIP-seq analysis supported the model of KLF4 as a central transcription factor of chondrogenesis. KLF4 bound to large numbers of the putative regulatory regions of major chondrogenic genes, and many of the bound sites included the KLF motif. All these findings indicated direct regulation of the cartilage signature genes by KLF4. We also revealed a novel link of KLF4 with RAP1 signalling. The PKA-RAP1-MEK-CREB signalling axis is involved in modulation of various types of genes,[54–56] and Prg4 expression in articular cartilage is induced by fluid flow shear stress via this signalling pathway.[11] Our results showed that the KLF4- or KLF2-effects on cartilage ECM genes were dependent on each molecule in the PKA-RAP1-MEK-CREB axis.

Because KLF4 upregulated more chondrogenic and anabolic genes, and downregulated more inflammatory and catabolic genes than KLF2 in our in vitro experiments, we assumed KLF4 to be more promising as a therapeutic target for OA. Intraarticular Ad-KLF2 had previously shown to protect against cartilage damage induced by monoiodoacetate.[21] Therefore, we tested intraarticular injection of AAV-KLF4 in the mouse DMM model. AAV-KLF4 improved pain behaviours, and reduced severity of OA histopathological changes in cartilage, meniscus and synovium. These results indicated that KLF4 had therapeutic and protective effects against OA-associated tissue damage and pain.

Several limitations should be noted in this study. First, all mouse experiments were done with only male animals, and sex differences were not addressed. Second, ageing-associated spontaneous OA model was not performed in our study. Third, only experiments using *Aggrecan-Cre^{ERT2};Klf2$^{flox/flox}$* mice were performed, because *Klf4* deletion in articular cartilage of *Aggrecan-Cre^{ERT2};Klf4$^{flox/flox}$* mice was not sufficient. Finally, because KLF4-overexpressed cells were used in our ChIP-seq analysis, more sites could be detected as peaks than in ChIP-seq of endogenous KLF4.

In conclusion, this study identifies KLF4 and KLF2 as new transcription factors that are important regulators of the chondrocyte phenotype, and that also have effects on other joint tissues by upregulating ECM components and by suppressing ECM-degrading enzymes and inflammatory mediators. KLF4 and KLF2 thus have a spectrum of biological activities with promise in treatment of joint diseases such as OA.

MATERIALS AND METHODS

Experimental procedures are provided in online supplemental materials and methods.

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Contributors MKL designed the study. MK and MO performed the experiments. MK, PN, AS and MO analysed the data. MK, TT, PO, SRH, PN, AS and MO provided expertise and/or materials. PO, SRH, HA and MKL supervised the project. MK and MKL drafted the paper, which was approved by all coauthors. MKL acts as guarantor.

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Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article. The GEO accession numbers for all the datasets used in the present study are available in online supplemental materials and methods.

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EPIDEMIOLOGICAL SCIENCE

Colchicine prophylaxis is associated with fewer gout flares after COVID-19 vaccination

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ABSTRACT

Objectives COVID-19 vaccination often triggers a constellation of transitory inflammatory symptoms. Gout is associated with several comorbidities linked to poor outcomes in COVID-19, and gout flares can be triggered by some vaccinations. We analysed the risk of gout flares in the first 3 months after COVID-19 vaccination with inactivated virus, and whether colchicine can prevent gout flares following post-COVID-19 vaccination.

Methods A clinical delivery population-based cross-sectional study was conducted in the Gout Clinic at the Affiliated Hospital of Qingdao University between February and October 2021. Study participants were selected using a systematic random sampling technique among follow-up patients with gout. We collected data, including vaccinations and potential risk factors, using a combination of interviews, health QR codes and medical records. Logistic regression was used to adjust for covariates.

Results We enrolled 549 gout participants (median age 39 years, 84.2% vaccinated). For the 462 patients who received COVID-19 vaccine, 203 (43.9%) developed at least one gout flare in the 3 months after vaccination. Most of these flares were experienced within 1 month after the first (99/119 (83.2%)) or second (70/115 (60.9%)) dose of vaccine. Compared with unvaccinated participants, COVID-19 vaccination was associated with higher odds of gout flare within 3 months (adjusted OR 6.02; 95% CI 3.00 to 12.08). Colchicine use was associated with 47% less likelihood of postvaccine gout flare.

Conclusion COVID-19 vaccination was associated with increased odds of gout flare, which developed mainly in month 1 after each vaccine dose, and was negatively associated with colchicine prophylaxis.

Key message

What is already known about this subject?
⇒ Gout is associated with several comorbidities linked to poor outcomes in COVID-19, and gout flares can be triggered by some vaccinations.

What does this study add?
⇒ This study showed that COVID-19 vaccination was associated with increased odds of gout flare, which developed mainly in month 1 after each vaccine dose, and was negatively associated with colchicine prophylaxis.

How might this impact on clinical practice or future developments?
⇒ These findings warrant further investigation into whether mRNA vaccines have the same effect, which ideally prospectively and with multicentre collaboration.
⇒ This study may inform discussions with patients with gout about the risks of gout flare around the time of COVID-19 vaccination.

INTRODUCTION

Vaccines against SARS-CoV-2 represent a pivotal and effective countermeasure to contain the COVID-19 pandemic. Given gout is associated with many risk factors (eg, age and comorbidities) for poor COVID-19 outcomes,[1] vaccination of patients with gout is of high priority.

Risk factors for gout flare include dietary factors, medications and comorbidities. Recently, a case-crossover study conducted by Yokose *et al*[2] revealed that recombinant herpes zoster vaccine (RZV) in the prior 2 days was associated with a twofold increased odds of gout flare when compared with no vaccine periods (adjusted OR 1.99; 95% CI 1.01 to 3.89). A study conducted by the European Alliance of Associations for Rheumatology Coronavirus Vaccine physician-reported Registry reported 4.4% of flare following mRNA vaccination in inflammatory/autoimmune rheumatic and musculoskeletal disease cases from 30 countries.[3] However, to date, no systemic analysis, even real-world data, investigated the association between COVID-19 vaccine and gout flare.

We analysed the risk of gout flares in the first 3 months after COVID-19 vaccination with inactivated virus, and whether colchicine associates with reduced gout flares following post-COVID-19 vaccination.

METHODS

Study design and participants

We performed a clinical delivery population-based observational and cross-sectional study, based on face-to-face electronic questionnaires and individual medical records from the Biobank Information Management System (BIMS; Haier, China), which took place in the Gout Clinic at the Affiliated Hospital of Qingdao University between February

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2021 and October 2021. All participants had gout according to the 2015 American College of Rheumatology/European League Against Rheumatism gout classification criteria.[4]

A sample size of 544 was calculated by using a formula for calculating sample size for cross-sectional study, assumed a 90% CI, 5% non-response rate, 70% COVID-19 vaccination rate in Shandong Province[5] and 40% proportion of gout flare as estimated by the reports.[6]

Assessments and procedures

After written informed consent was obtained, all eligible participants completed a structured interview questionnaire, with the help of trained research staff. The questionnaire includes a Gout Assessment Questionnaire (GAQ 2.0), data of gout flare and the type and date of each vaccine.

Gout-specific health-related quality of life (HRQoL) was assessed by the Gout Impact Scale (GIS) of the GAQ 2.0 (Chinese version), a 24-item instrument with five scales: gout concern overall, gout concern during gout flare, medication side effects, unmet gout treatment need and well-being during flare, each with a 0–100 score (higher score indicating more concern/need).

Patients were asked to recall the data of gout flare regarding times of onset, clinical signs and symptoms within 3 months before the first dose vaccine and after any of the vaccines. These data were double-checked in each patient's standardised medical record to ensure the reliability and accuracy.

The type and date of each vaccine were collected by uploading their personal health QR code, a vaccination certificate, which was launched by Chinese government.[7]

Body mass index (BMI) was calculated as weight in kilograms divided by height in metres squared. A positive family history of gout was defined as ≥1 of the patient's first to second degree relatives affected by gout.[8] Comorbidities were defined as present if formally recorded in the past history of the medical record, or if the patient was currently receiving comorbidity specific drug treatment, and included hypertension, renal disease, hyperlipidaemia, tumour, fracture, cardiovascular, digestive, respiratory or mental diseases. Last serum urate (SU) done before the first vaccine and the urate-lowering treatment at the time of the first vaccine were traced by the BIMS. Prophylactic colchicine usage was defined as taking 0.5 g colchicine once or twice daily ≥1 month at any time during the vaccination period (online supplemental figure 1).

In total, we enrolled 2983 patients with 2036 follow-up patients as estimated by the last year's numbers of patients who visited our clinical centre. New patients, who were the first time to visit our clinic, were excluded. Eligible patients were invited to participate using a systematic random sampling technique.[9] The sampling fraction was determined by dividing the total 8-month follow-up numbers by the sample size. The first participant was selected as his or her first visiting sequence on our research initiating date and every second interval was included using systematic random sampling. Six hundred and seventy-eight were selected for questionnaire interview. Completed questionnaires were returned by 646 out of 678 respondents, with a response rate of 95.3%. There were 96 patients excluded because of insufficient medical record data. Finally, 549 were enrolled for the analysis (online supplemental figure 1).

This study was initiated at the very beginning of COVID-19 vaccination in China. The vaccination programme was started from December 2020 and greatly expanded in April, May and June 2021. We matched the data of gout flare by the first vaccination date of vaccinated patients in the non-vaccination participants. Most patients in non-vaccination group were vaccinated subsequently as government advocacy and sufficient vaccine supply developed. All clinical data were corrected and confirmed by personal medical records.

Statistical analysis

All analyses were performed using SPSS V.26.0 (IBM). Continuous variables were expressed as mean (SD) or median (IQR) and were compared by independent sample t-test or Wilcoxon sign-rank test. Categorical variables were expressed as number (percentage) and were compared by χ^2 test. Logistic regression analyses were used to assess the association of variables with gout flare. P value <0.05 was considered statistically significant.

RESULTS

Study participants

The study included 549 participants (531 (96.7%) men), with a median (IQR) age of 39 years (32–49) (table 1). Four hundred and sixty-two (84.2%) received COVID-19 vaccine, in which 400 (86.6%) had received two doses. Most patients (250 (54.1%)) received the Sinovac Life vaccine, 174 (37.7%) had the Sinopharm BIBP and 38 (8.2%) the others (recombinant COVID-19 vaccine (CHO cell) or recombinant COVID-19 Vaccine (adenovirus type 5 vector)). Both Sinovac Life and Sinopharm BIBP vaccines contain aluminium hydroxide adjuvant. Mean (SD) time between the first and second dose of the vaccine (if applicable) was 36 (13) days. The non-vaccination and vaccination participants displayed comparable means (SD) of BMI (kg/m^2; 26.95 (3.59) vs 27.67 (4.15), p=0.15), duration of gout (year; 7.74 (6.33) vs 6.40 (5.34), p=0.11), gout flare *per* person within last 3 months before the first vaccine (0.56 vs 0.56), last SU done before the first vaccine (mg/dL; 7.63 (2.22) vs 7.45 (1.97), p=0.55) and proportions of comorbidities (46.0% vs 50.7%, p=0.48), prophylactic colchicine usage (32.2% vs 24.7%, p=0.14), achieving the treatment urate target in recent year (29.9% vs 24.9%, p=0.35) and urate-lowering drugs use at the time of the first vaccine (p=0.44). However, the vaccinated patients had a higher proportion of patients with gout flares within last 3 months after vaccination (43.9% vs 32.2%, p=0.04) and a lower mean (SD) score of well-being during attack of GIS (57.30 (26.18) vs 63.43 (23.65), p=0.04) compared with the non-vaccinated ones. Detailed characteristics are shown in table 1.

Gout flares following vaccination

For the 462 patients who received COVID-19 vaccine, 203 (43.9%) developed at least one gout flare in the 3 months after any vaccine. Most of these flares were reported within 1 month after the first (99/119 (83.2%)) or second (70/115 (60.9%)) dose of vaccine (table 2). Though the mean (SD) visual analogue score was higher in the second-dose vaccinated patients than the first-dose vaccinated ones (5.65 (2.34) vs 5.02 (2.31), p=0.04), the flares were mild or moderate. The main joint sites of gout flare were comparable and most occurred in first metatarsophalangeal, ankle or heel (the first dose: 85.7%; the second dose: 94.8% in total, table 2).

We analysed the odds of a gout flare in the 3 months following COVID-19 flare within the first 3 months after vaccination (model 1: adjusted OR 6.02; 95% CI 3.00 to 12.08) indicating receiving COVID-19 vaccine was associated with sixfold higher odds of gout (table 3). Specifically, patients received Sinovac Life got a significant higher odd of gout flare (model 2: adjusted OR 3.13; 95% CI 1.12 to 8.72, table 3). The OR tended to be higher

Table 1 Demographic characteristics of the participants

Characteristics	Total (n=549)	No vaccination (n=87)	COVID-19 vaccination (n=462)	P value
Demographics				
Age (years), mean (SD)/median (IQR)	41.01 (12.65)/39 (32–49)	40.75 (14.50)/37 (28–52.5)	41.05 (12.36)/39.5 (32–48)	0.86
Male sex, n (%)	531 (96.7)	82 (94.3)	449 (97.2)	0.18
Body mass index (kg/m²), mean (SD)	27.55 (4.06)	26.95 (3.59)	27.67 (4.15)	0.15
Completed college (n, %)	358 (65.2)	50 (57.5)	308 (66.7)	0.81
Smoking, n (%)				0.08
Never	286 (52.1)	44 (50.6)	242 (52.4)	
Sometimes	74 (13.5)	10 (11.5)	64 (13.9)	
Regular	114 (20.8)	26 (29.9)	88 (19.1)	
Quit	75 (13.7)	7 (8.5)	68 (14.7)	
Alcohol drinking, n (%)				0.37
Never	130 (23.7)	19 (21.8)	111 (24.0)	
Sometimes	259 (47.2)	42 (48.3)	217 (47.0)	
Regular	80 (14.6)	17 (19.5)	63 (13.6)	
Quit	80 (14.6)	9 (10.3)	71 (15.4)	
Comorbidities*, n (%)	274 (49.9)	40 (46.0)	234 (50.7)	0.48
COVID-19 vaccination				
Vaccination, n (%)	462 (84.2)	–	–	–
Completed	–	–	400 (86.6)	
Not completed	–	–	62 (13.4)	
Vaccination received, n (%)				–
Sinovac Life	250 (54.1)	–	250 (54.1)	
Sinopharm BIBP	174 (37.7)	–	174 (37.7)	
Others	38 (8.2)	–	38 (8.2)	
Gout specific indicators				
Age at onset (years), mean (SD)	35.90 (11.1)	35.57 (13.7)	35.97 (10.5)	0.81
Duration of gout (years), mean (SD)	6.64 (5.5)	7.74 (6.3)	6.40 (5.3)	0.11
Positive family history, n (%)	101 (18.4)	14 (16.1)	87 (18.8)	0.55
Palpable tophus, n (%)	66 (12.0)	8 (9.2)	58 (12.6)	0.38
Gout impact scale scores†, mean (SD)				
Gout concern overal	82.62 (22.36)	82.54 (20.60)	82.63 (22.7)	0.97
Gout concern during attack gout	71.65 (20.55)	73.61 (19.96)	71.29 (20.66)	0.33
Medications side effects	71.47 (19.9)	72.72 (19.22)	71.23 (20.03)	0.52
Unmet gout treatment need	60.93 (15.82)	62.45 (16.76)	60.64 (15.63)	0.33
Well-being during attack	58.27 (25.88)	63.43 (23.65)	57.30 (26.18)	**0.04**
Colchicine prophylaxis, n (%)	142 (25.9)	28 (32.2)	114 (24.7)	0.14
Achieving the treatment urate target in recent year, n (%)‡	141 (25.7)	26 (29.9)	115 (24.9)	0.35
Last serum urate done before first vaccine (mg/dL), mean (SD)	7.51 (2.01)	7.63 (2.22)	7.45 (1.97)	0.55
Gout flares *per* person within last 3 months before first vaccine, mean	0.56	0.56	0.56	–
Gout flares within last 3 months after any vaccine, n (%)	231 (41.1)	28 (32.2)	203 (43.9)	**0.04**
Urate-lowering drugs use at the time of the first vaccine, n (%)				0.44
Febuxostat	304 (55.4)	53 (60.9)	251 (54.3)	
Benzbromarone	48 (8.6)	8 (9.2)	40 (8.7)	
No or missing	197 (35.9)	26 (29.9)	171 (37.0)	

*Comorbidities include diabetes, hypertension, renal disease, hyperlipidemia, tumour, fracture, cardiovascular, digestive, respiratory or mental diseases.
†Gout impact scale scores: 0~100 where 100 indicates worse condition.
‡Achieving the treatment urate target in recent year indicates serum urate < 6 mg/dL.

among participants with high last serum urate levels before the first-dose vaccine (adjusted OR 1.14; 95% CI 1.02 to 1.27). On the contrary, colchicine prophylaxis was associated with 47% less likelihood of having an increase in gout flare burden after vaccination (model 1: adjusted OR 0.53; 95% CI 0.31 to 0.92, model 2: adjusted OR 0.53; 95% CI 0.30 to 0.92, table 3).

DISCUSSION

The findings of this clinical delivery population-based cross-sectional study provide important implications for COVID-19 vaccine administration in people with gout. Our data indicate a higher odds of gout flare within 3 months after COVID-19 vaccine and that colchicine prophylaxis is associated with markedly reduced odds of postvaccine gout flare. The percentage of medically confirmed flares reported within 3 months after COVID-19 vaccination was 203/462 (43.94%). Most patients developed a flare within 1 month after the first (99/119 (83.19%)) or second (70/115 (60.87%)) vaccine. Notably, Sinovac Life vaccine received as well as higher SU levels before the first vaccine are all associated with increased risk of the postvaccine gout flare.

The main limitation of this study is its observational and cross-sectional nature, with retrospective collection of gout flare data that may be subject to recall bias. Substantially, personal medical records were checked to verify the gout flare data, thereby minimising the bias. Also, we could not fully account for confounding

Table 2 Characteristics of patients after the first and second dose COVID-19 vaccines

Characteristics	Total (n=462)	First dose	Second dose	P value
Vaccination completed, n (%)	400 (86.6)	–	–	–
Vaccination received, n (%)		–	–	–
Sinovac Life	251 (54.3)	–	–	–
Sinopharm BIBP	173 (37.5)	–	–	–
Others	38 (8.2)	–	–	–
Side effects after any vaccination*, n (%)	141 (30.5)	–	–	–
Gout flare after any vaccination, n (%)	203 (43.9)	–	–	–
Flare ratio, n/N (%)	–	119/203 (58.6)	115/203 (56.7)	0.65
VAS, mean (SD)	–	5.02 (2.3)	5.65 (2.3)	**0.04**
Without other specific triggers†, n/N (%)	–	67/119 (56.3)	61/115 (53.0)	0.70
Timing of gout flare (days), n/N (%)	–			**<0.001**
Within 1 week	–	36/119 (30.3)	22/115 (19.1)	
1 week ~1 month	–	63/119 (52.9)	48/115 (41.7)	
1~3 months	–	20/119 (16.8)	45/115 (39.1)	
Main joint site of gout flare, n/N (%)	–			0.07
First MTP	–	49/119 (41.2)	58/115 (50.4)	
Ankle and heel	–	53/119 (44.5)	51/115 (44.4)	
Knee	–	15/119 (12.6)	4/115 (3.5)	
Wrist	–	2/119 (1.7)	2/115 (1.7)	

*Side effects after vaccination includes cold symptoms (fatigue, cough, fever, muscle pain nd headache); wheezing or shortness of breath; nausea, vomiting or diarrhoea; flustered and chest tightness.
†Other specific triggers include cold, exercise, alcohol consumption, diuretic use and purine intake.
VAS, visual analogue score; MTP, metatarsophalangeal.

Table 3 Variables associated with a gout flare in the 3-month period*

	Univariate analysis		Multivariate analysis			
			Model 1		Model 2	
Variables for gout flare	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
Demographics						
Sex (ref: female)	0.82 (0.32 to 2.10)	0.67	4.04 (0.68 to 24.04)	0.13	4.33 (0.73 to 25.82)	0.11
Age (*per* year)	1.00 (0.99 to 1.02)	0.49	1.01 (0.99 to 1.03)	0.16	1.01 (0.99 to 1.03)	0.18
Body mass index (*per* kg/m²)	1.03 (0.99 to 1.08)	0.16	1.02 (0.97 to 1.08)	0.44	1.02 (0.97 to 1.08)	0.45
Comorbidity† (ref: no)	0.92 (0.67 to 1.29)	0.64	–	–	–	–
Tophi (ref: no)	1.01 (0.59 to 1.70)	0.98	–	–	–	–
Disease duration (*per* year)	0.96 (0.92 to 0.99)	**0.007**	0.96 (0.92 to 1.01)	0.09	0.96 (0.92 to 1.01)	0.12
Flare within 3 months prior to first vaccine (ref: no)	0.17 (0.06 to 0.54)	**0.003**	1.27 (0.21 to 7.68)	0.79	1.27 (0.21 to 7.71)	0.79
Last serum urate done before first vaccine (*per* mg/dL)	1.22 (1.11 to 1.33)	**<0.001**	1.14 (1.02 to 1.27)	**0.021**	1.14 (1.02 to 1.27)	**0.025**
Vaccination						
COVID-19 vaccination (ref: no)	4.57 (2.66 to 7.84)	**<0.001**	6.02 (3.00 to 12.08)	**<0.001**	–	–
Sinovac Life vaccine (ref: no)	2.90 (1.28 to 6.56)	**0.011**	–	–	3.13 (1.12 to 8.72)	**0.029**
Sinopharm BIBP vaccine (ref: no)	0.55 (0.28 to 1.10)	0.09	–	–	0.48 (0.2 to 1.14)	0.09
Other vaccines (ref: no)	0.70 (0.34 to 1.41)	0.32	–	–	0.49 (0.20 to 1.19)	0.12
Treatment						
Colchicine prophylaxis (ref: no)	0.38 (0.24 to 0.61)	**<0.001**	0.53 (0.31 to 0.92)	**0.025**	0.53 (0.30 to 0.92)	**0.024**
Febuxostat use at the time of first vaccine (ref: no or missing)	0.89 (0.47 to 1.68)	0.71	–	–	–	–
Benzbromarone use at the time of first vaccine (ref: no or missing)	0.66 (0.36 to 1.21)	0.18	–	–	–	–

Model 1: fully adjusted by COVID-19 vaccination, sex, age, body-mass index, disease duration, flare within 3 months prior to the first dose vaccine, last serum urate done before the first dose vaccine, colchicine prophylaxis and urate-lowering drugs use at the time of the first dose vaccine.
Model 2: fully adjusted by vaccine types (Sinovac Life, Sinopharm BIBP and others including recombinant COVID-19 vaccine (CHO cell) or recombinant COVID-19 Vaccine (adenovirus type 5 vector)), sex, age, body mass index, disease duration, flare within 3 months prior to the first dose vaccine, last serum urate done before the first dose vaccine, colchicine prophylaxis and urate-lowering drugs use at the time of the first dose vaccine.
Bold values indicate P<0.05.
*Gout flare within 3 months after any vaccine in COVID-19 vaccination participants or the matched time period in the non-vaccination participants.
†Comorbidities include hypertension, renal disease, hyperlipidaemia, tumour, fracture, cardiovascular, digestive, respiratory or mental diseases.

factors affecting the relevant outcomes. Importantly, we only investigated the effects of inactivated virus COVID-19 vaccines on gout flare, the only approved vaccines currently in China. Other vaccine types (eg, mRNA, viral vector or protein subunit type) need to be studied for validation, ideally prospectively and with multicentre collaboration.

In conclusion, this study suggests that COVID-19 vaccination is associated with a higher odds of postvaccine gout flares. Colchicine prophylaxis was associated with marked reduction of gout flares after COVID-19 vaccine. Surprisingly, mention of gout, a disease so frequently linked with obesity, type 2 diabetes, hypertension and advanced age, has been omitted from recent rheumatology society recommendations for COVID-19 vaccination in patients with rheumatic disease.[10] The willingness to get vaccinated against COVID-19 in patients with rheumatic diseases is limited by the fear of vaccine side effects.[11] This study may inform discussions with patients with gout about the risks of gout flare around the time of COVID-19 vaccination.

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Severely impaired humoral response against SARS-CoV-2 variants of concern following two doses of BNT162b2 vaccine in patients with systemic lupus erythematosus (SLE)

Severe COVID-19 is associated with a poor prognosis among patients with systemic lupus erythematosus (SLE).[1] Accordingly, patients with SLE receiving immunosuppressive drugs have been prioritised for vaccination in France.[2] However, patients with autoimmune diseases—especially those receiving anti-CD20—are now known to mount a suboptimal humoral response following COVID-19 vaccination.[3] Furthermore, vaccine-induced humoral protection against omicron, the current dominant variant of concern (VOC) worldwide, is not known in SLE.

Patients with SLE were prospectively enrolled in the vaccine task force set up between March and May 2021 in our national centre for autoimmune diseases.[4] Humoral vaccine responses against B (ancestral), alpha, delta and omicron variants were assessed using a specific multiplex ELISA assay (CoViDiag kit, Innobiochips, Loos, France). Humoral response was defined by a specific SARS-CoV-2 anti-spike IgG (anti-S) level in serum >260 binding antibody units (BAU)/mL, according to French Health Authorities.[5] Data were compared between groups using Fisher's exact test for dichotomous variables and Mann-Whitney test for continuous variables.

Fifty-five patients with SLE were enrolled in the vaccine task force. Among them, 10 had prior COVID-19 and 10 were under immunosuppressive drugs and received three-dose primary series of BNT162b2 vaccine following national recommendation.[2] Eventually, 35 COVID-19-naive patients with SLE (43.4 (36.0, 48.6) years; 88.6% female; table 1) and 9 healthy volunteers (HV) (59.0 (56.0, 62.0) years; 88.8% female) who received two doses of BNT162b2 vaccine 4 weeks apart were screened for humoral vaccine responses at baseline, at second dose, and 2 and 5 months after second dose. The two-dose and three-dose primary series of BNT162b2 vaccine were performed following an interval of 4 weeks between doses as recommended.[2]

Patients with SLE and HV had no detectable anti-S and anti-nucleocapsid at baseline. Two months after the second-dose vaccine, 54.3% (n=19/35), 54.3%, 42.9% and 28.6% of patients with SLE had specific anti-S titres>260 BAU/ml against B, alpha, delta and omicron variants, respectively. At the same time point, the percentage of subjects able to mount humoral response against VOC were lower in SLE as compared with HV (100% of HV for B, p=0.016; 100% for alpha; p=0.016; 88.9% for delta; p=0.023; 55.6% for omicron, p=0.235). In patients with SLE, when the humoral responses against VOC was obtained 2 months after the second dose, it was maintained at 5 months in only 10.5% (n=2/19), 10.5% (2/19), 13.3% (2/15) and 10% (1/10) for B, alpha, delta and omicron variants, respectively. Overall, 5 months after the second-dose vaccine, the percent of patients with SLE with humoral response against B (n=2/35, 5.7% vs n=7/9, 88.9%p<0.001), alpha (5.7% vs 77.8%, p<0.001) and delta (5.7% vs 55.6%, p=0.002) variants were dramatically low as compared with HV. Almost all vaccinated subjects (n=34/35, 99% patients with SLE and n=7/9, 77.8% HV, p=0.101) failed to mount long-lasting humoral response against Omicron after two doses of BNT162b2 vaccine (figure 1). Of note, 50% (n=4/8), 50% (n=4/8), 37.5% (n=3/8) and 12.5% (n=1/8) of patients with SLE who were under immunosuppressive drugs (azathioprine, mycophenolate mofetil or anti-CD20) and received three-dose primary series of BNT162b2 had a preserved humoral response for B, alpha, delta and omicron variants, respectively, 5 months after the third dose. Moreover, all but one (83.4%, n=5/6) patients with SLE who had COVID-19 6.2 (3.9–11.6) months before the first BNT162b2 dose had a sustained humoral response 5 months after the second dose against all VOC including omicron (online supplemental figures S2 and S3; online supplemental table S2).

In this 6-month prospective monocentric study, we show that more than 90% of COVID-19-naïve patients with SLE failed to reach vaccine-induced humoral response after two doses of BNT162b2. To our knowledge, this is the first evaluation of long-lasting humoral responses against VOC including omicron variant in a cohort of patients with SLE. Neutralisation activity was not determined but the threshold of 260 BAU/mL for humoral response was consistent with previous published results and a strong correlation between anti-S IgG BAU/mL, live viral neutralisation and pseudo-neutralisation activity has been reported by our group.[6] Since our patients with SLE received no immunosuppressive drugs, the poor immune response observed

Table 1 Characteristics of patients with SLE

	Two-dose primary, n=35	Three-dose primary, n=10	Prior COVID-19 n=10
Age, years (median (IQR))	43.4 (36.0, 48.6)	44.6 (38.5, 49.88)	50.1 (40.2, 59.6)
Female gender, n (%)	31 (88.6)	8 (80.0)	9 (90.0)
Delay since diagnosis, years (median (IQR))	7.8 (4.0, 12.9)	18.8 (12.9, 21.3)	8.9 (3.6, 27.5)
SLE involvement, n (%):			
Cutaneous	22 (62.8)	9 (90.0)	4 (40.0)
Joint	25 (71.4)	9 (90.0)	5 (50.0)
Renal	12 (34.3)	7 (70.0)	5 (50.0)
Serosal	10 (28.6)	6 (60.0)	4 (40.0)
Haematologic	11 (31.4)	4 (40.0)	4 (40.0)
Neuropsychiatric	4 (11.4)	2 (20.0)	2 (20.0)
Antiphospholipid syndrome	6 (17.1)	1 (10.0)	2 (20.0)
SLE treatment at BNT162b2 vaccine, n (%)			
Hydroxychloroquine	30 (85.7)	10 (100.0)	9 (90.0)
(HCQ)<200 µg/L	2 (6.6)	2 (20.0)	2 (20.0)
(HCQ) 200–1500 µg/L	24 (80.0)	7 (70.0)	6 (60.0)
(HCQ)>1500 µg/L	4 (13.3)	1 (10.0)	1 (10.0)
Steroids	16 (45.7)	9 (90.0)	8 (80.0)
Daily dose, mg (median (IQR))	7(5,10)	7.5(7, 10)	6.5(5, 7)
Belimumab	1 (2.9)	0 (0)	0 (0)
Azathioprine	0 (0)	2 (20.0)	0 (0)
Methotrexate	0 (0)	0 (0)	0 (0)
Mycophenolate mofetil	0 (0)	7 (70.0)	1 (10.0)
Cyclophosphamide	0 (0)	0 (0)	0 (0)
Anti-CD20	0 (0)	3 (30.0)	0 (0)
Lymphocyte count, $\times 10^9$/L (median (IQR))	1.52 (1.28, 2.00)	1.10 (0.97, 1.91)	1.44 (1.20, 2.60)
Gammaglobulins, g/L (median (IQR))	11.9 (9.6, 15.6)	12.2 (10.3, 14.9)	14.0 (11.2, 15.5)
SLEDAI, median (IQR)	0 (0, 2)	2 (0, 2)	1 (0, 2)

HCQ:level measured in whole blood.
HCQ, hydroxychloroquine; SLE, systemic lupus erythematosus; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.

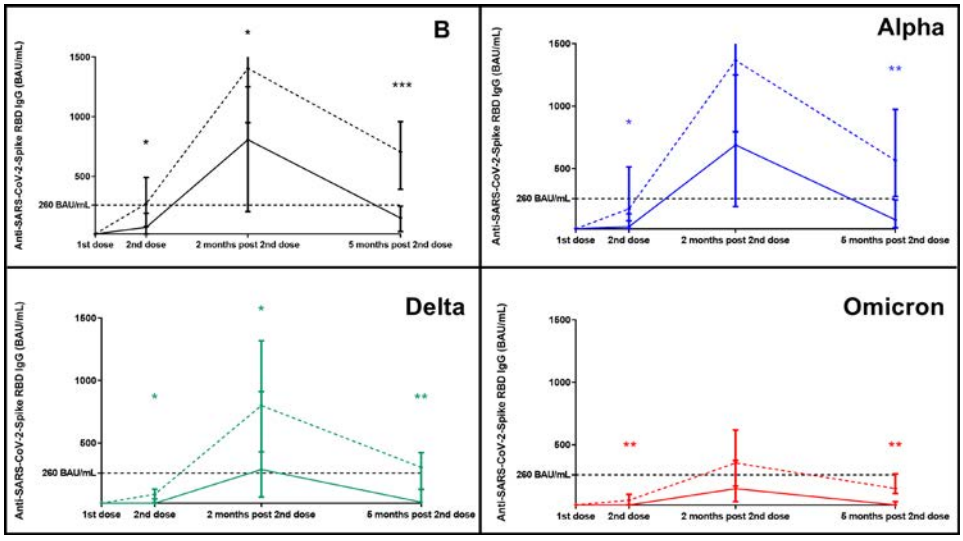

Figure 1 Humoral response following two doses of anti-SARS-CoV mRNA BNT162b2 vaccine in COVID-19-naïve patients with SLE and healthy volunteers. Serum SARS-CoV-2 anti-spike IgG level assessed overtime against B (ancestral), beta, delta and omicron variants by using a specific quantitative ELISA assay (CoViDiag kit, Innobiochips, Loos, France) in patients with SLE (n=35, solid line) and healthy volunteers (n=9, dotted line). Medians and first and third quartiles were showed at second dose, 2 months and 5 months after second dose. P value was calculated using Mann-Whitney U test (*p<0.05; **p<0.01; ***p<0.001). The value of 260 indicated the threshold for humoral response. BAU, binding antibody units; SLE, systemic lupus erythematosus.

after two vaccine doses could not be ascribed to treatment. Such vaccine failure is worrisome considering the recent onset of highly contagious SARS-CoV-2 variant such as omicron. In patients who were under immunosuppressive drugs and received 3-dose primary series of vaccine, the highly variable humoral responses may reflect the different impact of immunosuppressive agents on the vaccine immunogenicity.

In conclusion, our data show the poor immune long-lasting protection conferred by two doses of BNT162b2 vaccines in patients with SLE. Screening for humoral response to vaccination based on anti-S titres should be performed in all patients with SLE, including those who are not receiving immunosuppressive drugs. The absence of anti-SARS-Cov-2 antibodies after full vaccination might help to identify patients who are candidates for additional strategies, including anti-Sars-COV-2 monoclonal antibody prophylaxis, to protect them from COVID-19

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Routine treatment of rheumatoid arthritis with biologics and targeted agents: changes in patients and their response over 15 years

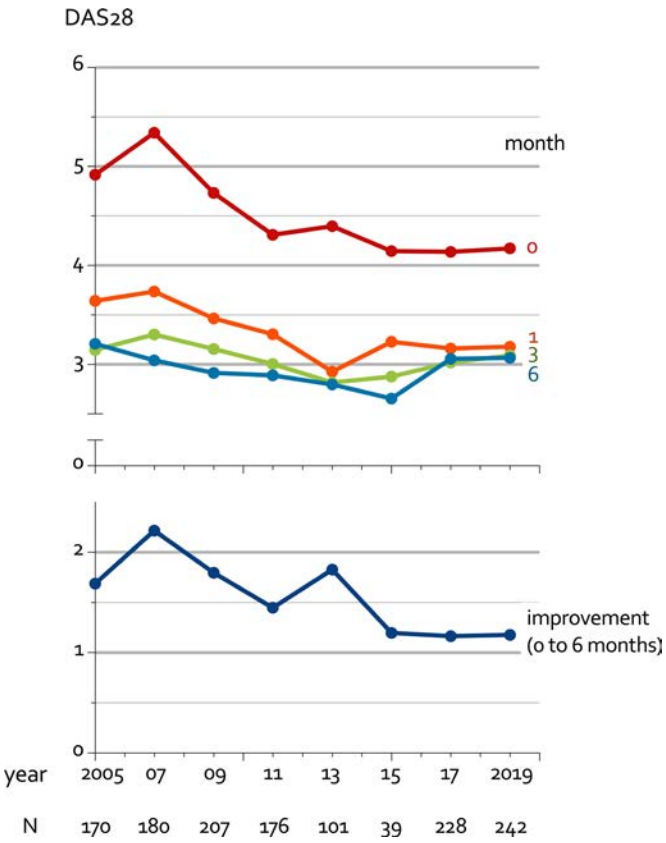

Figure 1 Development of DAS28 response over time. The top series indicate the absolute levels at baseline (red), 1 (orange), 3 (light green) and 6 months (dark green) and the bottom series (blue) shows change in DAS28 during the first 6 months of treatment over 15 years (calculated as the improvement in DAS28 between 0 and 6 months). The curves are smoothed by plotting the mean values over 2 years. DAS28, Disease Activity Score, 28 joints; N, number of patients included in the period.

Biologicals and later, targeted synthetic disease-modifying anti-rheumatic drugs (bDMARD, tsDMARD) have been approved for rheumatoid arthritis (RA) for more than 15 years.[1 2] Initially, most patients starting biologics had severe and active, long-standing disease with substantial damage: they had run out of treatment options. As time progressed, treatment options increased, strategies were intensified, and patients were treated earlier in their disease course.[3] In our ongoing registry comprising first bDMARD or tsDMARD therapy patients at Reade (the 'Biologicals cohort', NL59549.048.16), Amsterdam, we examined changes in the initial response (DAS28, during the first 6 months) related to changes in patient and disease characteristics in patients starting bDMARDs or tsDMARDs between 2004 and 2020.

To determine the Disease Activity Score (DAS28) change over 2004–2019 in the first 6 months of treatment, a linear mixed model analysis was used with DAS28 as dependent variable, and assessment time (time on drug), inclusion year (coded as 0–15), and the interaction between assessment time and inclusion year as independent variables. Subsequently, linear and logistic regression analyses were used to analyse changes over time in baseline DAS28, DAS28 at 6 months, response (change in DAS28 during the first 6 months of treatment), Minimal Disease Activity (MDA) (defined as DAS28 <2.60), disease duration, and rheumatoid factor, RF), respectively, anti-cyclic citrullinated peptide antibody (aCCP) positivity over time.

We included 1398 consecutive patients, 78% female, with a median disease duration of 5 (inner quartiles, 2–9) years, 71%RF and 76% aCCP positive; most (92%) started anti-tumour necrosis factor (TNF) alpha therapy. In the observation period the initial response (change in DAS28) decreased by 0.90 points, from –2.01 to –1.11. (mixed model interaction term: $p<0.0001$; figure 1). The baseline DAS28 value decreased by 1.08, from 5.09 to 4.01 ($p<0.00005$), whereas the 6-month value remained stable: a decrease of 0.08, from 3.04 to 2.96 ($p=0.60$). The components of the DAS28 and PROMs showed corresponding changes (online supplemental table 1). After 6 months, 255 (31%) patients achieved MDA, 127 (20%) achieved American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR)/Simplified Disease Activity Index (SDAI) remission (and 111 (14%) achieved ACR/EULAR/Boolean remission. Only the percentage of patients in MDA changed significantly over time, (OR 1.039 (95% CI 1.005 to 1.075) $p=0.026$), SDAI and Boolean remission did not ($p=0.39$ and 0.41, respectively). Disease duration on entry also strongly decreased, from median 9 (4–17) to 3 years (1–8; $p<0.0001$). The proportion of patients who were RF or aCCP positive decreased marginally ($p=0.91$).

A likely explanation for these findings is the introduction of early referral and treatment with rapid escalation in case of non-response. The fact that the DAS28 at 6 months remained constant is both good and bad news: many patients can expect to reach low disease activity levels after starting treatment with bDMARDs or tsDMARDs, but early treatment (and a more favourable starting position) apparently does not lead to more remissions. We previously described this effect before in a subselection of this cohort starting adalimumab,[4] where we showed that patients selected according to the selection criteria of various trials with adalimumab showed levels of improvement matching those seen in the trials, but all ended up with comparable disease activity levels at 6 months (DAS28 between 3.5 and 4.0).

In conclusion, in our practice current RA patients starting their first bDMARD or tsDMARD have much lower disease activity than patients starting 15 years ago. The disease activity achieved

after 6 months stayed constant at a low level, a good result, but also confirming that current treatment modalities are unable to induce remission in most patients.

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No excess mortality in contemporary undifferentiated arthritis, in contrast to rheumatoid arthritis: a study with a follow-up of at least 10 years

Two decades ago, the terminology of undifferentiated arthritis (UA) was proposed with the hypothesis that UA is an earlier phase of rheumatoid arthritis (RA).[1] Consequently, management strategies of RA are often transferred to UA-patients, assuming outcomes are comparable. Mortality is a long-term outcome that is unknown in UA, but widely studied in RA.

In RA, excess mortality becomes apparent after 10 years of follow-up in both anticitrullinated protein antibody (ACPA)-negative and ACPA-positive RA, and is presumably mediated by long-term, insufficiently suppressed inflammation.[2–4] Recently, it has been shown that early and treat-to-target treatment resolved excess mortality in ACPA-negative-RA, but not in ACPA-positive-RA.[5] In UA, mortality rates are unknown. Moreover, the UA-population has changed during the last decade.[1] Conventionally, UA was defined as not fulfilling the 1987 RA-critera and absence of another clinical diagnosis. With the introduction of the 2010 RA-criteria, part of the conventional UA- population became classified as RA. The remaining contemporary UA-population (not fulfilling the 1987/2010 RA-criteria) is largely autoantibody-negative, presents with monoarthritis or oligoarthritis and progresses less frequently to RA.[6] Thus, this population of contemporary UA may no longer represent a group of patients in an early phase of RA, but a population with intrinsically different characteristics than RA. We, therefore, hypothesised that there is no excess mortality in contemporary UA, in contrast to RA. This prompted us to assess mortality-rates in contemporary UA-patients, and compare this to conventional UA and RA, all during at least 10 years of follow-up.

We studied 860 conventional UA-patients (no 1987 RA-criteria or other diagnosis at baseline) and 561 contemporary UA-patients (no 1987 and 2010-RA-criteria or other diagnosis at baseline), included in the Leiden Early-Arthritis-Clinic (EAC) between 1993 and 2008 to ascertain minimally 10 years of follow-up after inclusion. Patients who were included between 1993 and 2008, but deceased within 10 years were included. Mortality data were obtained from the civic registries (June–2018). Mortality rates were compared with the general Dutch population using standardised mortality ratios (SMRs) adjusted for age at time of death, gender and calendar-year. SMRs were additionally stratified for ACPA-positivity (anti-CCP2, Euro diagnostica, cut-off>10 mg/L) and autoantibody-positivity (ACPA-positivity and/or rheumatoid-factor-positivity (in-house ELISA)). Mortality rates in RA, which have been previously studied for 1987-RA,[5] were included for comparison. For this, all patients with a clinical RA-diagnosis and fulfilling the 1987/2010-RA-criteria (n=762/828), included between 1993 and 2008 in the Leiden-EAC, were studied. Stratification was applied for early, treat-to-target disease-modifying antirheumatic drug (DMARD)-therapy, as described previously.[5]

The contemporary UA-population was predominantly autoantibody-negative; median swollen joint count was 1 and tender joint count 2, which presentation was milder than conventional-UA (table 1). The contemporary UA-population was also

Table 1 Characteristics of patients studied

	Contemporary UA N=561	Conventional UA N=860	RA according to 1987 RA-criteria N=762	RA according to 2010 RA-criteria N=828
Age (years), mean (SD)	49 (17)	51 (17)	56 (16)	56 (16)
Gender (female), %	54	59	67	68
Symptom duration (weeks), median (IQR)	13 (5–29)	17 (7–33)	19 (10–37)	20 (11–36)
ACPA-positive, %	7	19	52	54
RF-positive, %	9	21	59	61
Swollen joint count (0–28), median (IQR)	1 (1–2)	2 (1–3)	7 (4–12)	7 (4–12)
Tender joint count (0–28), median (IQR)	2 (1–4)	3 (1–7)	13 (7–21)	14 (8–21)
VAS (0–100), median (IQR)	28 (12–50)	31 (13–52)	44 (20–60)	40 (20–59)
CRP (ug/mL), median (IQR)	8 (3–22)	8 (3–23)	17 (7–38)	15 (6–37)
DAS28CRP, median (IQR)	3.2 (2.6–3.9)	3.7 (2.9–4.5)	4.8 (4.1–5.7)	4.7 (4.0–56)
HAQ-DI, median (IQR)	0.5 (0.1–1.0)	0.6 (0.2–1.1)	1.0 (0.6–1.5)	1.0 (0.5–1.5)
DMARD-therapy during follow-up	39%	57%	100%	100%
RA-progression* after 1-year follow-up	20%	44%	–	–

Characteristics of the conventional and contemporary UA-population and the RA-populations. Compared with conventional UA, contemporary UA patients were largely autoantibody negative and presented with lower disease activity at baseline. In conventional UA, more patients progressed to RA after 1 year of follow-up and DMARD-treatment was more frequently required.
*RA-progression was defined as clinical diagnosis of RA and fulfillment of the 1987 and/or 2010-criteria for RA.
ACPA, anticitrullinated autoantibody protein; CRP, C reactive protein; DAS, Disease Activity Score; DMARD, Disease-modifying antirheumatic drug; HAQ-DI, Health Assessment Questionnaire Disability Index; RA, rheumatoid arthritis; RF, rheumatoid factor; SJC, swollen joint count; UA, undifferentiated arthritis; VAS, Visual Analogue Scale.

smaller than conventional-UA (figure 1) and progressed less frequently to RA (online supplemental file 1). Median follow-up for conventional UA-patients was 17.2 years (IQR 13.0–22.1), for contemporary UA 17.3 years (IQR 12.7–22.3), for 1987 RA patients 16.0 years (12.8–20.4) and for 2010 RA patients 16.2 years (IQR 13.0–20.8). Mortality rates were increased in RA, both when defined according to the 1987-criteria or 2010-criteria. Additionally, ACPA-stratification revealed that early initiation of DMARD-treatment and treat-to-target-strategies did not resolve excess mortality in ACPA-positive-RA (figure 1).[5] In conventional-UA, a trend towards excess mortality was observed: SMR 1.11 (95% CI 0.96 to 1.27). However, part of this UA-population is

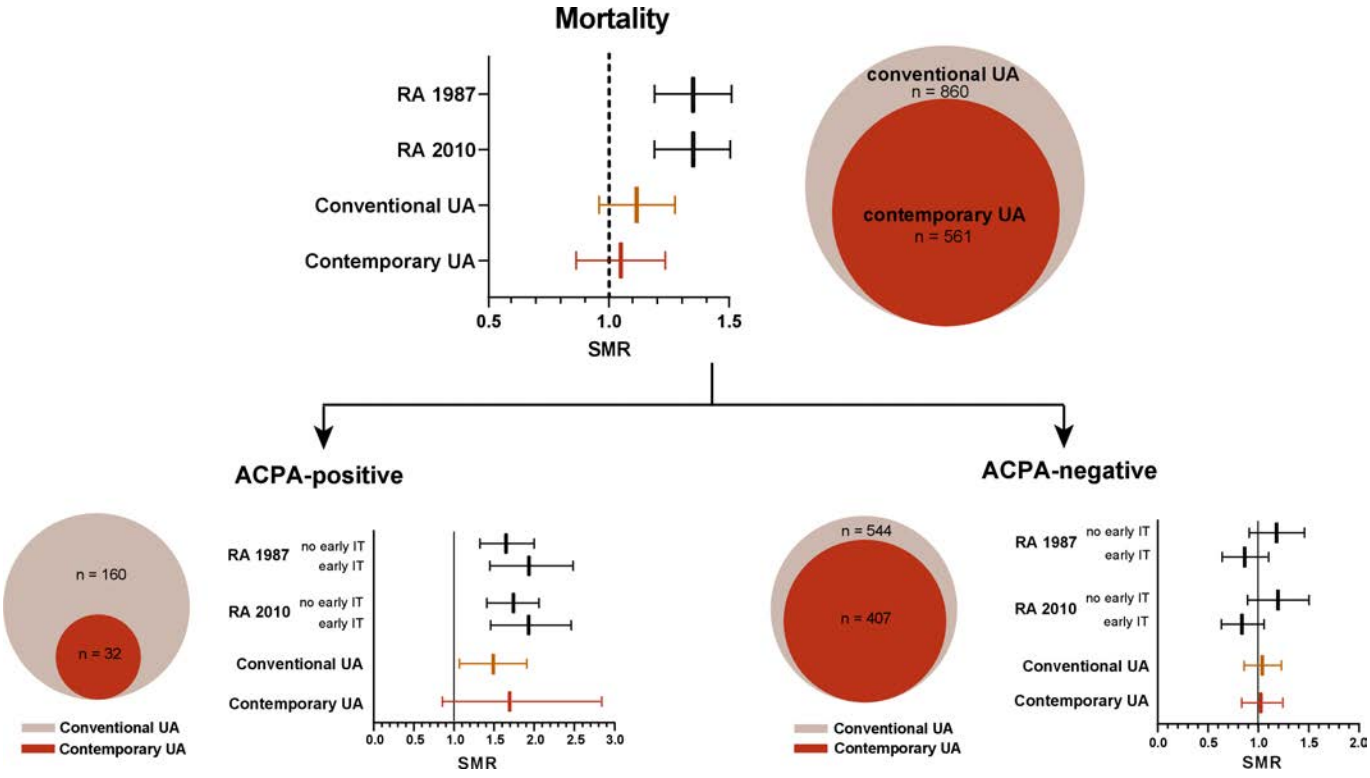

Figure 1 Standardised mortality ratios in undifferentiated and rheumatoid arthritis. Standardised mortality ratios in undifferentiated arthritis and rheumatoid arthritis patients with minimally 10 years of follow-up. Additionally, mortality was stratified for ACPA-status. In RA, SMRs were also stratified for early treat-to-target DMARD-therapy, which has been shown to be relevant for mortality analysis in RA.[5] ACPA, anticitrullinated-protein-antibody; DMARD, disease-modifying antirheumatic drugs, IT, intensive treatment; RA, rheumatoid arthritis; SMR, standardised mortality rate; UA, undifferentiated arthritis.

currently classified as 2010-RA. Subsequently, in contemporary-UA no excess mortality was observed: SMR 1.05 (95% CI 0.87 to 1.26; figure 1). ACPA-stratification provided data suggestive for excess mortality in ACPA-positive contemporary UA, although this group was small. No excess mortality was present in ACPA-negative contemporary UA (figure 1). Results were similar when stratification was performed for autoantibody-status (online supplemental file 1). Mortality rates in contemporary UA patients who did and did not receive DMARD-treatment were comparable (SMR 0.93 (95% CI 0.66 to 1.24) and SMR 1.08 (95% CI 0.82 to 1.36), respectively).

In conclusion, contemporary UA has no excess mortality, which is in contrast to RA. Only a few per cent of patients presenting with contemporary UA are autoantibody-positive; these patients may be considered at increased risk to progress to RA. Interestingly, the estimate of the SMR of this subgroup resembled that of RA, but the confidence interval was broad. The large majority of contemporary UA- patients, in contrast, is autoantibody-negative and presents with few inflamed joints. This is the first large study on mortality in UA. Differences between conventional UA and RA have been shown in the past for other outcomes. This study on mortality suggests that the differences have increased for contemporary UA and RA. These results should be considered together with results from other studies and other outcomes in contemporary UA.[6] Further research and discussions are needed as to whether the management of contemporary UA should be similar to or different from that of RA.

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Comparison of ultrasound attenuation by calcium pyrophosphate, hydroxyapatite and monosodium urate crystals: a proof-of-concept study

Over the past decade, ultrasound (US) has been extensively used for the diagnosis of crystal arthropathies, in particular gout and calcium pyrophosphate deposition disease.[1 2] US has proven to be accurate and reliable for the diagnosis of these diseases, and validated definitions for monosodium urate (MSU) and calcium pyrophosphate (CPP) crystal deposition in and around joints have been released by the Outcome Measures in Rheumatology US working group.[3–5] On the other hand, although routinely used in the diagnosis and management of calcific tendinitis/periarthritis, the precise role of US in hydroxyapatite (HA) deposition disease, in particular its validation and reliability assessment, remains to be determined.

The propensity to attenuate the US beam and generate acoustic shadowing are important US imaging features in distinguishing between the different types of crystal deposition. Generally, HA crystals are considered to generate acoustic shadowing, while MSU crystals tend to do the same depending mainly on the concentration and size of aggregates. In contrast, CPP crystals typically do not attenuate the US beam.[3–5] Therefore, this proof-of-concept study aimed to investigate the US attenuation characteristics of increasing concentrations of CPP, HA and MSU crystals.

Sixteen synthetic crystal suspensions with known concentrations of CPP (26–109 mg/mL), HA (31–153 mg/mL) and MSU (90–500 mg/mL) were prepared. These specific concentrations

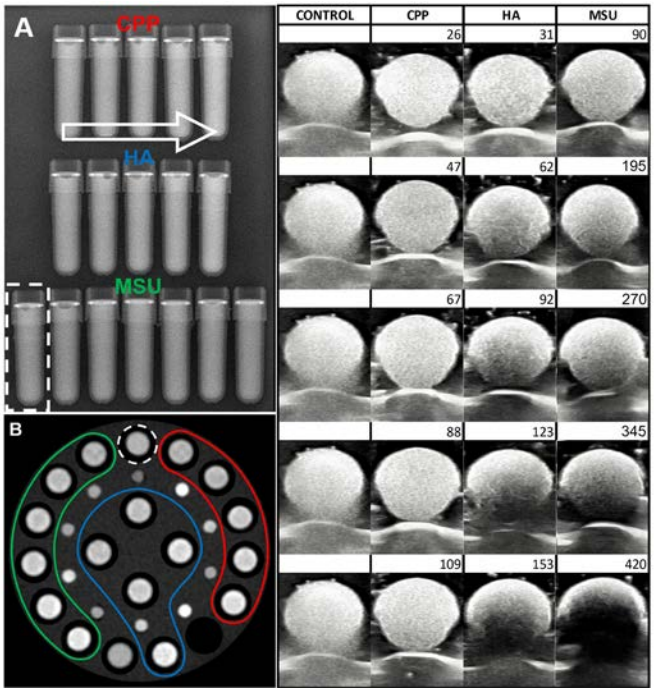

Figure 1 (A) Conventional radiography, (B) CT and (C) ultrasound (US) of the various synthetic crystal suspensions with known increasing (from left to right in A, arrow; and from top to bottom in C) concentrations of calcium pyrophosphate (CPP) (26–109 mg/mL; red in B), hydroxyapatite (HA) (31–153 mg/mL; blue in B) and monosodium urate (MSU) (90–500 mg/mL, green in B). These specific concentration ranges were selected to replicate the X-ray attenuation characteristics of those crystals in vivo.[6] the dashed line represents the crystal-free (0 mg/mL) agar-based lipogel background for reference. (C) Short-axis views comparing the US attenuation characteristics, including acoustic shadowing, of increasing concentrations of CPP, HA and MSU crystals. The left column shows the US attenuation of the crystal-free agar-based lipogel background for reference. (MSU at 500 mg/mL is not shown here as it generated clear acoustic shadowing similar to 420 mg/mL).

were selected to replicate the X-ray attenuation characteristics of those crystals when imaged by conventional radiography, CT and dual-energy CT in vivo (figure 1).[6] The density of the agar-based lipogel background was intentionally increased to mimic the X-ray attenuation of hyaline cartilage (ie, 100–120 HU at 120 kVp). Each crystal suspension was removed from its Eppendorf tube and placed in a plastic container filled with US gel, next to the control (ie, crystal-free background) calibration phantom. We acquired all US images using a Samsung RS80A system equipped with a high-frequency linear array transducer (4–18 MHz) set at the maximum frequency, by applying the same settings. US scans were performed by a single experienced sonographer (GF), blinded to the crystal type and concentration. For each of the 16 crystal suspensions, at least 2 images were recorded both in the long-axis and short-axis views, the latter including the control phantom. Interpretation of US images for the extent of US beam attenuation and the presence of acoustic shadowing was performed in consensus with a second experienced sonographer (FB). No patients were involved in this study.

Conventional radiography and CT confirmed the comparable and representative X-ray attenuation of the three crystal suspension types (figure 1). None of the five CPP suspensions generated acoustic shadowing or substantial US attenuation, regardless of crystal concentration. In contrast, both HA and MSU suspensions substantially attenuated the US beam and generated acoustic shadowing from a different given crystal concentration. While HA did not attenuate the US beam at 31 mg/mL, US attenuation gradually increased with increasing crystal concentration from 62 to 123 mg/mL, eventually generating clear acoustic shadowing at 153 mg/dL. Similarly, MSU at 90 mg/mL showed no US attenuation, which gradually increased from 195 up to 345 mg/mL to finally generate clear acoustic shadowing from 420 mg/mL (figure 1).

This proof-of-concept study provides initial evidence that, at crystal concentrations encountered in and around patient joints (ie, with a CT attenuation range of 150–300 HU[6]), CPP does not generate substantial acoustic shadowing, unlike HA and MSU which attenuate US in proportion to crystal concentration. Being an in vitro pilot study, US beam attenuation was assessed empirically and not quantitatively or semi-quantitatively. However, our findings highlight the potential ability of US to distinguish between CPP, HA and MSU crystal deposition based on their appearance and variable attenuation on grayscale images. Future larger studies should then be performed with various US systems and settings, multiple ultrasonographers, and in correspondence with ex vivo samples in order to validate and assess the reliability of these initial findings because the surrounding human tissues and equipment characteristics could impact the attenuation level of the US beam. If validation studies were to confirm the different patterns of US attenuation of the various crystal deposits, this would not only enhance the diagnostic performance of US in crystal arthropathies overall, but also enable the development of a grading system for US attenuation of crystal deposition—which is currently lacking—and thereby to semiquantitatively estimate crystal concentration for monitoring purposes.

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Eosinopenia to differentiate crystal-induced and septic arthritis

Septic and crystal-induced arthritis (related to calcium pyrophosphate deposition disease or gout) are the two main causes of acute knee arthritis. The features of septic and crystal-induced arthritis are very similar, with a high level of acute pain, sharp inflammatory syndrome and occasionally fever, so the differential diagnosis is difficult.[1 2]

Pyogenic septic arthritis of the knees is associated with high mortality and morbidity and is considered a medical emergency.[3] In case of severe sepsis, probabilistic antibiotic therapy and close monitoring are recommended.[4] Gram staining and culture of synovial fluid (SF) are the gold standard for the diagnosis of septic arthritis, but identifying the bacterium often takes several days. Examining SF for the identification of crystals allows for a definite diagnosis of gout and CPP crystal arthritis but requires skills and facilities that are not always present in the primary care setting or in emergency departments. Apart from procalcitonin, we lack a reliable, simple biomarker for the early discrimination of these two conditions.

Several studies demonstrated the value of eosinopenia as a diagnostic tool for severe pyogenic infections, notably in patients hospitalised in intensive care units.[5 6] The value of eosinopenia as a marker of bacterial infection of native joints remains unknown.

The aim of our study was to compare the eosinophil count during the early phase of septic and crystal-induced knee arthritis and to determine the metrological property of eosinopenia to differentiate the two conditions.

This retrospective study included data for consecutive patients hospitalised between May 2009 and June 2020 in two tertiary care hospitals. All patients had acute knee monoarthritis and demonstration of crystals (monosodium urate or CPP) or bacteria within the SF. Patients with diseases that can modify the eosinophilic cell count were excluded as were patients treated with steroids.

Demographic, biological and microbiological characteristics were collected for each patient. Blood cell count at admission was recorded. We chose an eosinophil count $<50/mm^3$ as a cut-off value to define eosinopenia[5 6], then calculated the sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) of eosinopenia for the diagnosis of septic versus crystal-induced arthritis, estimating 95% CIs. We also assessed

Table 1 Characteristics of patients with septic and crystal-induced arthritis

	Septic arthritis, n=114	Crystal-induced arthritis, n=104	P value
Male sex, n (%)	66 (57.9)	51 (49)	0.24
Age (years)	65.9 (17.2)	72.3 (15.5)	<0.005
Fever (temperature≥38.5°C), n (%)	44 (38.6)	35 (33.7)	<0.005
Comorbidities, n (%)*	42 (38.3)	48 (46.1)	<0.005
Bacteria, n (%)			
Staphylococcus†	52 (45.6)	–	
Others‡	62 (54.4)	–	NA
Crystals, n (%)			NA
MSU	–	34 (32.7)	NA
CPP	–	70 (67.3)	NA
Blood leucocyte count (cells/mm^3)	11 075 (4518)	9250 (3263)	<0.005
Neutrophil count (cells/mm^3)	8683 (4265)	6505 (3014)	<0.0005
Eosinophil count (cells/mm^3)	72.7 (84.1)	144.7 (152.9)	<0.0005
CRP level (mg/L)	165.1	100.5	<0.0005
Died, n (%)	3 (2.6)	0	<0.005
Eosinophil count≤50/mm^3, n (%)	62 (54)	26 (25)	<0.0005

Data are mean (SD) unless indicated.
*Diabetes, cancer, haematological malignancies.
†Methicillin-sensitive *Staphylococcus aureus* (39.5%), methicillin-resistant *Staphylococcus aureus* (6.1%); coagulase-negative Staphylococci (19.3%).
‡Streptococcus (21%); Gram-negative bacteria (7%); others (7.1%).
CPP, calcium pyrophosphate; CRP, C reactive protein; MSU, monosodium urate.;

the receiver operating characteristic (ROC) curve of eosinophil count for discriminating infected and non-infected patients.

Records for 4662 patients were analysed. We finally included 218 patients; 114 had a septic arthritis and 104 crystal-induced arthritis (table 1). Mean (SD) eosinophil count was lower in patients with septic than crystal-induced arthritis: 72.7/mm^3 (84.1) vs 144.7/mm^3 (152.9) (p<0.005).

Eosinopenia yielded a specificity of 76.9% (95% CI, 67.9% to 84%) and PPV 63.4% (95% CI, 55.3% to 71.6%) for the diagnosis of septic arthritis. Sensitivity was 50% (95% CI, 41% to 59%) and NPV 65.5% (95% CI, 55.3% to 75.6%); positive and negative likelihood ratios were 2.17 (95% CI, 1.46 to 3.22) and 0.65 (95% CI, 0.53 to 0.80), respectively. The area under the ROC curve was 0.68 (95% CI, 0.61 to 0.76).

Combining eosinopenia with different C reactive protein level thresholds did not improve the specificity or the PPV (data not shown). By contrast, the association of eosinopenia with a neutrophil count≥10 500 cells/mm^3 improved the specificity to 88.5% (95% CI, 80.7% to 93.4%) and PPV to 73.9% (95% CI, 61.2% to 86.6%) but lowered the sensitivity to 29.8% (95% CI, 22.2% to 33.8%). The NPV was 53.5% (95% CI, 46% to 60%) and positive likelihood ratio 2.59 (95% CI, 1.41 to 4.71). The area under the ROC curve was 0.59 (95% CI, 0.54 to 0.64).

The present study is the first to compare the eosinophil count in patients with septic and crystal-induced arthritis. In patients with acute knee arthritis, the combination of eosinopenia and neutrophil count>10 500 cell/mm^3 is evocative of septic arthritis.

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COVID-19 in patients with rheumatic diseases: what is the real mortality risk?

We read with great interest the paper published by D'Silva *et al*[1] regarding the main outcomes in rheumatic patients with COVID-19. According to this cohort, there was a mortality rate of 6%, slightly similar to the frequency observed in individuals without rheumatic diseases (RDs) (around 4%). On the other hand, there was a threefold higher risk of intensive care admission and mechanical ventilation in patients with RDs, suggesting some difficulties to handle this subgroup. More recently, Favalli *et al*[2] stated that the incidence of COVID-19 was also quite similar between patients with RD and individuals from the general population in Lombardia (0.62% vs 0.66%, respectively). Moreover, the disease activity was not worsened or triggered by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in rheumatic patients.

Brazil had its first confirmed case of COVID-19 on 26 February, and, to date, it accounts for more than one million cases and more than 50 000 deaths. The lethality rate has been estimated in 4.7%.[3] ReumaCoV Brasil is the official Brazilian Registry of Rheumatic Patients with COVID-19, supported by the Brazilian Society of Rheumatology. The inclusion phase started on 19 May with recruitment of 130 patients from 38 centres spread in five regions of the country. After the 4-week enrolment, we observed 12 deaths with a lethality rate of 9.2%, almost twice more than the general Brazilian population. Our data are more closely in accordance with the Global Rheumatology Alliance Global Registry[4] than D'Silva's cohort.[1]

Considering our 12 patients with RD, more than 83.3% were women with a mean age of 49.9 years (±17.2). Only three patients were older than 60 years. Four patients had systemic lupus erythematosus (SLE); two had rheumatoid arthritis; two had systemic sclerosis; one had systemic vasculitis; one had psoriatic arthritis; one had axial spondyloarthritis; and one had overlap syndrome. Oral corticosteroids were being used by 58.3% of cases, with daily dosages between 10 and 20 mg, and only four patients used hydroxychloroquine. The main symptoms related to COVID-19 were cough (91.7%), dyspnoea (83.3%) and fever (75.0%), quite similar to the previously published data.[4 5] Another relevant thing to address is the lack of biological therapy, higher prevalence of active SLE and recent combined methylprednisolone and cyclophosphamide pulse among our hospitalised patients. All of them were hospitalised in the intensive care unit with severe hypoxia (table 1).

Interestingly, we did not found any pattern related to death caused by COVID-19, suggesting neither RD itself (remission, disease activity, years since the diagnosis) nor the current immunosuppression status was associated with poor outcome. Nonetheless, the frequency of comorbidities was high, including hypertension (63.6%), diabetes (27.3%), chronic interstitial lung disease (27.3%) and renal failure (36.4%). Also, it is worthy emphasising the low adherence to international measures to mitigate the viral contagion, including social distancing (around 50%) in our cases, differently of reporting by Favalli *et al*.[2]

Thus, several other aspects, including immunosenescence, epigenetic modifications, viral load, genetic background, changes in T-cell diversity, inflammaging and comorbidities,[6] seem to be more involved with the uncontrolled inflammatory and immune response, as well as the cytokine storm, and higher mortality rate in patients with COVID-19 than the underlying RDs.

Although our data are preliminary, it is important to highlight the huge spectrum of RD, as well as the heterogeneity

Table 1 Clinical characteristics of 12 patients whose outcome was death, out of 130 patients included in the ReumaCoV Brazil cohort

	Age	Sex	Diagnosis	Disease duration	Systemic involvement	Disease activity	Symptoms	Concomitant medications related to RD	Drug withdrawal	Comorbidities	COVID treatment	Social isolation	Epidemiology*	COVID-19 diagnosis
1	24	F	SLE	4 years	No	No	Dyspnoea, fever, cough	Oral CS (>20 mg/day), HCQ	No	No	Unknown	Yes	No	RT-PCR
2	27	M	SLE	17 months	Nephritis, APS	Yes (haematological, ILD)	Dyspnoea, cough, haemoptysis	Oral CS (>20 mg/day), HCQ, CYC (pulse), warfarin	CYC	Drug addiction	Azithromicin, heparin (full dosage), HCQ, IVIG, IVCS (pulse)	No	Yes	RT-PCR
3	36	M	Ax-SpA	2 years	No	No	Dyspnoea, fever, anosmia, cough	Adalimumab	No	Hypertension	Azithromicin, HCQ	Yes	Unknown	RT-PCR
4	38	F	Overlap syndrome	1 year	Skin, haematological, nephritis, serositis	Yes (joint, skin, haematological, nephritis, serositis)	Asthenia, dyspnoea, fever, cough	Oral CS (10–20 mg/day), IVCS (pulse)	No	DM, hypertension, osteoporosis	HCQ, IVCS (pulse), meropenem, vancomycin	No	Yes	Serology
5	40	F	SLE	9 years	Nephritis	No	Headache, fever, cough	Oral CS (<10 mg/day), HCQ	No	Hypertension, renal failure on hemodialysis	Azithromicin, oral CS (<10 mg/day), meropenem, vancomycin	Yes	Unknown	RT-PCR
6	47	F	SLE	22 years	Skin, joint, lymphopaenia, neuropsychiatric	Yes (psychosis)	Dyspnoea, fever, cough	Oral CS, HCQ, CYC (pulse), IVCS (pulse)	CYC	No	Heparin (half-dosage), HCQ, IVCS	Yes	Yes	RT-PCR
7	55	F	SS	14 years	ILD, pulmonary hypertension	Yes (ILD)	Asthenia, headache, dyspnoea, cough	MMF	MMF	Chronic pulmonary embolism, splenectomy	Vancomycin	Yes	No	RT-PCR
8	58	F	SS	20 years	ILD, pulmonary hypertension	Yes (ILD)	Asthenia, headache, cough, fever, ageusia, cough	MMF, nintedanib, sildenafil, bosentan, IVIG	MMF	DM, hypertension, hypercholesterolaemia,	IVIG	No	Unknown	RT-PCR
9	58	F	RA	20 years	ILD	No	Joint pain, asthenia, headache, dyspnoea, fever, anosmia, ageusia, cough	RTX, oral CS (10–20 mg/day)	No	Heart disease, hypertension, ILD	IVIG	Yes	Unknown	RT-PCR
10	70	F	RA	12 years	Peripheral neuropathy	No	Asthenia, dyspnoea	MTX	MTX	Heart disease, renal failure, obesity	Heparin (half-dosage), ceftriaxone meropenem vancomycin	No	Unknown	RT-PCR
11	72	F	PsA	14 years	No	No	Asthenia, dyspnoea, fever, cough	LEF	LEF	Heart disease, DM, renal failure, hypertension, obesity, gout	Unknown	No	Unknown	RT-PCR
12	74	F	Systemic vasculitis	11 years	Nephritis, peripheral neuropathy	Yes	Dyspnoea, fever, cough	AZA, oral CS (<10 mg/day)	No	No	Heparin (full dosage), azithromicin, IVCS (pulse)	No	Yes	RT-PCR

*Epidemiology defined as positive close contact with confirmed COVID-19 case.
APS, antphospholipid syndrome; ax-SpA, axial spondyloarthritis; AZA, azathioprine; CS, corticosteroid; CYC, cyclophosphamide; DM, diabetes mellitus; F, female; HCQ, hydroxychloroquine; ILD, interstitial lung disease; IVCS, iliac vein compression syndrome; IVIG, intravenous immunoglobulin; LEF, leflunomide; M, male; MMF, mofetil mycophenolate; MTX, methotrexate; PsA, psoriatic arthritis; RA, rheumatoid arthritis; RD, rheumatic disease; RT-PCR, reverse transcription polymerase chain reaction; RTX, rituximab; SLE, systemic lupus erythematosus; SS, systemic sclerosis.

of these patients, regarding the risk of death, especially in those with recent use of corticosteroids, reinforcing that protective measures need to keep on being recommended, and the rheumatologists should weigh the peculiarities of each disease and the immunosuppression to better manage them.

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Response to: 'COVID-19 in patients with rheumatic diseases: what is the real mortality risk?' by Marques *et al*

We appreciate the comments by Marques *et al* in response to our manuscript.[1] Their description of a cohort of patients with rheumatic disease and COVID-19 is another important contribution to the literature during this quickly evolving pandemic. In this report of 130 patients with rheumatic diseases, the authors estimated a mortality rate of 9.2%, higher than the 4.7% mortality rate reported in the general population in Brazil.[1] It is important to note that one must be cautious when comparing these two rates because the composition of these cohorts according to demographics and other confounding features likely differs but was unable to be accounted for in the analysis. However, there were several important features in this cohort which may explain the higher mortality rate including a large comorbidity burden among the deceased, frequent use of glucocorticoids and less frequent use of biologic disease-modifying antirheumatic drugs (DMARD).[2] The authors hypothesise that factors other than the underlying rheumatic disease explain the higher mortality rate.

We agree that there are scant data to explain why some patients with rheumatic diseases may have worse outcomes compared with the general population. Potential explanations for reported differences may include differences in the distribution of comorbidities, use of immunosuppressive medications or the underlying diseases themselves. The COVID-19 Global Rheumatology Alliance observed a lower hospitalisation rate among patients receiving biologic and targeted synthetic DMARDs,[2] and Marques *et al* reported that none of the deceased patients in their registry were on biologic DMARDs.[1] In our study of a cohort in Boston, MA, USA, we observed a higher risk of mechanical ventilation and intensive care unit admission in patients with rheumatic disease after adjusting for several relevant covariates including demographic features and key comorbidities, although residual confounding is still possible.[3] We did not have sufficient power to adjust for differences in immunosuppressive medications as they relate to differences in outcomes within our cohort of patients with rheumatic diseases.

When interpreting reports of outcomes in patients with rheumatic diseases during the COVID-19 pandemic, it is important to consider how cohorts are assembled. It is not clear how patients were identified and enrolled in the registry that Marques *et al* describe.[1] If patients with COVID-19 were not systematically obtained (eg, from administrative databases containing data from patients in hospitals, clinics or healthcare systems), there is potential for selection bias since more severe cases are more likely to present for care and be reported by their providers, which may lead to an overestimation of the mortality rate. Selection bias may explain why the mortality rates reported by the COVID-19 Global Rheumatology Alliance[2] and Marques *et al*[1] are higher than those observed in our cohort which was assembled from the population of patients with positive PCR testing within a large healthcare system.[3] The mortality in our rheumatic disease cohort was slightly higher numerically than that of the control population though this difference was not statistically significant (6% vs 4%, respectively; p=0.69).[3]

We thank Marques *et al*[1] for their contribution to help us all better understand the impact of COVID-19 on patients with rheumatic diseases. Additional studies of larger cohorts with appropriate comparators identified systematically from the same data set are necessary to clarify any association between rheumatic disease and outcomes of COVID-19 infection.

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Comment on 'Implication of baseline levels and early changes of C-reactive protein for subsequent clinical outcomes of patients with rheumatoid arthritis treated with tocilizumab'

We read with interest the article 'Implication of baseline levels and early changes of C-reactive protein for subsequent clinical outcomes of patients with rheumatoid arthritis treated with tocilizumab' by Shafran *et al*.[1] This was an exploratory analysis of multiple studies and concluded that baseline C-reactive protein (CRP) and its early course may inform, to some extent, the estimation of potential therapeutic success in patients with rheumatoid arthritis (RA) treated with tocilizumab. Although we appreciate the pursuit of personalised treatment decisions in RA, this conclusion is not the only interpretation, and previously published analyses of these data further characterise the relationship between CRP and clinical outcomes with tocilizumab treatment in patients with RA.[2]

Shafran *et al*[1] concluded that baseline and early changes in CRP levels are predictive of tocilizumab response. Although CRP levels were affected differently by tocilizumab than rituximab or methotrexate treatment, decreasing to near zero in tocilizumab-treated patients, the data show some independence between CRP and clinical outcomes and demonstrate the potential influence of disease activity on clinical outcomes. Figure 1 presents mean percentage change in CRP levels and Clinical Disease Activity Index (CDAI) over time.[1] In the tocilizumab group, percent change in CRP was numerically larger than percent change in CDAI, implying some independence of the two variables. In the methotrexate group, percent change in CRP was smaller than percent change in CDAI, also suggesting some independence of the two variables. In the rituximab group, percent changes in CRP and CDAI were similar.

Figure 2 presents mean CRP values at baseline and week 4 for patients achieving remission/low/medium/high CDAI at week 24.[1] The error bars overlap within treatment groups, implying no statistical differences; however, the authors conclude that early CRP levels predict response to tocilizumab.

Figure 3 presents mean CRP values over time for patients achieving remission/low/medium/high CDAI at week 24.[1] In the tocilizumab group, the curves are close together (error bars would be expected to overlap) because CRP levels are reduced in nearly all patients. This implies that the impact of CRP on clinical outcomes is removed early with tocilizumab treatment and that clinical response is based on treatment effectiveness and other factors, as shown in Figure 4.[1] In all treatment groups, mean disease activity is consistent with the week 24 outcome category; for example, patients with high disease activity at week 24 tend to have the highest mean disease activity throughout. This suggests a relationship between baseline and week 24 disease activity, as recently quantified.[3]

Figure 5 presents ORs for achieving CDAI remission at various CRP level cutpoints (a), comparison between treatments of ORs for achieving CDAI remission at various CRP cutpoints (an approach that cannot be clearly interpreted for these nonrandomised treatment groups) (b), and comparison between randomised treatment arms from a validation sample of ORs for achieving CDAI remission at various CRP level cutpoints (c).[1] In (a), the 95% CIs cross 1.0 for all CRP cutpoints with tocilizumab, suggesting that there is an equal likelihood of attaining CDAI remission above or below threshold. In (c), the likelihood of achieving CDAI remission is higher with tocilizumab than methotrexate, regardless of baseline CRP level, and, based on overlapping CIs, there is a similar likelihood of achieving CDAI remission in patients treated with methotrexate combined with tocilizumab versus tocilizumab alone at all baseline CRP levels.

Overall, these results are consistent with previous analyses of randomised controlled trials in which relationships between biomarkers and outcomes were summarised at the group level and at individual patient levels.[2] Serum CRP level changes after tocilizumab dosing are expected for most patients but occur independently of changes in other clinical signs and symptoms, and tocilizumab can be efficacious across a broad range of baseline CRP concentrations. Decrease in CRP levels is a pharmacodynamic marker of tocilizumab treatment but neither a surrogate for clinical activity nor a reliable predictor of clinical outcomes. This is relevant to the practising clinician, who should continue to make decisions for patients with RA treated with tocilizumab based on clinical and symptomatic response.

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Response to: 'Comment on 'Implication of baseline levels and early changes of C-reactive protein for subsequent clinical outcomes of patients with rheumatoid arthritis treated with tocilizumab" by Pethoe-Schramm *et al*

We would like to thank Dr Pethoe-Schramm and colleagues for their comment on our paper[1] and the diligent assessment of the data we presented.[2] The authors addressed each of our Figures sequentially and, therefore, it will be a pleasure to respond here accordingly.

Before doing so, we would like to mention that in contrast to other studies, like the one by Wang *et al*[3] which we also referred to in our paper, we approached the analyses by using Clinical Disease Activity Index (CDAI) remission criteria (endorsed by ACR-EULAR for this purpose) rather than "remission" criteria by the Disease Activity Score using 28 joint counts (DAS28) .[4 5] As often described, DAS28 <2.6 is an inappropriate remission endpoint because many patients still have residual swollen joints which are drivers of joint damage.[6 7] Moreover, acute phase reactants are highly weighted in the formula.[8 9] Consequently, DAS28 remission rates are exaggerated and misleading in the context of interleukin-6 (IL-6) inhibition by cytokine receptor inhibitors like tocilizumab or pathway inhibitors like Janus kinase inhibitors;[10 11] moreover, these issues cannot be overcome by lowering the threshold for the remission cutpoint.[5 12] Finally, since DAS28 includes an acute phase reactant, using it as an outcome to evaluate the role of C-reactive protein (CRP) cannot be seen as independent of CRP and, therefore, may be circular.

Equally importantly, we did not start by asking the question of the 'distribution of baseline… concentrations of…CRP' in relation to achieving DAS28 <2.6 or not, but rather asked the question, which CRP-levels those patients who achieved stringent remission at endpoint had at baseline and compared this with baseline CRP levels in patients who had other disease activity states at endpoint and not just non-remission. Thus, our research question, although covering similar aspects, tackled the data differently than other studies.

Figure 1 of our paper[1] shows a parallel reduction of levels of CRP and CDAI for rituximab (RTX) but not for tocilizumab (TCZ) where CRP-levels decrease much more dramatically than CDAI levels. As Pethoe-Schramm *et al* mention, this is an implication of some independence of the two variables, and also reiterates what is already known from the tocilizumab clinical trials[13 14] and our previous work[10] that on IL-6R blockade CRP may normalise independently of clinical improvement, which is neither the case for RTX nor methotrexate (MTX).

In Figure 2 of our paper the CIs indeed, overlap, but as indicated in the results and figure legend, the differences across the disease activity states were highly significant by Kruskal-Wallis test across the three disease activity groups for all variables and all treatment types. Importantly, however, while all other variables and all treatment types had the same direction of association across the various disease activity states, the direction was the reverse only for the CRP data in the TCZ treated population, while in RTX and MTX patients CRP association was again the same as seen with all other measures. Indeed, during the review process we were asked to show the results for other single variables and have chosen pain as an example, because pain, like CRP is not included in the CDAI. As expected and in line with the CDAI data, pain changes behaved opposite to CRP changes for TCZ but not RTX or MTX.

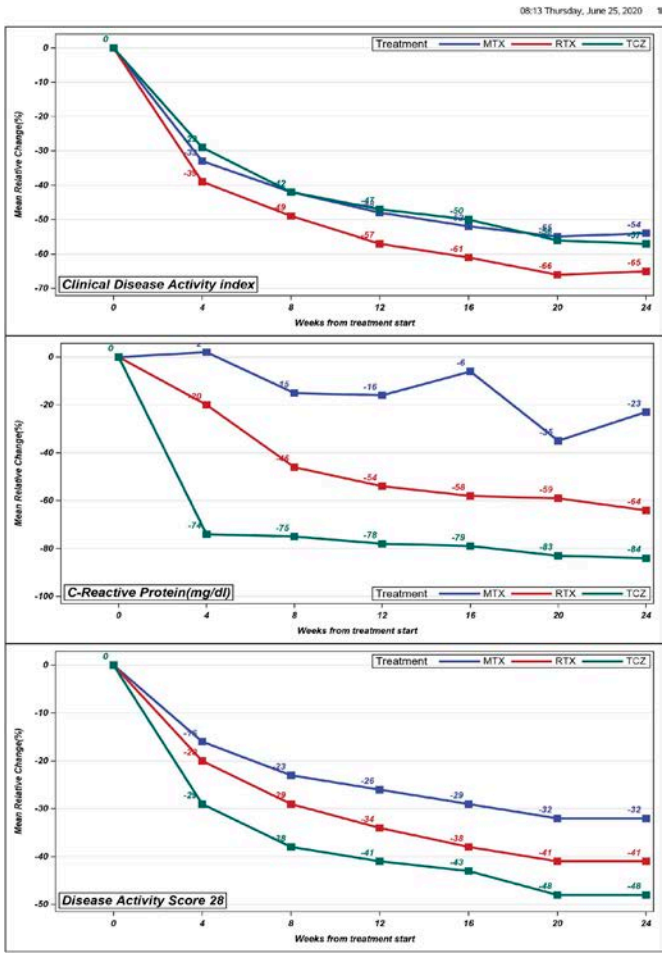

Figure 1 Changes of CDAI, CRP and DAS28 from baseline to 24 weeks for tocilizumab (TCZ), rituximab (RTX) and methotrexate (MTX). As can be seen, DAS28 changes parallel CRP changes for TCZ but not for MTX and RTX. This is not a comparative analysis of efficacy, but just to illustrate the differences of using different instruments. RTX and MTX come from the same trial in early rheumatoidarthritis patients and TCZ data come from a pool of three trials in patients with insufficient response to MTX, as detailed in the original paper.[1] Please note that for reasons of better clarity the scale of each panel is different. CDAI, Clinical Disease Activity Index; CRP, C-reactive protein; DAS28, disease activity score using 28 joints and erythrocyte sedimentation rate.

With respect to Figure 3 of our publication, let us please reiterate what this Figure has been developed for, namely to show the different behaviours of CRP levels in patients who reach different states at endpoint when being treated with TCZ versus other agents. In those reaching remission, TCZ led to a most dramatic and early change of CRP compared with other states and compared with other drugs (Figure 3 B, D, F in our paper).[1] This is in stark contrast to CDAI changes which were shown there in Figure 4. Just to reiterate: patients in remission start with the highest CRP levels and end with the lowest CRP levels when TCZ is used, while on RTX and MTX patients reaching remission have the lowest CRP level from beginning to end. And this is true for all drugs when the CDAI is used, thus, again, revealing the difference between CRP changes and clinical changes when TCZ is applied

We agree with Pethoe-Schramm *et al* that in Figure 5 CIs cross 1 which is a matter of power.[1] However, our analyses revealed consistently that patients treated with TCZ had the best odds of

achieving remission with high CRP levels compared with lower ones while this was not the case for RTX and MTX. We had determined that with increasingly higher definitions of 'elevated' CRP (eg, cutpoint of ≥4 mg/dL) TCZ efficacy increased relative to patient with not or less 'elevated' CRP levels, which was also confirmed in a sensitivity analysis from a separate trial (Figure S1).[1]

We would like to come back to the issue of using DAS28 or DAS28-based states as outcomes in trials of tocilizumab. In the FUNCTION trial TCZ monotherapy conveyed significant differences compared with MTX monotherapy when using DAS28 remission, but not CDAI, Simplified Disease Activity Index (SDAI) or even ACR responses,[15] and we have pointed out the fallacy of this score in a recent review.[16] To provide Pethoe-Schramm *et al* as well as the readers with some additional information why we feel the value of baseline CRP levels for TCZ treatment may have escaped previous investigations, we have now complemented the data of our paper with DAS28 changes over time. As depicted in figure 1 accompanying this response, we saw a similar pattern for DAS28 as for CRP, but not CDAI, with TCZ which was different from the data observed with RTX and especially MTX (Figure 1).

In summary, we fully agree with Pethoe-Schramm *et al* that rheumatologists should make their treatment decisions based on clinical disease activity (and, as exemplified again here, by using the CDAI). Indeed, regular *clinical* assessment is part and parcel of the treat-to-target and EULAR rheumatoid arthritis (RA) management recommendations,[17 18] where the use of ACR-EULAR remission criteria is also addressed. While on the group level of all patients enrolled in clinical trials of RA TCZ has comparable efficacy to other biologics,[19 20] our data suggest that those with the highest CRP values will fare even better on TCZ than they might do on other drugs. This is the essence and novelty of our findings, and a step into precision medicine to support treatment selection in clinical practice, which—although only weak overall—is ultimately shown for CRP here: positive association with later outcomes for one drug, and an inverse (negative) one for other drugs. Whether this finding also holds up in clinical practice or is just a result of a post-hoc analysis of clinical trial data, will have to be seen in future in prospective investigations.

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Comment on: 'EULAR recommendations for the management of psoriatic arthritis with pharmacological therapies: 2019 update' by Gossec *et al*

We read with great interest the published paper by Gossec *et al*[1] which provided updated European League Against Rheumatism (EULAR) recommendations for pharmacological therapies for psoriatic arthritis (PsA). In this full-scale, evidence-based guideline, the authors proclaimed 6 overarching principles and 12 recommendations for PsA. Recently, the field of pharmacotherapy for PsA has progressed rapidly and is replete with competing clinical trials working on the efficacy of plenty of drugs. However, in light of some evidence on therapeutic efficacy still being scarce today, there are a few points in this study that are worth mentioning.

First, for PsA with relevant skin involvement, the authors recommended methotrexate (MTX) as the standard treatment superior to other conventional synthetic disease-modifying antirheumatic drugs (csDMARDs). However, the evidence on the efficacy of MTX remains limited to date.[2] Furthermore, the medication trends for psoriasis in Asia seemed to be quite different from the EULAR recommendations. In our previous population-based study in Taiwan, we found that ciclosporin (CsA) was more commonly used for patients with psoriatic diseases in the past decade.[3] Likewise, an epidemiological study from Korea reported higher use of CsA than MTX for patients with psoriasis, and the use of CsA showed a 32.2% increase during the period of 2006–2015. The preference of CsA over MTX may be explained by the nature of CsA, which allows for easier monitoring and management of adverse effects.[4] It is noteworthy that previous studies from Japan revealed improved quality of life for patients with psoriasis after low-dose and short-term use of CsA.[5 6] Although CsA might pose unwanted side effects, nephrotoxicity caused by short-term use of CsA has been investigated to be reversible. Moreover, for patients with psoriasis, higher dose of CsA as induction therapy and lower dose of CsA as maintenance therapy have been reported to be a feasible way to control and diminish symptoms. The laboratory abnormalities found during induction therapy have been documented to return to normal ranges during maintenance therapy.[7] Considering the insufficient evidence for MTX to be prior to other csDMARDs, we suggest that CsA should have a different weight on the discussion.

Second, the EULAR recommendations classified PsA into oligoarticular and polyarticular joint involvement. However, a previous study pointed out that the activity states of oligoarticular PsA cannot be accurately evaluated without assessing full 66/68 joint counts. The Disease Activity States of the PsA score, which is calculated by a comprehensive overview of the 66/68 joint counts, has been proven to have high validity for clinical endpoints of PsA.[8] As a result, to prevent misclassification and make treatment strategy for PsA more robust, thoroughly running through 66/68 joint counts to evaluate active PsA instead of simply accounting for the affected joint counts is recommended.

Third, the authors provided the concept of cautious tapering of biologic disease-modifying antirheumatic drugs (bDMARDs) to the smallest effective dose when patients have reached sustained remission. Nonetheless, the article did not provide a valid algorithm for tapering down bDMARDs. A Denmark clinical trial published in 2019 displayed a tapering guidance for bDMARDs meanwhile maintaining stable disease activity for PsA.[9] In our opinion, the EULAR recommendations should have an introduction on the tapering strategy in an effort to help alleviate the burden on patients with PsA.

Last but not least, the EULAR recommendations undoubtedly provided the most detailed and multifaceted guideline for the treatment of PsA. However, we think the therapeutic concerns for elderly patients with PsA deserve some attention. Based on a recently published study, there is obvious discrepancy in disease severity between late-onset and early-onset PsA.[10] To date, evidence on pharmacological approaches to elderly patients with PsA is still lacking. Therefore, further research is needed to underpin therapeutic precautions and modification on the treatment dosage for elderly patients with PsA.

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Response to: 'Comment on: 'EULAR recommendations for the management of psoriatic arthritis with pharmacological therapies: 2019 update' by Gossec *et al*' by Wei *et al*

We thank Wei *et al*[1] for their correspondence on the recently published European League Against Rheumatism (EULAR) recommendations for the management of psoriatic arthritis (PsA).[2] The authors of this correspondence raise some interesting points.

The first point relates to the role of cyclosporine A (CsA) in the treatment of patients with psoriatic disease. The authors mention that this drug is often used in Asia, in particular in skin psoriasis. They suggest that CsA could be proposed for PsA. While CsA is recommended for the management of skin psoriasis,[3] EULAR's systematic literature searches did not reveal a convincing efficacy in PsA based on two small randomised controlled trials[4 5]; indeed, even a randomised trial on the addition of CsA in patients who had an insufficient response to methotrexate failed to meet its primary endpoint.[6] Therefore, CsA is not put forward in the EULAR recommendations for the management of PsA,[2] and these recommendations do not deal with patients who only have psoriasis.

The second point of the authors' comments relates to the definition of oligoarticular disease and, more specifically, the necessity for a full 66/68 joint count. We fully support using the 66/68 joint counts in PsA, as studies have shown that more limited joint counts may miss significant proportions of inflammation.[7] The authors mentioned the Disease Activity in Psoriatic Arthritis (DAPSA) score, which is also recommended in the updated EULAR recommendations and, indeed, uses the 66/68 counts.[8]

Wei *et al* support the new recommendation for tapering of biological DMARDs in patients reaching sustained remission, but suggest that an algorithm or a tapering guidance would be useful.[1 2] Currently, given the lack of data on this subject, it is difficult to propose formal tapering strategies.[4] In the absence of any data for PsA, Wei *et al* may be referred to the EULAR RA management recommendations[9] for a potential orientation, given that in RA tapering schedules have been studied.[10] Tapering should be further investigated in PsA. The last point regards elderly patients and we agree with the authors that research is warranted on the management of elderly patients with PsA.

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Association of subcutaneous belimumab and long-term antimalarial treatment reduces antiphospholipid antibodies levels in systemic lupus erythematosus: post-hoc analysis of a randomised placebo-controlled trial—comment on: 'Effect of belimumab treatment on antiphospholipid antibody levels: post-hoc analysis based on two randomised placebo-controlled trials in systemic lupus erythematosus' by Chatzidionysiou *et al*

We read with interest the article by Chatzidionysiou *et al*,[1] which reported a post-hoc analysis of two trials on intravenous belimumab in systemic lupus erythematosus (SLE), showing a significant reduction of antiphospholipid antibodies (aPL) only in patients cotreated with antimalarials.

However, this post-hoc analysis focused on anticardiolipin antibodies (aCL) only, with no data on anti-β2-glycoprotein I (antiβ2GPI) antibodies, which display the best diagnostic/prognostic value in antiphospholipid antibodies syndrome (APS). Moreover, although long-term use of antimalarials was reported to reduce aPL levels by itself,[2] no information on the duration of antimalarials cotreatment was reported.[1] Interestingly, treatment with belimumab was recently reported to induce aCL and antiβ2GPI disappearance in a small case series of patients with SLE-associated APS.[3] We also reported a significant reduction in aCL and antiβ2GPI titres in 12 patients with SLE on belimumab, either alone or associated with antimalarials.[4]

This study aimed to assess the effect of subcutaneous belimumab (200 mg/week) versus placebo, in association with standard therapy +/- antimalarials, on aCL and antiβ2GPI titers in

a)

	No antimalarials co-treatment			Antimalarials co-treatment		
	Belimumab (N=62)	Placebo (N=28)	p-value	Belimumab + Antimalarials (N=112)	Placebo + Antimalarials (N=47)	p-value
aCL IgA, n	5	3		12	4	
Median	-40.0	8.6	0.136	-19.9	0.0	0.504
aCL IgG, n	8	4		22	8	
Median	-21.9	43.8	0.270	-13.3	-8.6	0.573
aCL IgM, n	15	6		27	11	
Median	-26.7	34.9	0.259	-26.7	-12.5	**0.026**
antiβ2GPI IgA, n	28	10		52	19	
Median	-23.4	-15.5	0.304	-25.7	0.0	**0.002**
antiβ2GPI IgG, n	5	5		8	6	
Median	-31.6	6.5	0.403	9.2	-16.3	0.847
antiβ2GPI IgM, n	9	4		17	7	
Median	0.0	-42.4	0.485	-52.4	-16.7	0.525
	Duration of antimalarials treatment <12 months			Duration of antimalarials treatment ≥12 months		
	Belimumab + Antimalarials (N=43)	Placebo + Antimalarials (N=16)	p-value	Belimumab + Antimalarials (N=69)	Placebo + Antimalarials (N=31)	p-value
aCL IgA, n	2	2		10	2	
Median	32.4	-12.6	0.699	-26.5	0.0	0.160
aCL IgG, n	5	2		17	6	
Median	6.3	60.4	1.000	-17.1	-8.6	0.420
aCL IgM, n	9	6		18	5	
Median	-21.4	-19.6	0.193	-26.9	20.7	**0.023**
antiβ2GPI IgA, n	16	6		36	13	
Median	-26.0	7.7	0.105	-25.7	0.0	**0.012**
antiβ2GPI IgG, n	0	0		8	4	
Median		21.2	---	9.2	-46.6	0.552
antiβ2GPI IgM, n	7	4		10	3	
Median	-60.5	-8.3	0.298	-26.9	-36.6	0.933

b)

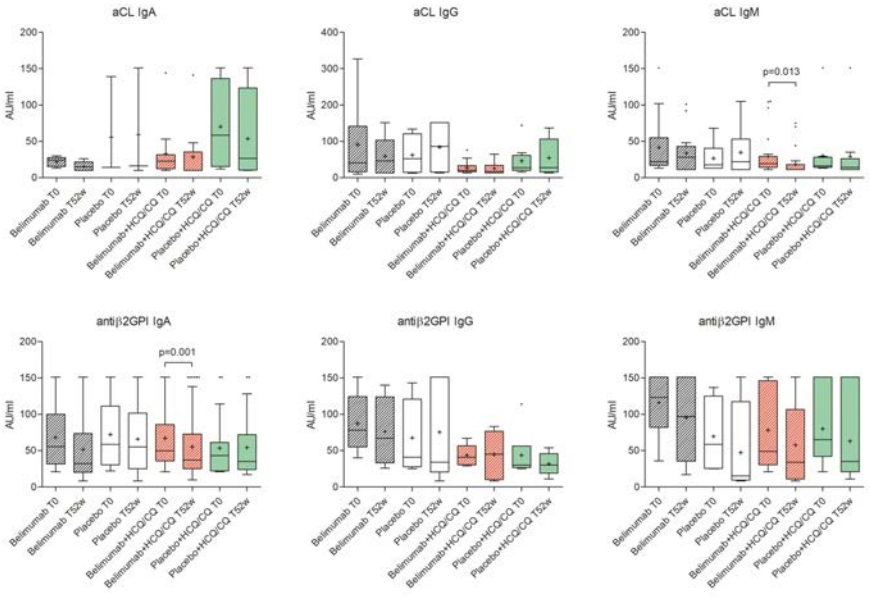

Figure 1 (A) Median per cent change in anticardiolipin antibodies (aCL) and anti-β2-glycoprotein I (antiβ2GPI) antibodies titers at 52 weeks of follow-up as compared with the baseline level in patients treated with belimumab or placebo, stratified according to the cotreatment with antimalarials hydroxychloroquine (HCQ) or chloroquine (CQ) and to the duration of antimalarials treatment. (B) Anticardiolipin and antiβ2GPI antibodies titres in patients treated with belimumab or placebo, alone or associated with antimalarials, at baseline and at 52 weeks of follow-up.

patients with SLE, using data from the randomised controlled trial "A study of belimumab administered subcutaneously in subjects with systemic lupus erythematosus (SLE)" (BLISS-SC).[5]

The median values and median per cent change from the baseline levels of IgG/IgM/IgA aCL and antiβ2GPI antibodies were evaluated at 24 and 52 weeks in the BLISS-SC trial[5] and compared between treatment arms by the Mann-Whitney test. Analyses were stratified according to the antimalarials cotreatment and its duration.

A total of 249 patients with SLE from the BLISS-SC trial tested positive for aPL using different commercial ELISA kits; 174 received belimumab and 75 placebo; antimalarials were coadministered in 112 and 47 patients, respectively.

A significant reduction in antiβ2GPI IgA titres was observed in the belimumab group as compared with the placebo one (median per cent change of −22.0 vs −4.4, p=0.012) at 24 weeks. A significantly greater median per cent change in the belimumab group was confirmed for antiβ2GPI IgA (−25.6 vs −4.8, p=0.002), and for aCL IgM (−26.7 vs −7.1, p=0.017) at 52 weeks (data not shown). No aPL disappearance was found in the treatment arms. When stratifying the analysis according to antimalarials cotreatment, we found no significant difference in aPL titers reduction between the belimumab and the placebo groups in patients not receiving antimalarials, neither at 24 weeks (data not shown) or 52 weeks (figure 1A,B). Conversely, cotreatment with antimalarials significantly reduced median levels and was associated with a significantly greater change in antiβ2GPI IgA levels in the belimumab +antimalarials group as compared with the placebo +antimalarials group at both 24 weeks (median per cent change of −22.1 vs −2.2,p=0.010) and 52 weeks (−25.7 vs −0.0; p=0.002). Comparable results were found in aCL IgM levels at 52 weeks (−26.7 vs −12.5, p=0.026) as well. Notably, reduction in antiβ2GPI IgA and in aCL IgM titers in the belimumab +antimalarials group was statistically significant only for patients treated with antimalarials for ≥12 months (figure 1A).

This post-hoc analysis suggests a beneficial synergistic effect of subcutaneous belimumab and antimalarials in reducing not only aCL, but also antiβ2GPI antibody levels. Interestingly, this effect was significant in patients on long-term antimalarials treatment only.

The main limitations of this analysis are the relatively small sample size, and the fact that only data related to two follow-up time points were available, thus, despite the presence of a control group, spontaneous aPL fluctuations over time cannot be excluded.

Nevertheless, aPL are associated with an increased risk of cardiovascular events, which represent the leading cause of mortality in SLE. Since there is evidence that IgA aPL display a predictive value for thrombosis in patients with SLE[6] the combined treatment with belimumab and antimalarials is a promising therapeutic tool that deserves further studies.

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Response to: 'Association of subcutaneous belimumab and long-term antimalarial treatment reduces antiphospholipid antibodies levels in systemic lupus erythematosus: post-hoc analysis of a randomised placebo-controlled trial–comment on: 'Effect of belimumab treatment on antiphospholipid antibody levels: post-hoc analysis based on two randomised placebo-controlled trials in systemic lupus erythematosus' by Chatzidionysiou *et al*' by Bettiol *et al*

We thank Dr Bettiol *et al*[1] for their interest in our article.[2] Dr Bettiol *et al* performed a post-hoc analysis on a different randomised controlled trial of subcutaneous belimumab in systemic lupus erythematosus (SLE). The authors evaluated the effect of treatment with belimumab on the levels of anticardiolipin antibodies (aCL) (IgG, IgM and IgA) but also of anti-β2-glycoprotein I (antiβ2GPI) antibodies. The latter was unfortunately not available in BLISS-76 and BLISS-52 trials, as acknowledged in the limitations of our post-hoc analysis. The authors have also extended our observation about the effect of co-treatment with antimalarials on antiphospholipid antibody (aPL) titres in the belimumab versus the placebo group, by examining the duration of their use. We agree with the authors that the duration of antimalarials is of high clinical significance. In addition to previous retrospective studies,[3] we have recently shown in a pilot randomised controlled trial comparing hydroxychloroquine (HCQ) plus standard care versus standard care in patients with primary antiphospholipid syndrome that the long-term HCQ use was associated with a significant decrease in aPL titres over an average 2.6-year follow-up.[4] We had also previously shown that the duration of HCQ, and not just the use of HCQ, had a protective role against thrombosis in both aPL-positive and aPL-negative patients with SLE.[5]

Regarding the effect on aPL levels, Dr Bettiol *et al* report a significant median per cent change in the IgM aCL and IgA antiβ2GPI levels in the belimumab versus placebo group only in the subgroup of patients with concomitant antimalarials treatment and not in the overall comparison between the entire belimumab and placebo groups. No significant effect on aCL or antiβ2GPI titres was observed in patients not receiving antimalarials from the belimumab and the placebo groups, supporting the predominant role of antimalarials in aPL levels reduction in accordance to our results. In our post-hoc analysis, we did not find any significant overall effect of belimumab treatment on the titres of aCL antibodies, apart from a significant effect of belimumab versus placebo on IgG and IgA aCL titres over time only in the subgroup of patients treated with antimalarials.

As the authors acknowledge, the number of patients is small and therefore the risk of type II error is present. In many subgroups the number of patients was <10, which makes the application of statistical methodology very difficult. Additionally, since we know that aCL fluctuate with time, it is unlikely to be able to draw safe conclusions when only two measurements at two different time points are available. In our study, we undertook a longitudinal data analysis so as to include multiple time points. Finally, no adjustment for potential confounders was performed in the data analysis by Dr Bettiol *et al* in contrast with our analysis.

Despite the limitations, these results add a valuable information about the potential effect of belimumab, especially in combination with antimalarials, in reducing aPL levels in SLE patients. In both our study and in the study by Dr Bettiol *et al*, a reduction of the titres of some isotypes of aPL (especially of IgA type) was seen in the belimumab + antimalarials group, but no clear overall effect was seen. The independent role of belimumab on aPL titre reduction, and the clinical significance of its effect on IgA isotype of aPL, needs to be further evaluated in larger, long-term prospective studies.

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Can telerheumatology improve rheumatic and musculoskeletal disease service delivery in sub-Saharan Africa?

We note with great interest the paper by Costa *et al*,[1] who submits that telemedicine has emerged as a key tool for managing psoriatic arthritis (PsA) among patients in Italy during the COVID-19 pandemic. The WHO has emphasised that universal health coverage cannot be achieved without the support of e-health of which telemedicine is a subset.[2] In sub-Saharan Africa, there is a dearth of rheumatologists. Rheumatology services are mostly centred in urban tertiary academic hospitals despite the greater rheumatic and musculoskeletal disease (RMD) burden in rural communities.[3] In an attempt to link technology and healthcare in the region, innovative tools for electronic data capture and clinical decision support have been developed with varying degrees of success.[4 5]

Telemedicine (also used interchangeably with telehealth) can be defined as the use of medical information that is exchanged from one site to another through electronic communication to improve a patient's health.[6] It has wide use in radiology, dermatology and psychiatry. Its usage in specialties like rheumatology (telerheumatology) appears to be increasing. Different telemedicine services exist. These include teleconsultation (synchronous or asynchronous), remote patient monitoring, as well as tele-education (online continuing medical education). Key advantages include convenience, improved care access, shorter waiting times and long-term cost savings, among others. However, potential limitations such as impersonal interaction, technology requirements, data privacy concerns, absent/poorly defined regulations, restricted medical licensure and insurance coverage have been noted.[2 7]

In sub-Saharan Africa, issues such as suboptimal internet access, poor infrastructure, unstable power supply, absent regulation and competing health needs raise further challenges, especially in rural areas. Notwithstanding, telerheumatology can be of practical benefit even in resource-constrained settings as demonstrated in Iran, where roughly 4800 patients were treated remotely by a US-based rheumatologist over 5 years using Skype—a videoconferencing tool.[8] This can be replicated in sub-Saharan Africa, where the relatively few rheumatologists at tertiary academic centres can collaborate with primary care physicians or nurses who will act as remote patient presenters during teleconsultations. The latter will receive in-person or online training in basic rheumatic diseases, patient evaluation, musculoskeletal examination and minor procedures. Apart from providing an opportunity for continuing medical education, it will enhance 'brain gain' for the continent.

In Nigeria, over the last 5 years, rheumatology tele-education has enhanced specialist training via the web-based European League Against Rheumatism (EULAR) online rheumatology course which is compulsory for all trainees. As residency training has slowed during the pandemic, the commencement of once-weekly nationwide interactive teleconferencing trainee presentations using Zoom has been quite beneficial. Free applications like WhatsApp are quite popular with African Smartphone and tablet users, permitting patients and providers to communicate via video or chats. With a reduction in hospital visits from COVID-19, patients now send their test results or images directly to their doctors or a hospital platform via a chat application or email. E-consults are also done similarly between rheumatologists and their other colleagues in a bid to protect patients and staff.

Telerheumatology has also been suggested as a triage tool to identify patients requiring in-person visits. For virtual consultations to succeed, proper patient selection is essential with better benefits for those with an established diagnosis, stable disease and the need for screening before an in-person visit. This was demonstrated by Costa *et al*[1] with their choice of patients with PsA in Italy. It may be less ideal for those who have a flare, need a procedure or have complex diseases.[9] For our setting, rheumatic conditions such as inflammatory arthritis, degenerative arthritis and musculoskeletal pain can be assessed using telemedicine, whereas complex active systemic diseases like lupus and vasculitis are better evaluated and treated in the hospital first. Stable lupus and vasculitis can subsequently have virtual follow-up appointments between spread-out face-to-face visits.

Multisectoral collaboration is essential between national rheumatology associations, medical regulatory authorities, insurance companies and other relevant stakeholders to develop regulatory guidelines and legislation for telerheumatology practice.[2] In this regard, South Africa is ahead of most African countries regarding telemedicine regulations, although significant hurdles remain.[7] Establishment of regulatory frameworks will address ethical issues such as confidentiality, as well as administrative concerns such as licensure, legal liability, insurance and data security. Liability and jurisdiction should remain with the referring doctors even after specialist consultation especially in cross-border consultations.[7] Regional licensure such as that of the West African College of Physicians will be ideal for cross border practice across member countries. Data encryption and security should be developed with future capabilities for linkage to electronic medical records. Overall, these measures will not only encourage provider participation but boost patient and public confidence in telerheumatology.

To conclude, the benefits of telerheumatology must be balanced by recognising its limits with an adjunctive role to usual care advocated over outright replacement. Its introduction should be gradual, following frequent audits including feedback from patients and providers.[10] Despite the various challenges faced so far, the current pandemic has brought rheumatology practice to the forefront globally. Beyond COVID-19, telerheumatology has the potentials to improve RMD service delivery across Africa if properly harnessed.

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Response to: 'Can telerheumatology improve rheumatic and musculoskeletal disease service delivery in sub-Saharan Africa?' by Akpabio *et al*

The letter by Akpabio and colleagues[1] raises an important question and describes an interesting scenario related to their geographical area. However, this scenario can easily be applied to several other experiences. In fact, the situation described by Akpabio and colleagues[1] with regard to rheumatological centres being located in great urban areas as compared with the shortage of these specialists within their periphery is quite frequent.

The impact of the COVID-19 pandemic on everyday practice gives us an opportunity to speed up the development of the process already known as telemedicine, which may be an attempt to correct some of these disparities.

The authors of the letter indicate many and relevant suggestions which we find very adequate and deserve immediate consideration. In particular, during this phase, we feel that it is necessary to manage this development. What can this mean? First of all, it should be outlined that telemedicine does not mean reduced need for specialists. Each one of us has experienced the burden of this approach. Telemedicine, as compared with the traditional inperson medical consultation, means greater effort in terms of more complex visit planning, of time spent, of organisation (detailed patient charts and imaging adequately saved and readily available), of mental engagement (need for greater effort in attention) and finally in terms of responsibility.

Dr Akpabio and coworkers outlined the dearth of rheumatologists in sub-Saharan Africa.[1] This is in line with several evidence from other countries where, despite the rising number of rheumatology fellowship programmes, the presumed need for rheumatology personnel expected by 2030 will not be satisfied.[2]

These data are also in line with those from Southern Italy regions such as Campania, where rheumatologists are lacking both in the peripheral areas and city clinics and hospitals. Already in 2019, the Italian Society for Rheumatology outlined that the number of physicians in Italy to be trained as specialists in rheumatology is inadequate and has to be defined on the basis of care needs and of the number of patients with rheumatic diseases, which in Italy is about nine million.[3]

In more detail, in Campania, despite the increase in medical students in recent years, the number of rheumatology fellowship programmes remains insufficient.[3] For example, in our medical school (University of Naples Federico II), over 300 medical students start a bachelor's programme in medicine each year and only two rheumatology fellowship positions (0.66%) are provided yearly.

The COVID-19 pandemic has highlighted the need for more specialists in rheumatology for two further reasons: first, more rheumatologists are required due to increasing physician turnover, and as said for implementing and improving both telemedicine and inperson visits.

In addition, as COVID-19 vaccination is planned, increasing the number of rheumatologists could be essential for the management of antirheumatic therapies in severe COVID-19 cases experiencing cytokine storm conditions, in collaboration with the first medical line involved in the pandemic.[3 4]

Dr Akpabio and coworkers have also highlighted how rheumatology services are mostly centred in urban tertiary academic hospitals.[1] Telehealth has to be focused on providing care, and the use of telemedicine to support long-distance or the practical impossibility of inperson care has increased strongly in parallel with the COVID-19 pandemic.[5–7]

Each Italian region, including Campania, has tried to implement remote assistance systems above all to manage patients with chronic and rare diseases and to guarantee continuity of care even remotely—in some cases with telephone instant messaging or email-based and video consultations and in other cases with the help of ad hoc platforms.

Telemedicine and virtual software have decreased emergency room visits, safeguarding healthcare resources and decreasing the spread of COVID-19 by remotely treating patients during the COVID-19 pandemic.[5 6]

On the other hand, the current focus of healthcare systems on the COVID-19 pandemic, the non-availability of telemedicine in several places and delayed infusions have seriously reduced the quality of care for chronic rheumatic diseases, especially for severe ones that need strict monitoring.[8]

The COVID-19 pandemic has dramatically changed several aspects of rheumatology and of other branches of care. We think that in this era there is a need more than ever of experienced rheumatologists to improve telemanagement and face-to-face management of rheumatic and autoimmune diseases and for the management of patients with COVID-19.

Finally, this innovative approach can be critical in the general effort aimed at early recognition and treatment of rheumatic diseases. It could facilitate the identification of adequate clinical profiles that require such an approach. Identification and description of these profiles could be implemented with the aim of using them for telemedicine consultation. For example, we could easily imagine such an application in the field of osteoporosis. The clinical profiles already identified for drug reimbursement in primary and secondary osteoporosis by the Italian Agency for Drugs could easily represent a template to generate, in analogy, similar clinical profiles deserving early treatment in other rheumatic diseases.[9]

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Impact of COVID-19 pandemic on hospitalisation of patients with systemic lupus erythematosus (SLE): report from a tertiary hospital during the peak of the pandemic

We read with great interest the article by Mathian *et al*[1] who first described COVID-19 in patients with systemic lupus erythematosus (SLE). Subsequent reports[2 3] focused on the clinical course of COVID-19 among patients with SLE. We were interested in the impact of COVID-19 pandemic on hospitalisation of patients with SLE.

The first case of COVID-19 was reported in Malaysia on 25 January 2020. Cases spiked in early March 2020 until Malaysia recorded the highest cumulative number of confirmed COVID-19 infections in South-East Asia. Malaysia instituted lockdown from 18 March 2020 until 9 June 2020 (a 12-week period) as a public health measure to curb the pandemic.[4]

Recent studies[5 6] have reported increased mortality in out-of-hospital acute coronary syndromes, not fully explained by COVID-19 cases alone, and potentially related to the patients' reluctance to seek medical care out of fear of the infection threat. Bromage *et al*[7] reported incident acute heart failure hospitalisation significantly declined in their centre during the COVID-19 pandemic, but hospitalised patients had more severe symptoms at admission. Monti *et al*[8] reported higher percentage of irreversible bilateral visual loss due to giant cell arteritis induced by delayed referral during the COVID-19 pandemic. Our aim is to examine the impact of COVID-19 pandemic on SLE hospitalisation rates, clinical characteristics and management of patients admitted to a tertiary hospital during the peak of the pandemic.

We performed a detailed comparison of patients hospitalised during the lockdown (from 18 March 2020 to 9 June 2020) period and patients presenting in the same period in 2019 with respect to clinical characteristics and management during the index admission. Patient demographics, disease history and medication lists were obtained from hospitalisation and clinic visit notes. The primary cause for hospitalisation was determined by a physician based on review of hospitalisation records. In-hospital morbidity was determined by the length of hospitalisation and intensive care unit (ICU) requirement.

There were a total of 18 patients with SLE hospitalised during the lockdown period and six of them were newly diagnosed with SLE as illustrated in table 1. In contrast, the total SLE hospitalisations during the same period in 2019 were 52 admissions by 34 patients with SLE. We found a decline (65.4%) in SLE hospitalisation rate in our centre during the COVID-19 pandemic. There were increased numbers of new cases (6 vs 2) during the lockdown period. Five out of six new cases presented late to our hospital, during the eighth week of lockdown period. Among the six new cases, two patients had lupus myocarditis and one patient had severe lupus pancreatitis which required intravenous methylprednisolone, cyclophosphamide and immunoglobulin. Mean Systemic Lupus Erythematosus Disease Activity Index among patients with active lupus was higher (13 vs 10) during the COVID-19 pandemic as compared with the previous year.

The most common reason for hospitalisation was disease flare (38.9%) where haematological and mucocutaneous flares were the most frequent manifestations. Infection (22.2%) was the next most common cause. Only one patient with lupus had COVID-19 infection. There was no hospitalisation for renal biopsy and haemodialysis-related training, as compared with previous year. There was increased ICU admission during this period (4 vs 2). Of the 18 hospitalisations, there were 3 (16.7%) deaths reported. One died of severe pulmonary tuberculosis and two died of overwhelming sepsis. These three deaths occurred among our pre-existing patients with lupus. No death was reported in the same period of previous year.

Table 1 Clinical characteristics of patients with SLE by year of presentation

	Previous year period (18 March 2019 to 9 June 2019)	COVID-19 period (18 March 2020 to 9 June 2020)
Age (years), mean±SD	34±15	38±11
Female, n (%)	30 (88)	16 (89)
Total admission	52	18
Total patients	34	18
New case	2	6
Death	0	3
Length of stay, mean±SD	8.81±11	8.39±6
ICU stay	2	4
Clinical presentation		
Flare	20 (38.5%)	7 (38.9%)
Flare and infection	6 (11.5%)	3 (16.7%)
Infection	11 (21.2%)	4 (22.2%)
Others	15 (28.8%)	4 (22.2%)
Renal biopsy, n (%)	7 (13.5%)	0
Haemodialysis training, n (%)	9 (17.3)	0
Type of flare, n		
Renal	21	3
Mucocutaneous	10	8
Haematological	5	9
Serositis	1	4
Arthritis	0	2
Neurological	1	0
SLEDAI, mean±SD	10±5.6	13±8.9

ICU, intensive care unit; SLE, systemic lupus erythematosus; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.

This report highlights that SLE hospitalisation rate declined during the COVID-19 pandemic, but hospitalised patients had more severe symptoms needing more intensive treatment. Active disease and infection remain the main causes of admission in patients with SLE. Pattern of hospitalisation among our patient with lupus was consistent with other study,[9] where disease flare and infection contributed the main reason of hospitalisation. Our data suggest that patients with SLE have avoided coming to hospital. The national lockdown and social distancing restrictions may have reduced respiratory tract infections, which are a common trigger for SLE flare. Mortality rate was higher because of the delayed presentation during COVID-19 pandemic.

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Response to 'Impact of COVID-19 pandemic on hospitalisation of patients with systemic lupus erythematosus (SLE): report from a tertiary hospital during the peak of the pandemic' by Chuah *et al*

We thank Chuah *et al*[1] for their interest in our study reporting on the course of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) disease 2019 (COVID-19) in a case series of patients with systemic lupus erythematosus (SLE) under long-term treatment with hydroxychloroquine.[2] Chuah *et al* point to an indirect consequence of the COVID-19 pandemic, the reluctance of patients to come to the hospital because of the fear to contract COVID-19 that is reinforced by the awareness of the contagious nature of SARS-CoV-2. The experience of Chuah *et al* is instructive in this regard. The authors observed a decline of 65.4% in the SLE hospitalisation rate in their medical centre during the containment period, as compared with the same period in 2019. Moreover, five out of the six new patients with SLE presented to the hospital long after the first clinical manifestations. This finding should be put into perspective with the increase in the number of in-hospital deaths of patients with SLE related to infections apparently independent of SARS-CoV-2, which suggests a harmful increase in the diagnostic delay and/or in the medical care of these patients. We would like to suggest that the authors do not limit their comparison with the number of hospitalisations in 2019 but rather extend their study to the previous years to give more weight to their observations, given the important statistical variability of small sample size. We also do acknowledge that our case series does not provide any information in this area.

Since the start of the pandemic, several other issues have emerged regarding patients with SLE. The first difficulty was the shortage, or the fear of shortage, of antimalarial drugs, commonly used in the treatment of these patients, due to their off-label use to treat COVID-19.[3 4] However, given that these drugs have not yet been shown to be effective against SARS-CoV-2 infection, their prescription rate will most likely decrease and patients with SLE will again have unrestricted access to antimalarial medication for continued treatment of their disease. The second problem for patients with SLE is the question whether they are more susceptible to infection with SARS-CoV-2, and, if infected, whether they progress to a more severe form of the disease with a poorer outcome. Although the prevalence of confirmed or suspected SARS-CoV-2 infection has been reported to range from 4% to 8% in patients with SLE in regions severely impacted by the pandemic such as Northern Italy, Belgium and New York City,[5–7] it is almost impossible to proceed to a reliable comparison with the prevalence of the infection in the general population. Yet, studies of small or larger case series of patients with SLE and COVID-19 have suggested that associated comorbidities such as arterial hypertension, diabetes, chronic kidney disease, chronic obstructive pulmonary disease, congestive heart failure and obesity might be risk factors for progression to severe form of COVID-19, similar to what has been observed in the general population.[2 8–11] However, whether patients with SLE on glucocorticoids and/or immunosuppressants are at an increased risk for hospital admission during the course of SARS-CoV-2 infection, as has been suggested in immune-mediated inflammatory disease in general,[12–14] is unclear to date and further studies are needed to identify additional specific risk factors for severe COVID-19 in SLE. Third, the pursuit of individualised clinical and laboratory monitoring, in addition to the renewal of drug prescriptions, has been challenged in many countries by the population confinement, as well as the restructuring of their usual medical team and/or facilities toward the care of patients with COVID-19. It is probable that the use of telemedicine, as pointed out by several authors, has made it possible to not considerably disrupt the monitoring of disease and consequently prevent and/or detect certain complications for chronic patients already in the outpatient circuit.[15 16] However, teleconsultation is of limited value in terms of laboratory screening for, often clinically asymptomatic, abnormalities, such as proteinuria, elevated serum creatinine, thrombocytopenia and anaemia, all of which are common manifestations of SLE. Teleconsultation is also inadequate for new patients and for the diagnosis of acute events such as cardiovascular or infectious complications for which urgent treatment is often needed. Unfortunately, these complications are very frequent during SLE and it is important for medical teams to reflect on this issue. With respect to home proteinuria screening, the use of urine dipsticks may be practical value. The improvement of rapid and direct lines of communication between patients and physicians that, in the event of unusual symptoms, will allow the latter to convince reluctant patients to be hospitalised when the medical situation requires it, is also probably one of the solutions to these recurrent problems.

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Comment on Ye *et al* 'Presence of respiratory failure of COVID-19 infection in rheumatism patients in Wuhan, China'

We read with great interest the article by Ye et *al*,[1] which reported the clinical features of COVID-19 in patients with rheumatic diseases, most notably that respiratory failure developed more commonly in patients with rheumatic disease infected with COVID-19 than those without rheumatic disease (38% vs 10%, p<0.001). The authors collected data from a total of 2326 patients with COVID-19 from 13 January to 15 March 2020, including 21 cases in combination with rheumatic disease, and conducted a retrospective case series study of their laboratory indices. However, some conclusions and findings in the study need to be further clarified.

First, the sample size of patients with rheumatic disease infected with COVID-19 was only 21—too small to extrapolate results with statistical confidence. Rheumatic diseases often affect the lungs, which can cause further damage to the respiratory system during flares and this has been reported to lead to respiratory failure.[2] The COVID-19 infection can also result in lung disease progression,[3] often culminating in respiratory failure. Therefore, when assessing whether patients with rheumatic diseases and COVID-19 are more prone to respiratory failure, it is necessary to first determine whether any of the study patients had a history of lung disease, for example, interstitial lung disease.

Second, the different drugs used to treat rheumatic diseases—including glucocorticoids, antimalarial drugs, non-steroidal anti-inflammatory drugs and disease-modifying antirheumatic drugs (DMARDs)—have different effects on the condition of patients with rheumatic disease infected with COVID-19. Previous studies have suggested that long-term use of glucocorticoids may increase the risk of both serious and opportunistic infections.[4] Sharmeen *et al*[5] reported that various DMARDs or biological agents may affect the disease course differently. Notably, taking hydroxychloroquine, tumour necrosis factor antagonists or tocilizumab may result in the patient presenting with a mild viral illness, whereas rituximab or secukinumab could possibly worsen the disease. Meanwhile, Borba *et al*[6] reported that the use of high doses of hydroxychloroquine may present potential safety issues, which is not conducive to the treatment of COVID-19 infection. In Ye's study, patients with rheumatic diseases include: rheumatoid arthritis, systemic lupus erythematosus, primary Sjögren's syndrome and so on. The types and dosages of drugs taken by these patients are different, and the effects on diseases after COVID-19 infection are also different. This indicates that the discrepancy in the drugs used by patients with rheumatic disease may also lead to different presentation of respiratory failure.

Lastly, figure 3 in Ye's study showed several laboratory indices, but not all 21 patients with rheumatic disease infected with COVID-19 had results for every test. This should be noted and the estimated value and interpretation of the data percentage should be adjusted accordingly. Missing data due to current circumstances are understanding as patients may present an emergency condition which prevents indices from being obtained in time, or clinicians may not see the need for certain tests due to patients' presentation.

For the above-mentioned reasons, it might be too early to draw conclusions regarding the features of COVID-19 in patients with rheumatic diseases. We recommend that future studies increase the sample size of patients with rheumatic disease, and take into account both the patients' disease activity level and the pharmacological treatments used. This will allow a thorough assessment of the clinical features of patients with rheumatic disease infected with COVID-19.

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Response to: 'Comment on Ye *et al* 'Presence of respiratory failure of COVID-19 infection in patients with rheumatism in Wuhan, China" by Chen *et al*

We appreciate the valuable comments from Chen *et al* on our recent study about the clinical characteristics of COVID-19 cases with rheumatic disease.[1] They highlight an important point about the sample size of patients with rheumatism infected with COVID-19 in our study. We agree that the sample size was relatively small, although we have included all such cases in Tongji Hospital, the hospital that admitted the most patients with COVID-19 in China. We acknowledged this limitation in the discussion of the paper.[2] With the urgent pandemic of COVID-19, the aim of this study is to provide a timely report to present the clinical features of patients with rheumatism infected with COVID-19. The clinical manifestation of COVID-19 cases with rheumatic disease in Wuhan, the first COVID-19 epicentre in the world, may provide important information for disease management in patients with rheumatic disease in other areas of the world. In our report, we noticed that respiratory failure was more common in patients with rheumatism (38% vs 10%, p<0.001) infected with COVID-19, although the duration of hospital stay and mortality were similar between patients with rheumatic disease and those without rheumatic disease.[2] These results were confirmed by subsequent studies in other areas of the world.[3 4] An analysis of COVID-19 cases in Massachusetts, USA showed that there were higher rates of intensive care admission and mechanical ventilation, but not mortality, in patients with rheumatism infected with COVID-19, compared with those without rheumatic disease (48% vs 18%; OR: 3.11, 95% CI: 1.07 to 9.05, p=0.01).[3] Fredi *et al* also reported that patients with rheumatism infected with COVID-19 in northern Italy tend to be treated more often with high-dose glucocorticoids (65% vs 48%, p=0.14) and tocilizumab (23% vs 18%, p=0.55), due to the 'worsening of respiratory condition'.[4]

Regarding the effects of antirheumatic drugs, we agree that different disease and different medication may have different effects on disease condition. Due to the limited sample size, we did not subcategorise the patients into different groups based on different rheumatic disease or use of different disease-modifying antirheumatic drugs (DMARDs) when analysing some parameters. Although the use of high-dose glucocorticoids and chloroquine/hydroxychloroquine as a therapeutic approach in patients with COVID-19 is controversial, whether their use in patients with rheumatic disease as an antirheumatic medication (usually at lower doses and in a long-term manner) could affect COVID-19 susceptibility and severity remains elusive. By including 600 cases with rheumatic disease and COVID-19 from 40 countries, Gianfrancesco *et al* reported that use of tumour necrosis factor inhibitor was associated with a lower hospitalisation rate.[5] In contrast, use of non-steroidal anti-inflammatory drugs or conventional DMARDs was not associated with hospitalisation.[5] However, the effects of DMARDs use on other clinical characteristics of COVID-19 are not completely understood. We currently continue collecting data from other centres in Hubei province, aiming to increase the sample size and investigate the potential effects of DMARDs on COVID-19 using a larger dataset in Hubei, China.

Chen *et al* also raised an important point of missing values for some parameters in figure 3. We agree that the reasons for these missing data may due to the fact that clinicians may not see the need for certain tests or the patients' conditions may prevent certain tests. In Figure 3 of our study, the tests, such as white blood cell (WBC) count, lymphocyte count, neutrophil count, platelet count, haemoglobin, alanine aminotransferase, aspartate aminotransferase, direct and total bilirubin, creatinine, uric acid, activated partial thromboplastin time, prothrombin time, fibrinogen, high-sensitivity C-reactive protein and procalcitonin, had data from all patients. We found that leucopenia was rarely seen in patients with rheumatic disease (5%), despite the fact that lymphopenia was more common (57%). D'Silva *et al* reported similar findings in a recent study.[3] While WBC count in patients with rheumatism infected with COVID-19 was higher than that in those without rheumatic disease, lymphocyte count was similar in both the groups.[3] However, there were a few missing values in some tests including creatine kinase (CK, missing eight values) and cardiac troponin I (cTn-I, missing one value) in our study. We noticed in our study that the patients, who had highest level of CK (1248 U/L) and cTn-I (124.8 pg/mL), eventually passed away. An earlier report on patients with COVID-19 in Wuhan has indicated that deceased patients with COVID-19 had higher levels of cTn-I (22.2 vs 3.0 pg/mL, p<0.0001) and CK (39.0 vs 18.0 U/L, p<0.0001), compared with survivors.[6] These results suggest that clinicians should pay close attention to the injury of other organs. However, we agree that it requires further investigation to confirm the value of these parameters in determining the prognosis of COVID-19 in patients with rheumatism.

Due to the immunosuppressive effect of a number of antirheumatic drugs and the immune dysregulation underlying rheumatic diseases, patients with rheumatic disease have received great attention during the pandemic of COVID-19.[7] As the first epicentre of COVID-19 in the world, the clinical data of COVID-19 cases with rheumatic disease may provide first-hand information for the clinical practice of rheumatologists during the pandemic. With the rapid increase in the number of COVID-19 cases, there will be more investigations to supplement and revise our findings in patients with rheumatism infected with COVID-19.

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Clinical features of patients with rheumatic diseases and COVID-19 infection in Sarawak, Malaysia

We read with great interest the article by Ye *et al*[1] describing the clinical features and outcomes of patients with rheumatic diseases and COVID-19 in Wuhan, China. It concluded that length of hospital stay and mortality were similar between patients with rheumatic diseases and non-rheumatic groups, while respiratory failure was more common in patients with rheumatic diseases infected with COVID-19. D'Silva *et al*[2] and Zhao *et al*[3] subsequently highlighted the differences of clinical severity and outcomes in their respective cohorts of patients with rheumatic diseases and COVID-19. Fredi *et al*[4] presented data from northern Italy which supported an association of elderly age and the presence of comorbidities with a poor outcome of COVID-19 infection, rather than the type of rheumatic disease or background medications. The Global Rheumatology Alliance[5] has recently published data of characteristics associated with hospitalisation for COVID-19 among patients with rheumatic diseases. We would like to share the clinical course of COVID-19 among patients with rheumatic diseases in Sarawak.

We reviewed the medical records of all the patients with rheumatic diseases diagnosed with COVID-19. Sarawak recorded 569 cases of COVID-19 with 17 deaths as of 24 June 2020.[6] The confirmation of the diagnosis of COVID-19 was based on positive reverse transcriptase PCR from the nasopharyngeal swab that detected severe acute respiratory syndrome coronavirus 2 from a nasopharyngeal swab specimen. There were four patients with rheumatic diseases among the 569 patients with COVID-19 (0.7%). There were two systemic lupus erythematosus (SLE), one dermatomyositis and one scleroderma. Patient 1 had stable SLE treated with hydroxychloroquine and sulfasalazine. She also had hypertension and obesity and she was a smoker. She was diagnosed with severe pneumonia, ventilated and subsequently succumbed to the disease. She was diagnosed with COVID-19 posthumously. Patient 2 was also a patient with stable SLE on hydroxychloroquine and azathioprine, and she developed an SLE flare at admission. Patient 3 was 80 years old with stable polymyositis on azathioprine. She also had diabetes mellitus. Patient 4 was 72 years old with systemic sclerosis not on any immunosuppressant. Her comorbidities were lung fibrosis, pulmonary arterial hypertension and a caecal malignancy which was resected 2 years prior. She recovered from COVID-19 infection and was discharged home; however, during a follow-up call it was discovered that she had died at home. All patients with rheumatic diseases had a stable disease prior to COVID-19 infection, and none of the patients were on glucocorticoids. Table 1 shows the clinical features of the patients with rheumatic diseases and COVID-19 infection.

When we reviewed the data of the 17 COVID-19 deaths from Sarawak, there were more men (10 patients) and more patients were aged below 65 years (12 patients). The most common comorbidities present among the patients who died were hypertension (six patients) and diabetes mellitus (five patients). One patient had congestive heart failure and one patient had chronic kidney disease. There was only one patient with rheumatic disease among the 17 COVID-19 mortalities in Sarawak. Although most publications quoted elderly age as a major risk factor for COVID-19 mortality, we are unsure of the reason there were more younger patients in our COVID-19 mortality cohort.

In conclusion, data from Sarawak showed a low proportion of patients with rheumatic diseases among the COVID-19 cases mirroring the findings by other authors. These findings seem to support that the presence of rheumatic disease does not affect the susceptibility of COVID-19 infection or mortality. The risk factor for COVID-19 mortality is the presence of comorbidities. Flare of rheumatic disease is possible during the COVID-19 infection. As more data are collected, we hope to have a clearer picture of rheumatic diseases and COVID-19.

Table 1 Patients with rheumatic diseases and COVID-19 infection, n=4

Clinical features	Patients, n (%)
Age >65	2 (50)
Gender	
Female	4 (100)
Male	0
Rheumatological diagnosis	
Systemic lupus erythematosus	2 (50)
Polymyositis	1 (25)
Systemic sclerosis	1 (25)
Comorbidities	
Smoking	1 (25)
Obesity	1 (25)
Hypertension	1 (25)
Diabetes mellitus	1 (25)
Lung fibrosis and pulmonary arterial hypertension	1 (25)
Malignancy	1 (25)
Flare of rheumatological disease	
Flare	1 (25)
No flare	3 (75)
Background rheumatological treatment	
Glucocorticoids	0
Hydroxychloroquine	2 (50)
Azathioprine	2 (50)
Sulfasalazine	1 (25)
Clinical features of COVID-19 infection	
COVID-19 pneumonia	3 (75)
Supplemental oxygen via nasal cannula	2 (50)
Ventilation	1 (25)
Treatment of COVID-19	
Steroids	4 (100)
Hydroxychloroquine	4 (100)
Lopinavir/ritonavir	3 (75)
Outcome of COVID-19 infection	
Alive	2 (50)
Death	
Death from COVID-19	1 (25)
Death from underlying rheumatic disease	1 (25)

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Response to: 'Clinical features of patients with rheumatic diseases and COVID-19 infection in Sarawak, Malaysia' by Wan *et al*

We thank Dr. Wan and colleagues for their comments on our manuscript reporting the clinical features of COVID-19 pneumonia in patients with rheumatic disease.[1] Wan *et al* analysed 569 cases of COVID-19 in Sarawak, Malaysia and found 4 patients with rheumatic diseases (2 systemic lupus erythematosus, 1 dermatomyositis, and 1 scleroderma). Among these four patients with COVID-19 and rheumatic disease, they noted one case requiring ventilation (25%, 1/4) and one case developing an SLE flare at admission (25%, 1/4). These were in agreement with our findings that respiratory failure was more common in patients with COVID-19 with rheumatic disease (38% vs 10%, p<0.001) and some patients with COVID-19 and rheumatic disease may experience a flare of rheumatic disease during the clinical course of COVID-19 (19%, 4/21).[2]

Fredi *et al* and Wan *et al* raised an important question that whether the poor outcome and susceptibility of COVID-19 in patients with rheumatic disease are caused by the presence of rheumatic disease or the older age and comorbidities.[1 3] The overall effect of rheumatic disease on COVID-19 is complicated and both rheumatic condition and antirheumatic drugs may have potential roles in clinical course of COVID-19. Current evidence, including ours, showed that there were no significant differences in the duration of hospital stay and mortality between patients with rheumatic disease and those without rheumatic disease.[2–4] However, the presence of rheumatic disease may affect certain manifestation of COVID-19. As mentioned above, Wan's data indicate a considerable rate of respiratory failure (needs for ventilation), although the sample size was not large enough for statistical analysis. A recent report by D'Silva also confirmed that patients with COVID-19 with rheumatic disease in the greater Boston, Massachusetts area had higher rates of intensive care admission and mechanical ventilation compared with those without rheumatic disease (48% vs 18%; OR 3.11, 95% CI 1.07 to 9.05, p=0.01), although mortality was similar.[4] In the report of COVID-19 cases in northern Italy by Fredi *et al*, patients with rheumatic disease also had a higher, but not statistically significant, rate of high-dose glucocorticoids (65% vs 48%, p=0.14) and tocilizumab (23% vs 18%, p=0.55), which were used for the treatment of 'worsening of respiratory condition'.[3] Nevertheless, we agree that further investigations with larger sample size are required to assess the exact effects of rheumatic disease on COVID-19 outcome.

The COVID-19 Global Rheumatology Alliance has established a large database of global cases with COVID-19 and rheumatic disease.[5] Further analyses on those cases may provide a better understanding of the clinical manifestation of COVID-19 in patients with rheumatic disease.

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Should patients starting biologics be screened for COVID-19?

We read with great interest the European League against Rheumatism (EULAR) provisional guidelines regarding rheumatic and musculoskeletal disease management during the COVID-19 pandemic recently published by Landewé *et al*.[1] While the COVID-19 pandemic continues across the world, rheumatology care has been enormously impacted. In the strive to adapt, telemedicine and telehealth have taken a predominant role in our everyday practice. Additionally, our treatment schemes have changed and sometimes have been deferred. Initial questions from rheumatologists—such as 'are my patients with rheumatic diseases at higher risk of COVID-19?' and 'should I stop their anti-rheumatic treatment (biologic or not) during the COVID-19 pandemic?'—slowly get answered as evidence continues to accumulate.

However, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is here to stay for a long time, and a new question arises as outpatient rheumatology clinics and infusion centres are slowly reopened: should patients starting biologics be screened for COVID-19 too? Testing for SARS-CoV-2 before initiating biological therapy was not addressed in the recent guidelines published by the EULAR and the American College of Rheumatology for the management of adults with rheumatic diseases during the COVID-19 pandemic.[1 2]

Patients starting biological therapy are routinely screened for infections (testing for tuberculosis, HIV, hepatitis B and hepatitis C is a standard of care), and gastroenterologists have advocated for testing for SARS-CoV-2 before initiating biological therapy in patients with inflammatory bowel disease.[3]

So far, it appears the incidence or risk of contracting SARS-CoV-2 infection is similar between patients with rheumatic diseases and the general population.[1] Regarding COVID-19 complications, such as the risk of an intensive care admission or initiation of mechanical ventilation, evidence has been contradictory probably due to the heterogeneous samples, and different populations and treatments.[4]

The use of biological and targeted disease-modifying antirheumatic drugs (b/tsDMARDs) was associated with a lower rate of hospitalisation in the COVID-19 Global Rheumatology Alliance physician registry, which reported 600 cases of patients from 40 countries with rheumatic diseases and COVID-19 diagnosis.[5] Additionally, a study of 41 patients from Spain with rheumatic diseases treated with b/tsDMARDs did not find a higher risk of complications or mortality compared with the general population.[6] Whether these findings apply to those asymptomatic carriers or mildly symptomatic patients infected with COVID-19 in which biological therapy is being started remains unknown. We should analyse the current evidence carefully and recognise its limitations.

Advantages for testing include identifying asymptomatic carriers or mildly symptomatic patients for early isolation and vigilance and avoiding exposure of nurses administrating biological medication at home or personnel from infusion centres. Disadvantages include increased cost and lack of data regarding additional risk associated with b/tsDMARDs therapy. We believe that a pragmatic approach during these uncertain times is adequate and that screening for SARS-CoV-2 before biological therapy is reasonable.

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Response to: 'Should patients starting biologics be screened for COVID-19?' by Cardenas-de la Garza *et al*

Cardenas-de la Garza *et al*[1] ask a reasonable question that nowadays likely resonates in many rheumatological practices: "Should we screen our patients who start biologics for SARS-CoV-2?" As so often in medicine, the question is easily asked but is more difficult to answer.

First of all, screening implies the availability of a diagnostic test to be applied in a patient that does not have symptoms (yet). For SARS-CoV-2, we have two types of tests available: the PCR test, known as the 'swab-test', which proves the presence of the virus, and the antibody test, which proves the presence of antibodies against the virus.

The PCR test has as narrow window and is likely only positive for a while, when the patient is sick already or will get complaints soon. Asymptomatic carriership for SARS-CoV-2 is an arguable concept, if existent anyway. In addition, like every diagnostic test, the PCR test is not free of sensitivity and specificity problems. False negativity is a well-known issue, although manageable in a context of a patient with a typical clinical picture during an epidemic phase. The positive predictive value of the PCR test is likely very high during an epidemic phase, when many patients present with COVID-19 symptoms, but unknown when the epidemic is gone and the incidence is low.

Widely available and reliable antibody tests are lacking for the moment, and more importantly, we know far too little about the performance of these tests and about the consequences of a positive test for the patient. Does a positive test mean that you cannot start a biologic? Does it harm such a patient? Does a positive test indicate temporal or persistent immunity for SARS-CoV-2? What about cross-reaction with other coronaviruses?

We are of the opinion that we should learn a lot more before we can start thinking about such a delicate matter as screening. Screening may seem simple and straightforward, but it is awfully complicated and will lead both to false reassurance and unnecessary anxiety. For now, we strongly discourage screening for SARS-CoV-2 to be applied in patients with rheumatic and musculoskeletal diseases, whether or not they will start a biologic.

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Implications of SARS-CoV-2 infection for patients with rheumatic disease

We read with great interest the letter by Mathian *et al* in the *Annals of the Rheumatic Diseases*. In the letter, the authors reported that hydroxychloroquine (HCQ) did not protect patients with systemic lupus erythematosus (SLE) from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in the clinical treatment. We report similar results from our study of treatment of patients with SLE on HCQ. We wish to add supplemental evidence to some of the points not adequately addressed in Mathian *et al*'s[1] letter.

Mathian *et al* did not report the incidence rate of COVID-19 in patients with SLE. We agree with Mathian *et al*. that it is not practical to assess the actual incidence rate of SARS-CoV-2 in patients with SLE. However, we observed that during the outbreak, 6 (0.2%) cases had SLE in 3057 patients with COVID-19 who were admitted to Wuhan Huoshenshan Hospital (China). Taking that the prevalence of SLE is approximately 0.05%–0.1% in China, this may suggest that patients with SLE are susceptible to SARS-CoV-2 infection.[2 3] As HCQ is the first-line drug that will be prescribed to patients with SLE, our result confirmed the observations in Mathian *et al*'s letter that HCQ cannot prevent patients with SLE from SARS-CoV-2 infection.

The effect of myocardial damage in SARS-CoV-2-infected patients with SLE is another interesting observation represented in the letter by Mathian *et al*. Cardiac injury is frequent and often silent in patients with SLE.[4] However, it may cause cardiac arrest.[5] Notably, in our cohort, patient 2 had congenital heart

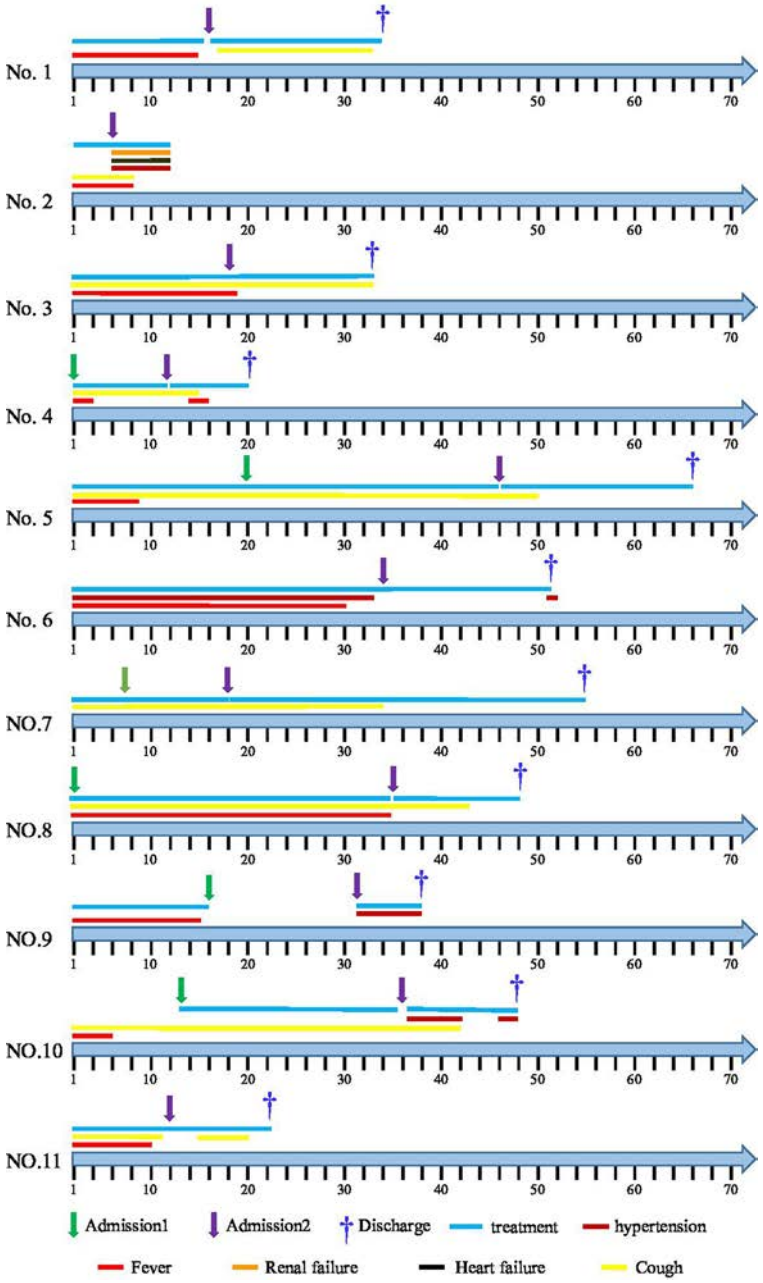

Figure 1 Timeline showing general and symptom onset, timing of hospital admission, timing of therapy and discharge of patients with systemic lupus erythematosus and rheumatoid arthritis.

Table 1 Characteristics of 11 COVID-19 patients with systemic lupus erythematosus and rheumatoid arthritis

	Patient 2	Patient 3	Patient 4	Patient 1	Patient 5	Patient 6	Patient 7	Patient 9	Patient 8	Patient 10	Patient 11
Age (years)	25	66	48	55	55	50	67	68	58	65	71
Sex	Male	Female	Female	Female	Female	Female	Female	Female	Female	Female	Female
Highest disease grade	Critical	Severe	Severe	Moderate	Moderate	Severe	Severe	Severe	Moderate	Moderate	Moderate
Disease history	SLE (>2 years); hypertension (1 year); congenital heart disease (15 years).	DM (>10 years); CRI (10 years); RA (>30 years); SLE (>20 years).	SLE (12 years); hypothyroidism (12 years).	SLE; LN.	SLE (13 years); hypothyroidism (3 years).	SLE (2 years).	RA (10 years); DM.	RA.	RA.	Hypertension (5 years); RA (5 years).	Hypertension (>20 years); RA (>10 years).
Initial symptom	Fatigue; fever (max, 38.8°C); cough.	Fever (max, 38.2°C); paroxysmal cough.	Fever (max, 39°C); occasional coughing.	Fever (max, 40°C); no cough.	Fever (max, 39°C); cough; fatigue.	Fever (max, 38°C); headache; dizziness; chest tightness.	Nausea; vomiting with panic; wheezing; a little cough.	Intermittent fever (max, 37.6°C); fatigue; anorexia; no cough.	Fever (max, 38°C); cough; shortness of breath; fatigue; muscle pain.	Fever (max, 39°C); cough; less sputum; chest tightness; fatigue.	Fever (max, 38.6°C); paroxysmal dry cough; fatigue; muscle pain.
Diagnosis	1. COVID-19. 2. SLE. 3. LN. 4. CRI (grade IV). 5. Renal hypertension. 6. Renal anaemia. 7. UCM. 8. HF.	1.COVID-19. 2. Bronchodilation. 3. RA. 4. SLE. 5. DM.	1. COVID-19. 2. SLE. 3. Hypothyroidism.	1. COVID-19. 2. SLE.	1. COVID-19. 2. SLE. 3. Hypothyroidism.	1. COVID-19. 2. SLE. 3. Hypertension. 4. LCI. 5. Hyponatraemia.	1. COVID-19. 2. Type 2 DM. 3. RA.	1. COVID-19. 2. RA.	1. COVID-19. 2. RA.	1. COVID-19. 2. Hypertension. 3. RA.	1. COVID-19. 2. Hypertension. 3. RA.
Treatment	Standard treatment	Standard treatment	Standard treatment	Standard treatment	Standard treatment	Standard treatment/ convalescent plasma transfusion	Standard treatment	Standard treatment	Standard treatment	Standard treatment	Standard treatment
Duration (days)	12	33	20	34	66	51	55	38	48	48	22
Outcomes	Death	Discharged	Discharged	Discharged	Discharged	Discharged	Discharged	Discharged	Discharged	Discharged	Discharged

Standard treatment: Chinese Clinical Guidance for COVID-19 Pneumonia Treatment.
CRI, chronic renal insufficiency; DM, diabetes mellitus; HF, heart failure; LCI, lacunar cerebral infarction; LN, lupus nephropathy; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; UCM, uraemic cardiomyopathy.

disease and other pre-existing diseases, with a short (12 days) and deteriorating course (figure 1, table 1). Prior to being admitted to the hospital, the patient's condition had already progressed to stage IV renal failure. The patient's condition did not improve and finally resulted in renal and heart failure. This case highlights the fact that COVID-19 may accelerate the progression to heart and kidney failure, which also supports the findings by Mathian *et al*.

Our cohort included 11 COVID-19 patients with SLE (patients 1–6) and rheumatoid arthritis (RA) (patients 7–11), respectively. One patient (patient 3) suffered from both SLE and RA. The COVID-19 course duration of patients with SLE was 36 (12–66) days. Patient 6 developed severe hypertension (170/120 mm Hg), lacunar infarction, hyponatraemia, moderate anaemia and hypokalaemia during hospitalisation. Furthermore, the nucleic acid test results for SARS-CoV-2 infection for this patient were repeatedly positive during the treatment period, and SARS-CoV-2 antibody was produced after a convalescent plasma transfusion. Five patients were discharged after antiviral and antibiotic treatment, while patient 2 died. All patients with RA were female, with an overall course of 40.6 (33–55) days, which was not different from that of patients with SLE (table 1). Patients 8 and 9 only suffered from RA and SARS-CoV-2 infection, while the other four patients also had other comorbidities, such as hypertension and diabetes. The biochemical indicators of myocardial injury and liver injury in patients with RA, such as brain natriuretic peptide and creatine kinase, were lower than those in the SLE patient group (online supplementary figures S1–S5). All patients with RA were discharged after symptomatic treatment.

Overall, our clinical data suggest that SARS-CoV-2 infection worsens the outcome of patients with SLE/RA, and the severity of SARS-CoV-2 infection is associated with pre-existing diseases, especially heart disease and kidney disease, which may cause severe failure or even death. Therefore, treatment of COVID-19 patients with RA/SLE needs to consider the use of drugs that can protect the heart and reduce the burden on the kidneys.

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Response to: 'Implications of SARS-CoV-2 infection for patients with rheumatic disease' by Lin *et al*

We thank Lin *et al* for their interest in our study reporting on the course of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) disease 2019 (COVID-19) in a case series of patients with systemic lupus erythematosus (SLE) under long-term treatment with hydroxychloroquine (HCQ).[1 2] Our work intended to report the failure of HCQ to prevent COVID-19 in a series of patients with SLE. Since our publication, several other studies have also shown the lack of efficacy of HCQ in the treatment of SARS-Cov-2 infection. Lin *et al* discuss whether patients with SLE are more susceptible to infection with SARS-CoV-2, and, when they are infected, whether they progress to more severe disease and a poorer outcome. Our study however was not designed to address these questions. Hence, both studies cannot really be compared.

First, Lin *et al* observed that 6 (0.2%) of 3057 patients with COVID-19 admitted to Wuhan Huoshenshan Hospital had been diagnosed with SLE. Based on the fact that the prevalence of SLE was reported to be approximately 0.05%–0.1% in other regions of China, the authors suggest that patients with SLE are more susceptible to SARS-CoV-2 infection than the general population. However, we consider that it is not possible to assess the prevalence of COVID-19 in patients with SLE based on these data. Indeed, as the risk of development of SLE varies according to occupational and environmental exposure[3] and because major differences in regional age-standardised prevalence rates are observed within the same country,[4] Lin *et al* had better taken the prevalence of SLE in the region of Wuhan instead of those of Hong Kong and the rural areas of Anhui Province. Furthermore, the number of patients with SLE admitted to hospital care for COVID-19 cannot be used as the sole estimate of the prevalence of COVID-19 in SLE. Indeed, due to the supposed susceptibility of patients with SLE to infections, physicians are likely more inclined to admit a patient with SLE with signs of COVID-19 to the emergency room. The real number of patients with SLE infected with SARS-CoV-2 is difficult to assess as well considering the high number of individuals with symptomatic or asymptomatic COVID-19 who have not been tested for the presence of the SARS-Cov-2, in addition to the considerable false-negative rate of the various testing approaches.[5]

Second, Lin *et al* suggest in their study, as we and others have reported previously in limited case series, that patients with SLE and associated comorbidities such as arterial hypertension, diabetes, chronic kidney disease and obesity are at risk of developing a severe form of COVID-19.[1 2 6 7] This hypothesis was recently confirmed in a study including a larger number of patients with SLE. Fernandez-Ruiz *et al* identified in a series of 41 patients with SLE in the New York area that the body mass index and the presence of one or more comorbidities, including malignancy, organ transplantation, hypertension, diabetes, chronic obstructive pulmonary disease, congestive heart failure and asthma, were independent predictors of hospitalisation in case of a confirmed COVID-19.[8] Whether patients with SLE treated with glucocorticoids and/or immunosuppressants are at risk for hospital admission during the course of SARS-CoV-2 infection, as has been suggested in immune-mediated inflammatory disease in general,[9–11] is as yet unclear and further studies are needed to understand the additional specific risk factors for poor COVID-19 outcome in patients with SLE.

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Emergency arising from patients' fear of taking antimalarials during these COVID-19 times: are antimalarials as unsafe for cardiovascular health as recent reports suggest?

We read with interest the paper of Graef *et al* recently published in your journal about the situation resulting from the massive use of antimalarials for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2/COVID-19), despite the fact that the evidence is controversial and there are concerns about its possible cardiotoxicity, leaving rheumatic patients who use them in a position of vulnerability due to medication shortages.[1] In the past few weeks, several papers have been published about the efficacy and safety of the antimalarials chloroquine (CLQ) and hydroxychloroquine (HCLQ) for the treatment of the different phases of infection by SARS-CoV-2/COVID-19, and the data are controversial. However, it is striking that some studies report high rates of cardiovascular events (CVEs) associated mainly with cardiac arrhythmias.[2] .

These findings of adverse CVEs reported in the aforementioned studies have unfortunately led to the emergency in this group of patients around fear of chronic use of antimalarials, and many users are abandoning these medications, which implies great clinical risk due to relapses that may appear.[3] On the other hand, the massive use of antimalarials for COVID-19 has resulted in medication shortages in some settings with potential consequences to patients users.

Antimalarials have been used for several decades for the treatment of malaria and some autoimmune diseases, mainly rheumatoid arthritis (RA) and lupus, with great utility and efficacy, and also great safety at conventional doses (250 mg per day of CLQ and 200–400 mg per day of HCLQ).[4]

We show a real-life experience in a rheumatic centre in Bogota, Colombia. We performed a retrospective cohort analysis of adverse events (AEs), adverse reactions (ARs) and medication-related problems (MRPs) presented in the last 16 months, according to the methodology of the Third Consensus of Granada. The severity of the events and reactions was evaluated using the Dader Method of therapeutic drug monitoring, and the Naranjo algorithm was used to characterise them as AEs, ARs or MRPs.

Here, we report the outcomes since 1 January 2019–30 April 2020. By the end of 2018, there were 1004 patients with RA using antimalarials; currently, there are 660 patients still using CLQ/HCLQ; 583 (88.3%) are using CLQ and 77 (11.7%) are using HCLQ; of them, 186 (28.2%) patients have cardiovascular comorbidities, previous to antimalarial use, like primary hypertension, cardiovascular disease or the two combined (table 1).

Table 2 Adverse events and reactions to antimalarials in patients with rheumatoid arthritis during observation period

AE/AR	Patients without CVD/PH (n=249)	Patients with previous CVD/PH (n=95)	Total n=344 (%)
Retinal toxicity	100	30	130 (37.9)
Gastrointestinal AEs	83	20	103 (30.0)
Dermatological ARs	65	45	110 (31.8)
Severe dizziness	1	0	1 (0.3)
CVD AEs/ARs	0	0	0 (0.0)

AE, adverse event; AR, adverse reaction; CVD, cardiovascular disease; PH, primary hypertension.

Regarding safety concerns during observation period, 344 patients presented with AEs and ARs that required the withdrawal of antimalarials; of them, 130 (37.9%) had retinal toxicity an expected AR; 103 (30%) had gastrointestinal intolerance, accepted as an AE; 110 (31.8%) had different types of dermatological ARs; 1 patient had severe dizziness, possibly an MRP; and there were no CVEs or arrhythmias, despite the fact that 95 (27.6%) patients previously had cardiovascular comorbidity (table 2).

At first glance, these results seem surprising; there are zero incidences of new, incidental CVE in this RA cohort using antimalarials, although of course, we are talking about different doses for RA and COVID-19; on the contrary, CLQ and HCLQ have been associated with a reduced risk of CVE in patients with rheumatic diseases through robust research,[5] in addition to the finding that the use of HCLQ is independently associated with decreased risk for cardiovascular morbidity among patients with RA.[6] AEs and ARs presented by these patients are consistent with data previously published but reinforce the fact that antimalarials are drugs that lack cardiotoxicity in rheumatic patients; therefore, education for doctors and patients in general must be reinforced to prevent antimalarials from being abandoned because of the past news.

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Table 1 Cardiovascular disease in patients with rheumatoid arthritis currently and previously using antimalarials

Currently users of antimalarials				Previous users of antimalarials withdrawn by adverse events		
Comorbidities	CLQ (n=583)	HCLQ (n=77)	Total n=660 (%)	CLQ (n=330)	HCLQ (n=14)	Total n=344 (%)
PH	140	16	156 (23.5)	74	1	75 (21.8)
CVD	10	4	14 (2.1)	3	0	3 (0.9)
PH and CVD	15	1	16 (2.4)	16	1	17 (4.9)
No CVD/PH comorbidities	418	56	474 (72.0)	237	12	249 (72.4)

CLQ, chloroquine; CVD, cardiovascular disease; HCLQ, hydroxychloroquine;PH, primary hypertension.

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Response to: 'Emergency arising from patients' fear of taking antimalarials during these COVID-19 times: are antimalarials as unsafe for cardiovascular health as recent reports suggest?' by Santos-Moreno *et al*

We thank Santos-Moreno *et al* for their response to our commentary.[1 2] The authors provide data on cardiovascular adverse events associated with maintenance antimalarial use for patients with rheumatoid arthritis (RA). In addition to our previous correspondence response[3] from Erre *et al*,[4] these additional data are reassuring to patients with rheumatic diseases on chronic doses of antimalarials regarding the continued safety of these medications.

However, as we have also addressed in a previous response, the safety profile of antimalarials in patients with COVID-19 infection compared with patients with rheumatic disease may differ widely. Observational data regarding cardiovascular events in hospitalised patients with COVID-19 treated with hydroxychloroquine (HCQ) with or without azithromycin have found variable rates of QTc prolongation and arrhythmias.[5–7] Lane *et al* recently used combined electronic health record and administrative data from five countries and a new user, active comparator cohort design to address two scientific questions among adults with RA[8]: (1) the risk of serious adverse events, particularly cardiovascular events, among individuals with RA initiating therapy with HCQ versus sulfasalazine; (2) the risk of these outcomes when azithromycin is started (compared with starting amoxicillin) among prevalent HCQ users with RA. The cardiovascular outcomes assessed in that study included cardiovascular mortality, cardiac arrhythmia, and heart failure, and were identified using ICD codes.[8] However, there was no statistically significant association between HCQ use and cardiac arrhythmia, compared with sulfasalazine use (calibrated HR 0.89, 95% CI 0.77 to 1.04) among individuals with RA.[8] Although a similarly designed study using population-level data could be conceived to assess the study question presented by Santos-Moreno *et al*, the surrogate outcome of QTc prolongation would be more difficult to ascertain using administrative/claims data.

In summary, we agree that these data offer additional support that the possible cardiotoxicity of antimalarials for treatment of COVID-19 should not be extrapolated to patients with rheumatic disease where its safety and efficacy is well established.

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Correction: *B cell subset composition segments clinically and serologically distinct groups in chronic cutaneous lupus erythematosus*

Jenks SA, Wei C, Bugrovsky R, *et al*. B cell subset composition segments clinically and serologically distinct groups in chronic cutaneous lupus erythematosus. *Ann Rheum Dis* 2021;80:1190–1200. doi:10.1136/annrheumdis-2021-220349

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