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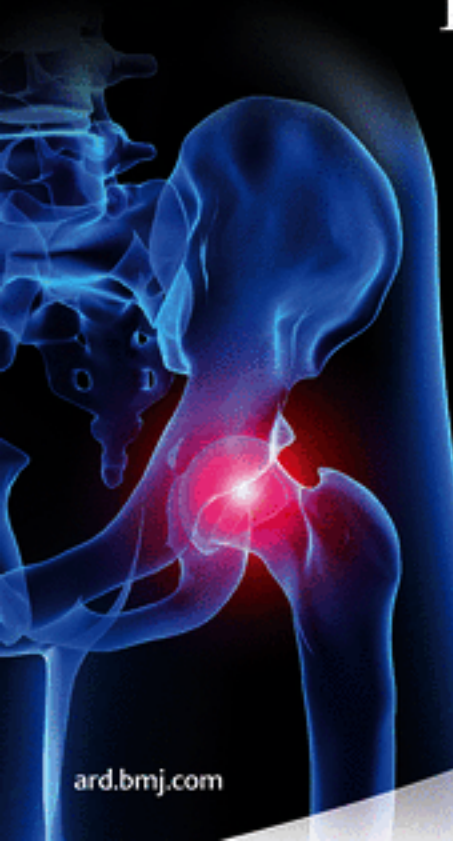

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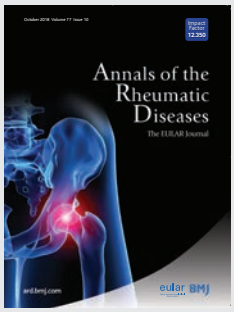

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Ultrasound assessment as predictor of disease relapse in children and adults with arthritis in clinical stable remission: new findings but still unmet needs

Elisa Gremese, Anna Laura Fedele, Stefano Alivernini, Gianfranco Ferraccioli

Several lines of evidence suggest that imaging plays an important role in the diagnosis and monitoring of patients with juvenile idiopathic arthritis (JIA) leading the European League against Rheumatism (EULAR)–Paediatric Rheumatology European Society (PReS) to develop as point to consider the use of imaging within the JIA assessment in clinical practice.[1] The use of ultrasound (US) may increase the sensitivity of clinical examination through its ability to detect joint inflammation (in terms of synovial hypertrophy, joint effusion and power Doppler (PDUS) activity) in case of clinically inactive disease.[2–4] In particular, it has been shown that the majority of patients with JIA in clinical remission may exhibit greyscale (GSUS) abnormalities and in half of them the presence of PDUS signal can be found,[4] without any significant difference on the basis of the pharmacological treatment scheme through which the remission was achieved[2–4] and even in case of drug-free remission (DFR).

In *ARD*, De Lucia *et al* analysed the predictive power of US assessment to foresee disease flare in patients with JIA in clinical remission.[5] The authors included 88 consecutive patients with JIA, stratified based on the joint involvement, undergoing US assessment (including GSUS and PDUS) of 44 joints, finding that nearly one-fourth of them showed US abnormal findings despite clinically inactive disease, mainly in patients with extended joint involvement and with shorter period of time from remission achievement (less than 6 months). Interestingly, during the 4 years of follow-up, almost half of patients with JIA experienced a disease flare with the higher rate of relapse in patients with JIA with residual PDUS synovitis at the time of remission achievement. These can be considered relevant findings since previous studies failed to confirm the predictive value of US assessment in foreseeing the chance of disease flare in JIA.[6 7] In particular, Magni-Manzoni *et al* evaluated 39 patients with JIA showing that US-detected synovitis is a common finding in children in clinical remission and its presence does not predict an early flare in the affected joints.[6] Similarly, Zhao *et al* recently confirmed in a comparable study cohort of children with JIA that nearly half of them have abnormal US findings whose presence is not associated with subsequent clinical flare in up to 2 years of follow-up.[7] Moreover, the EULAR/PreS points to consider do not include a proposal list of the joints sites eligible for US assessment to increase the ability to detect subclinical ongoing inflammation despite clinical sustained remission in children. In particular, Breton *et al*[8] assessed the II–V metacarpophalangeal and I–V metatarsophalangeal (MTP) joints in children with JIA and in healthy controls detecting features of synovitis (defined as synovial thickening with or without effusion and hypervascularity) mainly in MTP joints of children with JIA without any clinical sign of inflammation. The study by De Lucia *et al*[5] provides additional information having performed a more extensive US assessment, including 44 joints, which may significantly increase the accuracy of disease activity determination in children in whom subjective symptoms may be underestimated compared with adult patients. In adults with rheumatoid arthritis (RA), the EULAR/Outcome Measures in Rheumatology (OMERACT) group have developed a standardised combined scoring system, including both GSUS–PDUS components, in the evaluation of synovitis in multiple joints with high reliability when applied in scanning patients but still lacking a defined optimal reduced joint set to be evaluated in clinical practice as well as in clinical trials.[9 10] The manuscript by De Lucia *et al*[5] therefore differentiates itself from the previous ones in being much more stringent in its methodology and it raises the need to use GSUS and PDUS parameters in a complementary manner to detect more precisely the entity of residual inflammation of the target tissue in children with inactive disease, strongly supporting its use as a useful tool in clinical practice.

USE OF ULTRASONOGRAPHIC AND STRICT CLINIMETRIC COMBINED CRITERIA FOR THE IDENTIFICATION OF RESIDUAL SYNOVITIS IN ADULT PATIENTS WITH RA IN STABLE CLINICAL REMISSION

Stable clinical remission is the most important goal in children with arthritis as well as in adults with RA,[11] but despite apparent clinical remission, defined with composite indices such as DAS (Disease Activity Score),[12] joint damage progression can occur.[13] This issue is tightly related to the current lack of uniform definition of sustained remission in adults with RA.[14] The ACR/EULAR definition certainly has improved our confidence in defining a patient in remission in clinical trials, yet some issues still need to be defined in clinical practice.[14 15]

It is known that US assessment, through GSUS and PDUS evaluation, is able to identify residual synovitis in more than 50% of adult patients with RA in clinical remission on the basis of their DAS value.[13] When comparing the PDUS-negative and PDUS-positive patients with RA in DAS remission, in a real-world setting, significantly fewer PDUS-negative patients with RA in clinical remission experienced a flare during 12 months of follow-up, compared with patients with RA with PDUS positivity at the time of remission achievement.[16 17] Recently, Zufferey *et al*,[18] in a multicentre cohort study including adult patients with RA in DAS28-selected clinical remission, confirmed an incidence of US-detected residual synovitis in more than half of enrolled RA confirming the use of US, yet the assessment of US positivity yielded a moderate predictive power for loss of remission in a real-life setting. A longitudinal study evaluating the combined use of serial US assessment in long-standing adult patients with RA in clinical remission showed an increase

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in the success rate of maintaining disease control after anti-tumour necrosis factor tapering and discontinuation compared with only DAS-based selection.[19]

The histological characteristics of synovial tissue residual inflammation have been described in patients with RA in sustained DAS-defined and US-defined remission.[20] Moreover, the use of a stricter selection criterion as the Simplified Disease Activity Index (SDAI) enables a more precise identification of patients with RA in sustained disease control with histopathologically minimal synovitis and significantly lower chance of disease relapse after treatment de-escalation. These findings support the concept that the fulfilment of the SDAI-based remission status, combined or not with US remission criterion, reduces the relapse rate after treatment modifications in adults with RA. This should be demonstrated also in JIA at the same extent.

USE OF COMBINED SEROLOGICAL AND IMAGING BIOMARKERS FOR THE IDENTIFICATION OF PATIENTS WITH ARTHRITIS IN STABLE CLINICAL REMISSION WITH HIGH RISK OF DISEASE RELAPSE AFTER TREATMENT TAPERING/DISCONTINUATION

In addition to the use of US assessment for the identification of patients with RA eligible to treatment tapering and discontinuation, once stable clinical remission is achieved, serological biomarkers such as anticitrullinated peptide antibody (ACPA) positivity independently associated with disease flare after treatment discontinuation[21] and with lower chance of achieving (persistent) DFR.[22 23] To date, limited information are available on predictive biomarkers of disease relapse in ACPA/rheumatoid factor (RF)-negative patients with RA. An abnormal Multi-Biomarker Disease Activity panel arose to identify patients with RA in clinical remission, despite their serological status, with higher risk of disease relapse because of residual synovitis.[24] To date, no comparable studies have been conducted in patients with JIA in sustained remission; therefore, the results obtained by De Lucia *et al*,[5] showing a strong predictive value of US assessment, suggest its possible inclusion as instrumental criterion for remission definition in patients with JIA, which could increase the success rate of treatment de-escalation. However, it should be taken into account also that US is not able to predict disease flares in all patients, needing certainly further standardisation and mostly biomarkers capable of defining a deeper remission.

UNMET NEEDS FOR THE GLOBAL DEFINITION OF CLINICAL REMISSION AND FOR A WISER STRATEGY FOR MEDICATION TAPERING AND DISCONTINUATION IN JIA

More complicated is the issue about the definition of clinical remission in JIA since it consists of a heterogeneous group of conditions involving synovial tissue and even extra-articular domains. Moreover, the clinical course of JIA is unpredictable, with periods of low disease activity followed by recurrence of signs and symptoms on or off medications.[25] The probability of achieving inactive disease and/or clinical remission in JIA seems to be tightly related to the clinical subtype.[26] Children with RF-positive polyarthritis showed a lower overall remission rate after treatment compared with children with oligoarticular-persistent JIA.[27] These data are supported by the US findings, provided by De Lucia *et al*, showing a lower likelihood of US-detected residual synovitis in the latter group.[5] In fact, in the study by De Lucia *et al*,[5] US-detected residual synovitis was more significant in patients with JIA with more extended joint involvement despite no information provided on its incidence in relation to the presence of extra-articular manifestations. This is indeed a question that this study raises, suggesting the need to include multiple domain evaluation within the global assessment of children with JIA in remission. However, once disease remission is achieved in children, limited evidences are available about the predictors of disease flare. Among them, RF positivity was found to be an independent parameter associated with disease flare after attaining inactive disease together with a severe disease course (defined as an active joint count >4, use of biologics and patient global assessment >30 mm),[28] and abnormal C reactive protein.[29] Conversely, contradictory data are available on the predictive role of antinuclear antibody (ANA) positivity whose presence was associated with a higher risk of flare by Guzman *et al*,[28] whereas in a previous prospective study conducted by Miotto e Silva *et al*, patients with JIA who flared during the follow-up were not different in terms of ANA positivity compared with subjects who maintained disease control during the follow-up.[30] Limited data are available about the best strategy to taper or discontinue pharmacological treatment in children with JIA once clinical remission is achieved and stably maintained. Systematic literature analysis including published studies carried out on adults with RA showed that biological disease modifying anti rheumatic drugs (bDMARDs) discontinuation leads to an increased risk of losing remission or Low Disease Activity status and an increased risk of radiographic damage progression compared with treatment continuation.[31] The best modality of treatment tapering in adults with RA is still a matter of debate since both the reduction of drug dose and the increase of application interval (spacing) are suggested in adult patients with RA in persistent remission.[11] More exhaustive information will be provided by the analysis of currently ongoing trials, comparing different modalities of treatment reduction (early vs late reduction, respectively) in adults with RA in stringent clinical remission (Clinical Disease Activity Index ≤2.8),[32] despite no systematic data on this issue available so far for children with JIA.

In conclusion, the research agenda should aim to a more comprehensive definition of disease remission for children with arthritis. This goal could be reached through multiple comparison studies aiming at defining the global profile of 'the patient' with arthritis in remission and then eligible to treatment tapering or discontinuation reducing at minimum the risk of disease flare. Therefore, US assessment, which has been shown to be a useful tool to determine subclinical activity in patients in sustained clinical remission, should be included along with other possible biomarkers—in studies aiming at the definition of the best matrix able to identify the long-term drug-free remission prognosticators regardless of the patient's age.

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Overdiagnosis and overtreatment in rheumatology: a little caution is in order

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ABSTRACT

Overdiagnosis is a term coined by experts in cancer screening to point to indolent cancers detected by screening that would have never led to manifest health problems. Overdiagnosis leads to unnecessary medical care (overtreatment), anxiety and cost. In rheumatology overdiagnosis and overtreatment are hardly discussed but likely present. This viewpoint examines how our prevailing views on the management of inflammatory rheumatic diseases may relate to overdiagnosis and overtreatment. Six paradigms of modern rheumatology will be discussed: early diagnosis, intensive treatment, remission, prognosis and risk stratification, evidence-based rheumatology, and precision medicine. It is concluded that, in spite of the enormous progress that they have brought, all paradigms bear the intrinsic dangers of overdiagnosis and overtreatment. So a little caution is in order.

Overdiagnosis, a term coined by experts in cancer screening, is an unwarranted side effect of screening.[1] It points to cancers detected by screening that—if remain undiagnosed—will never lead to manifest health problems because of their indolent character. Overdiagnosis leads to overtreatment (unnecessary healthcare), psychological distress and huge costs.

While overdiagnosis and overtreatment show up everywhere in medicine,[2] there is remarkably little scientific attention. GB Welch[3] explained overdiagnosis and overtreatment as inherent consequences of medical doctors' attitude: 'Doctors attempt to mitigate a risk without considering how small or unlikely the potential benefit is, they try to fix a problem rather than monitor and cope with it, they prefer to act quickly rather than waiting for more information, and they prefer newer treatments over older' (*sic*). The influence of the public on their attitude should not be underestimated.

OVERDIAGNOSIS AND OVERTREATMENT IN RHEUMATOLOGY

For inflammatory rheumatic diseases, such as rheumatoid arthritis (RA), psoriatic arthritis (PsA) and ankylosing spondylitis (AS), the story of overdiagnosis and overtreatment is a tabula rasa. That is understandable. After a long history of therapeutic nihilism and acquiescence, the focus in rheumatology has shifted from caring for the disabled to actively finding the unrecognised, from a wait-and-see policy to early intervention, and from careful step-up treatment with poorly effective but toxic drugs to immediate intervention with powerful new drug combinations. To date, no one seems to bother about overdiagnosis and overtreatment since for the first time in history we have an opulence of effective drugs and more are coming. Critical questions about early diagnosis and treatment are *no-brainers*, since 'early' implies 'before irreversible damage has occurred', and isn't that at everybody's benefit? In addition, it is a fact of life that in many countries getting proper access to 'necessary health care' is still a more urgent problem than dealing with 'unnecessary health care'.

In cancer, early detection has always intuitively implied better prognosis, but this faith has recently been shaken. In some patients early detection has indeed saved lives, but in others an early cancer diagnosis has initiated an unnecessary chain of harms: they received a burdening diagnosis of cancer, but this cancer would have never impacted their lives if they had stayed ignorant.[3] Cancer screening advocates still consider this collateral damage of a greater good.

Inflammatory rheumatic diseases differ from cancer in many aspects, and overdiagnosis and overtreatment will take another shape, but there are peculiar similarities too. In this Viewpoint article I shall examine our current rather offensive approach to detect and treat inflammatory diseases against the potential hazards of overdiagnosis and overtreatment. I will do this by discussing six popular paradigms illustrative of our current vision. These six discussions reflect my personal opinion and are not (yet) underpinned by scientific data from the literature. To fully appreciate these issues, however, additional studies are required.

PARADIGM 1: EARLY DIAGNOSIS

A clinical diagnosis of an inflammatory rheumatic disease is the prerogative of the skilled rheumatologist. There is no proper alternative. Objective tests do not suffice for a diagnosis and a rheumatic disease may still exist in the absence of objective evidence. The quest to an *earlier* diagnosis therefore demanded a lot from the practising rheumatologist. Several initiatives have been taken to convince the rheumatologist of the relevance of early diagnosis: early arthritis and back pain clinics, referral criteria for general practitioners, new sophisticated imaging, smarter diagnostic tests, and on top of that a thorough rethinking of the classification criteria. Some have been recently reformulated to serve the demand for an earlier diagnosis better,[4–6] although we know that diagnosis and classification are distinct entities that can easily be muddled up.[7]

These initiatives may have had double-faced effects. Bad cases may have reached the office of the rheumatologist timelier, but in their wake many milder cases with less clear symptoms and a better prognosis will have shown up too. New diagnostic

tests (eg, ultrasound) that promised increased sensitivity may have contributed to a considerable rise in incidence. More inclusive classification criteria, inappropriately used to diagnose more patients, may have stretched the disease spectrum further. The net result is more *'grey-zone medicine'* with milder patients diagnosed earlier than before, prepared for our intensive treatments.

We have even gone beyond the limits of early diagnosis. Recent research aims at identifying risk groups of patients with a *propensity* to develop true inflammatory disease (eg, clinically suspect arthralgia[8]) in order to treat them pre-emptively. This is befuddling risk of disease with presence of disease, which may turn healthy people into patients and can cause collateral damage.

PARADIGM 2: INTENSIVE TREATMENT

It is beyond any doubt that the discovery and implementation of many new treatments during the last two decades have had a tremendous positive influence. The intensive treatment paradigm, when evaluated against the background of treatment results before 1990, has markedly improved the outcome of patients with classic RA, PsA and AS. Early diagnosis and intensive treatment have gone hand in hand. This response to the submissive 'go-low-go-slow' movement was welcomed and spearheaded by the successes of—among others—the COBRA trial in RA[9] and later on the BeST trial.[10] The surge of new effective medicines and innovative therapeutic strategies that has followed was justified by improving the outcomes in the most severe and active patients in trials. However, trial results are *average* group results that do not unveil that clinical superiority is often caused by only a minority in the trial. The majority could have done similarly well on usual therapy. The type of overtreatment that follows from this principle has apparently been broadly accepted in the medical community: 'Some need it some do not'.[2] This may be a justifiable approach if there is no alternative, but modern rheumatology has been gifted with an ample choice of very effective drugs. Only their hierarchical priority across the spectrum of disease has been poorly studied. To date, we still extrapolate trial results obtained in severe patients to those with milder disease, convinced that we do a good job, but we likely overtreat some of them.

PARADIGM 3: REMISSION

The paradigm that clinical remission is the best achievement for patients and doctors is a strong one, but one that easily leads to overtreatment in many patients. The paradigm of remission is based on the proven relationship between clinical disease activity and structural damage progression across inflammatory diseases[11 12]: 'the lower the disease activity the better the outcome'.

Admittedly, our belief in the virtues of clinical remission may be correct from the perspective of the patient with severe disease and high disease activity. However, the marginal effect of 'pushing toward remission' in a patient already in a low disease activity state may be of negligible value in some cases and may do more harm than good. Strategy trials may have proven the feasibility of intensive monitoring and treat-to-remission, but their surplus efficacy on radiographic progression was minimal or absent.[13–15] The question if the difference between zero and minimal structural progression really adds value to the patients, given the totality of factors that contribute to 'happy patients',[16] has not been answered by these trials. What we have learnt, though, is that drug use in patients who were intensively monitored and steered towards remission was markedly higher than in patients treated regularly—a subtle sign of overtreatment.

PARADIGM 4: PROGNOSIS AND RISK STRATIFICATION

Many publications have dealt with prognosis of outcome or prediction of treatment response. Imaging tests, biomarkers and translational 'signatures' have all been investigated for their prognostic potential. Most of them were not reproducible. If they were, their marginal effects over and above simple clinical measurements were, if tested at all, negligible and not clinically relevant.[17] Prognostic research is difficult in the absence of untreated patients that show the natural course of the disease. Unfortunately, many researchers, clinicians and the public are not appropriately educated to interpret prognostic (risk) information and grossly overestimate its value. We still do not know about the most important health outcomes to patients, namely those outcomes that truly add value and that we should actually be able to predict.[16] Diagnosis and treatment based on unreliable prognostic markers will inevitably lead to overdiagnosis and overtreatment in a number of patients.

PARADIGM 5: EVIDENCE-BASED RHEUMATOLOGY

Randomised clinical trials form the major source for evidence-based medicine (EBM). The field has made marked progress by systematising the culminating evidence into clinical guidelines (recommendations).[18–20] Systematic literature review and expert judgement of the evidence are at the basis of the guidelines, which intend to describe the state of the art and to help clinicians making treatment decisions in individual patients. Undoubtedly, guidelines are an asset to clinicians for many reasons, but are not always well understood and properly used. Guidelines reflect, in principle, the consensual opinion of medical experts regarding the available evidence in the literature and describe *best-practice* irrespective of the local situation of patients, healthcare systems and countries. Guidelines also breathe prevailing visions in the field, such as early diagnosis and intensive treatment towards remission. In addition, guidelines may have legislative and economical implications and can lead to defensive medicine. Sometimes, and alien to the intentions of the EBM movement, guidelines are too rigidly pursued by clinicians who may ignore the needs of individual patients. Such a practice may contribute to overdiagnosis and overtreatment.

PARADIGM 6: PRECISION MEDICINE

Precision medicine is a current hype, boosted by former President Obama's Precision Medicine Initiative from 2016.[21] Precision medicine suggests that patients with a particular diagnosis can be separated into subgroups based on genetic, cellular or molecular analysis, each with a special medical intervention. Advocates of precision medicine claim that current drug treatments, although effective in drug trials, fall short because so many of our patients do not reach remission or lose efficacy over time. While precision medicine appeals to the public and to funding bodies, because it breathes technological innovation, influential critics have warned that precision medicine will not lead to increased patient values and may cause overdiagnosis, overtreatment and cost explosions.[22] Even if precision medicine will succeed in elucidating and targeting multiple pathways of the same disease, the ultimate treatment success will not only depend on cellular and biological factors but rather on an intangible interplay of comorbidities, psychosocial factors, compliance, beliefs and non-scientific perceptions. In other words, while it is unlikely that precision medicine will eventually become the holy grail of

rheumatology, it may have caused already a lot of unnecessary medicine far before we have realised.

In summary, all prevailing paradigms of disease management bear—in my opinion—the potential risk of overdiagnosis and overtreatment. This appreciation does not intend to disavow the immense progress that has been made, on the contrary. It argues in favour of a more frugal and sustainable vision on how to best manage our inflammatory rheumatic diseases in the future, now that we have such a broad spectrum of effective drugs and strategies available. We may have to acknowledge in rheumatology that 'earlier' (diagnosis) is not always necessarily 'better', that 'more' (treatment) is not automatically 'more effective', and that 'increased sensitivity' (of diagnostic tests) is not synonymous to 'better yield'. We need to better distinguish 'disease' from the 'risk of disease'. We need trials investigating how to best manage all those milder cases that come to us as a result of our early diagnosis initiatives. We need to learn what does and does not really matter to patients with inflammatory rheumatic diseases.[16] We need to pay more attention to possible overdiagnosis and overtreatment in our clinical research. And we need to learn, without relinquishing all the good of early intensive interventions and our precious new treatments, how we can further increase the *value* of our healthcare for the sake of our patients with inflammatory rheumatic diseases and for society that has to pay the bill.

A little caution is in order.

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EXTENDED REPORT

Epidemiology and socioeconomic impact of the rheumatic diseases on indigenous people: an invisible syndemic public health problem

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ABSTRACT

Epidemiological studies in Latin America suggest indigenous people lack proper healthcare for musculoskeletal (MSK) and rheumatic diseases.

Objectives This study aimed to estimate the prevalence of MSK disorders and rheumatic diseases in eight Latin American indigenous communities, and to identify which factors influence such prevalence using network analysis and syndemic approach.

Methods This is a cross-sectional, community-based census study according to Community-Oriented Program for the Control of Rheumatic Diseases methodology. Individuals with MSK pain, stiffness or swelling in the past and/or during the last 7 days were evaluated by participating physicians. A descriptive, univariable and multivariable analysis was performed, followed by a network analysis.

Results We surveyed 6155 indigenous individuals with a mean age of 41.2 years (SD 17.6; range 18–105); 3757 (61.0%) were women. Point prevalence in rank order was: low back pain in 821 (13.3%); osteoarthritis in 598 (9.7%); rheumatic regional pain syndromes in 368 (5.9%); rheumatoid arthritis in 85 (1.3%); undifferentiated arthritis in 13 (0.2%); and spondyloarthritis in 12 (0.1%). There were marked variations in the prevalence of each rheumatic disease among the communities. Multivariate models and network analysis revealed a complex relationship between rheumatic diseases, comorbidities and socioeconomic conditions.

Conclusions The overall prevalence of MSK disorders in Latin American indigenous communities was 34.5%. Although low back pain and osteoarthritis were the most prevalent rheumatic diseases, wide variations according to population groups occurred. The relationship between rheumatic diseases, comorbidities and socioeconomic conditions allows taking a syndemic approach to the study.

INTRODUCTION

Musculoskeletal (MSK) and rheumatic diseases (RD) have important negative functional, socioeconomic, family and individual impacts on the general population, but they are not considered a public health concern. Various explanations for this have been offered, including a dearth of evidence on RD burden in local, regional and special risk groups and inadequate knowledge translation from researchers to health decision-makers.[1]

Recent publications highlighting the high impact of RD have been disseminated through the Global Burden of Disease 2010 study.[2–5]

Global health initiatives for socially disadvantaged populations mainly focus on infectious diseases and give little consideration to the fact that non-communicable diseases are on the rise both in low/middle-income countries and developed countries, causing the greatest impact in low/middle-income countries.[6 7] This impact is greater in indigenous populations, which are invisible to healthcare programmes and face limited access to healthcare. This situation produces the coexistence of infectious and chronic diseases, conditioning high levels of disability, socioeconomic and family impact that reduce the quality of life of indigenous populations.[8 9]

In 1980, the International League Against Rheumatism, together with the WHO, initiated the Community-Oriented Program for the Control of Rheumatic Diseases (COPCORD), a programme designed to detect and manage RD at the community level.[10–20]

There is a need for statistical, hard data reflecting the incidence, prevalence, mortality and burden of RD on the indigenous peoples of Latin America. Such data ought to be considered within a context of comorbidities, unequal healthcare access, geographical and linguistic barriers, low social support and a paucity of healthcare policies for providing culturally sensitive care.[15]

Recently, Singer *et al* have proposed the syndemic framework—a theoretical approach positing that the interaction of two or more biological diseases, in different sociocultural situations and in the context of varying healthcare standards, exacerbates their deleterious effects on the health of individuals, communities and societies.[21] It is ideal for the study of chronic diseases in disadvantaged patients and could be instrumental in helping understand the impact of RD in indigenous populations. Therefore,

Table 1 Demographic and socioeconomic characteristics of the study population

	Total	Mixtec	Chontal	Maya	Qom	Kariña	Chaima	Warao	Raramuri
	(n=6155)	(n=937)	(n=124)	(n=1521)	(n=1656)	(n=262)	(n=692)	(n=583)	(n=380)
Gender (female), n (%)	3757 (61.0)	570 (60.83)	72 (58.1)	916 (60.22)	1020 (61.6)	175 (66.8)	416 (60.1)	352 (60.4)	236 (62.1)
Age (years), mean (SD)*	41.50 (17.6)	46.94 (20.10)	47.07 (18.1)	45.21 (17.80)	35.39 (13.9)	41.57 (18.4)	44.27 (18.3)	37.95 (15.6)	33.57 (13.1)
Education (years), mean (SD)	5.18 (4.1)	7.10 (4.70)	4.74 (3.6)	4.53 (3.64)	4.85 (3.3)	7.38 (4.9)	5.51 (4.3)	2.12 (3.0)	–
Unemployed, n (%)*	1487 (24.2)	56 (5.98)	3 (2.4)	66 (4.34)	596 (36.0)	130 (49.6)	308 (44.5)	148 (25.4)	180 (47.4)
Type of healthcare									
None, n (%)	2043 (33.2)	27 (2.9)	13 (10.5)	86 (5.7)	0 (0.0)	262 (100.0)	692 (100.0)	583 (100.0)	380 (100.0)
National health insurance, n (%)	3682 (59.8)	723 (77.2)	108 (87.1)	1407 (92.5)	1444 (87.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total social coverage, n (%)	182 (3.0)	122 (13.0)	3 (2.4)	28 (1.8)	29 0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Private, n (%)	248 (4.0)	65 (6.9)	0 (0.0)	0 (0.0)	183 (11.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Type of healthcare professional									
Total number of times seeking care, n	(n=5018)	(n=2110)	(n=51)	(n=816)	(n=854)	(n=403)	(n=78)	(n=326)	(n=380)
Medical doctors, n (%)	2254 (44.9)	936 (44.4)	16 (31.4)	390 (47.8)	373 (43.7)	289 (71.7)	28 (35.9)	222 (68.1)	0 (0.0)
Traditional medicine†, n (%)	727 (14.5)	330 (15.6)	0 (0.0)	104 (12.7)	103 (12.1)	80 (19.9)	46 (59.0)	1 (0.3)	63 (16.6)
Not seeking care, n (%)	2037 (40.6)	844 (40.0)	35 (68.6)	322 (39.5)	378 (44.3)	34 (8.4)	4 (5.1)	103 (31.6)	317 (83.4)

*P<0.001.
†Spiritualist, bone setter, herbalist, traditional healer or chiropractor.

our proposal includes an ecological methodology with a network analysis approach within a syndemic framework to study the RD distribution in some Latin American indigenous communities.

OBJECTIVES

1. To estimate the prevalence of MSK disorders and RD, along with the factors associated with these conditions in eight Latin American indigenous communities utilising the COPCORD methodology.
2. To compare and describe factors associated with the prevalence of RD in general and with the four most prevalent diseases using network analysis.

MATERIALS AND METHODS

Population

Indigenous individuals over 18 years of age, and fulfil language criteria, self-identification, confirmed indigenous ancestry and residence in ancestral areas. The participants lived in indigenous communities from the following ethnicities: (A) Qom, Argentina; (B) Warao, Kariña and Chaima in Venezuela; and (C) Maya-Yucateco, Mixteco, Chontales and Rarámuris in Mexico (online supplementary table S1). Participants were identified as indigenous if they descended from indigenous peoples, lived in the indicated geographical area and maintained their indigenous cultural, social and economic identity.

The main outcome of this study was the presence of RD, which was assessed through a cross-culturally validated COPCORD questionnaire.[22] Bilingual community members trained as interviewers administered this questionnaire. The questionnaire explored MSK symptoms (eg, pain, stiffness, disability), treatments, coping mechanisms and the process of seeking biomedical and/or traditional medicine. Positive cases were defined as those who had experienced MSK pain in any region during the past 7 days or at least once during their lifetime. Internists and rheumatologists assessed all positive cases and diagnosed RD applying international criteria for rheumatoid arthritis (RA),[23] systemic lupus erythematosus,[24] gout,[25 26] osteoarthritis (OA) of the hands and knees,[27 28] rheumatic regional pain syndromes (RRPS),[29] fybromialgia[30] and spondyloarthritis.[31] For non-specific MSK we used the WHO's International Classification of Diseases 10th Revision.[32]

In addition, sociodemographic, socioeconomic, clinical and occupational outcomes were measured. These outcomes were selected due to their known association with RD and the details of these assessments have been described elsewhere.[16–20] Briefly, sociodemographic outcomes included gender and level of education; socioeconomic outcomes included employment status, health and social insurance coverage; clinical outcomes included self-reported comorbidities, smoking and alcohol intake, and physical functioning, through the well-validated Health Assessment Questionnaire–Disability Index (HAQ-DI)[33]; finally, occupational outcomes were assessed through a custom-made survey to define participants' lifetime occupations and the biomechanical joint loads associated with these.

Statistical analyses

An exploratory analysis of the variables included in the theoretical model was carried out. The analysis yielded measures of central tendency and dispersion for continuous variables, and absolute and relative frequencies for ordinal, nominal or categorical variables. We performed a bivariate analysis of each independent and confounding variable comparing the population with and without RD using one-way and two-way analysis of variance for continuous variables and X^2 analysis for ordinal, nominal or categorical variables. Only statistically significant variables (<0.05 and CI of the OR above 1.0) were included in various multiple logistic regression analyses with forward selection to differentiate variables with independent impact. These regression models used the presence of RD and the presence of the most prevalent RD (ie, RA, OA and back pain) as dependent variables. Significant independent variables were grouped into four blocks: (1) sociodemographic, (2) ethnicity, (3) comorbidities

Table 2 Characteristics of pain by indigenous group

	Total	Mixteco	Chontal	Maya	Qom	Kariña	Chaima	Warao	Raramuri
	(n=6155)	(n=937)	(n=124)	(n=1521)	(n=1656)	(n=262)	(n=692)	(n=583)	(n=380)
Pain 7 days, n (%)*	2127 (34.6)	432 (46.1)	51 (41.1)	591 (38.9)	471 (28.4)	70 (26.7)	290 (41.9)	146 (25.0)	76 (20.0)
Associated with trauma	509 (23.9)	98 (22.7)	14 (27.5)	214 (36.2)	95 (20.2)	6 (8.6)	48 (16.6)	12 (8.2)	22 (28.9)
	(n=2124)	(n=432)	(n=51)	(n=591)	(n=471)	(n=70)	(n=228)	(n=145)	
Pain intensity:	(n=2125)	(n=431)	(n=51)	(n=590)	(n=471)	(n=70)	(n=290)	(n=146)	(n=76)
1) None, n (%)	41 (1.9)	4 (0.9)	0 (0.0)	19 (3.2)	1 (0.2)	0 (0.0)	2 (0.7)	5 (3.4)	10 (13.2)
2) Little, n (%)	563 (26.5)	162 (37.6)	16 (31.4)	168 (28.5)	2 (0.4)	0 (0.0)	172 (59.3)	23 (15.8)	20 (26.3)
3) Regular, n (%)	625 (29.4)	142 (32.9)	13 (25.5)	146 (24.7)	77 (16.3)	40 (57.1)	115 (39.7)	76 (52.1)	16 (21.1)
4) Strong, n (%)	560 (26.4)	103 (23.9)	14 (27.5)	198 (33.6)	160 (34.0)	26 (37.1)	1 (0.3)	41 (28.1)	17 (22.4)
5) Severe, n (%)	336 (15.8)	20 (4.6)	8 (15.7)	59 (10.0)	231 (49.0)	4 (5.7)	0 (0.0)	1 (0.7)	13 (17.1)
Historical pain†, n (%)*	2778 (45.1)	612 (65.3)	81 (65.3)	777 (51.1)	668 (40.3)	105 (40.1)	319 (46.1)	160 (27.4)	56 (14.7)
Associated with trauma, n (%)	776	116	22	196	154	105	40	125	20
	(n = 2777)	(n=612)	(n=81)	(n=777)	(n=668)	(n=105)	(n=319)	(n=160)	(n=59)
Pain intensity:	(n=2777)	(n=612)	(n=81)	(n=775)	(n=668)	(n=105)	(n=319)	(n=160)	(n=22)
1) None, n (%)	73 (2.6)	29 (4.7)	5 (6.2)	24 (3.1)	8 (1.2)	0 (0.0)	3 (0.9)	22 (13.8)	6 (27.3)
2) Little, n (%)	581 (20.9)	220 (35.9)	16 (19.8)	186 (24.0)	2 (0.3)	0 (0.0)	127 (39.8)	98 (61.3)	8 (36.4)
3) Regular, n (%)	886 (31.9)	215 (35.1)	19 (23.5)	181 (23.4)	99 (14.8)	68 (64.8)	189 (59.2)	1 (0.6)	2 (9.1)
4) Strong, n (%)	628 (22.6)	121 (19.8)	25 (30.9)	239 (30.8)	225 (33.7)	3 (2.9)	0 (0.0)	38 (23.8)	4 (18.2)
5) Severe, n (%)	609 (21.9)	27 (4.4)	16 (19.8)	145 (18.7)	334(50.0)	34 (32.4)	0 (0.0)	1 (0.6)	2 (9.1)
Physical limitation	3207	815	121	797	756	113	329	191	85
Current physical limitation, n (%)	498 (15.5)	48 (5.9)	22 (18.2)	107 (13.4)	134 (17.7)	20 (17.7)	87 (26.4)	41 (21.5)	39 (45.9)
	2804	468	42	813	756	113	337	192	83
Not coping with discomfort, n (%)	273 (9.7)	10 (2.1)	4 (9.5)	119 (14.6)	19 (2.5)	27 (23.9)	48 (14.2)	29 (15.1)	17 (20.5)
Functional capacity (HAQ-DI), mean (SD)*	0.1 (0.2)	0.09 (0.3)	0.12 (0.3)	0.11 (0.3)	0.1 (0.3)	0.1 (0.2)	0.12 (0.3)	0.08 (0.3)	0.09 (0.2)
HAQ-Di <0.8	226 (3.7)	25 (2.7)	4 (3.2)	64 (4.2)	61 (3.7)	8 (3.1)	33 (4.8)	17 (2.9)	14 (3.7)

*P<0.001.
†Musculoskeletal pain experienced at least once during lifetime.
HAQ-DI, Health Assessment Questionnaire-Disability Index.

and (4) disability. Statistical analysis was performed using Stata SE V.11.0 for Mac.

To explore, describe and understand the relationship between sociodemographic, socioeconomic, clinical and occupational variables with the presence of RD, we conducted a network analysis, in which lines that connect nodes that represent population attributes represent relationships. These analyses are empirically based, built on mathematical or computational models and highly graphical.[34] The data collected in this study were processed to obtain quantified descriptors of the population through the following steps: (1) normalisation and organisation of variables in columns and rows as number of categories or range of numeric values; (2) columns were grouped using a second variable and algorithms were defined to perform specific queries; and (3) the data were used to build visible relationship networks. Network visualisation and statistical analysis was performed with the Yifan Hu method[35] in the Gephi software package.[36] The nodes table and edge relation were obtained by implementing a MATLAB[37] script; the solution used a software implemented[38] to calculate the OR,[39] which was used as a property of the nodes. Using the OR, the variables with the highest ratio with respect to RD diagnoses were obtained. Population description was completed with

Table 3 Prevalence of rheumatic diseases by indigenous community*

	Mixteco	Chontal	Maya	Qom	Kariña	Chaima	Warao	Raramuri
	(n=937)	(n=124)	(n=1521)	(n=1656)	(n=262)	(n=692)	(n=583)	(n=380)
	n (%; 95% CI)	n (%; 95% CI)	n (%; 95% CI)	n (%; 95% CI)	n (%; 95% CI)	n (%; 95% CI)	n (%; 95% CI)	n (%; 95% CI)
Osteoarthritis	117 (12.4; 10.4 to 14.7)	40 (32.2; 24.1 to 41.2)	144 (9.47; 8.0 to 11.0)	64 (3.8; 29.8 to 49.0)	40 (15.2; 11.1 to 20.2)	122 (17.6; 14.8 to 20.6)	46 (7.8; 5.8 to 10.3)	25 (6.5; 4.3 to 9.5)
Rheumatoid arthritis	11 (1.1; 0.5 to 2.0)	–	17 (1.0; 0.6 to 1.7)	40 (2.4; 1.7 to 3.2)	–	13 (1.8; 1.0 to 3.1)	3 (0.5; 0.1 to 1.4)	2 (0.5; 0.6 to 1.8)
Low back pain	157 (16.7; 14.4 to 19.3)	13 (10.4; 5.7 to 17.2)	153 (10.0; 8.5 to 11.6)	328 (19.8; 17.9 to 21.8)	19 (7.2; 4.4 to 11.0)	68 (9.8; 7.7 to 12.2)	77 (13.2; 10.5 to 16.2)	6 (1.5; 0.5 to 3.4)
Rheumatic regional pain syndromes	39 (4.1; 2.9 to 5.6)	14 (11.2; 6.3 to 18.2)	116 (7.6; 6.3 to 9.0)	70 (4.2; 3.3 to 5.3)	28 (10.6; 7.2 to 15.0)	62 (8.9; 6.9 to 11.3)	38 (6.5; 4.6 to 8.8)	1 (0.2; 0.0 to 1.4)
Fibromyalgia	4 (0.4; 0.1 to 1.0)		35 (2.3; 1.6 to 3.1)	1 (0.06; 0.0 to 0.3)		8 (1.1; 0.5 to 2.2)		1 (0.2; 0.0 to 1.4)
Spondyloarthritis	2 (0.2; 0.02 to 0.7)			4 (0.2; 0.06 to 0.6)	3 (1.1; 0.2 to 0.3)	7 (1.0; 0.4 to 2.0)	1 (0.1; 0.003 to 0.9)	3 (0.7; 0.1 to 2.2)
Systemic lupus erythematosus	1 (0.1; 0.002 to 0.5)			1 (0.06; 0.0 to 0.3)		1 (0.1; 0.003 to 0.8)		
Systemic sclerosis (scleroderma)				2 (0.1; 0.0 to 0.4)		1 (0.1; 0.003 to 0.8)		

X^2 test.

Table 4 Logistic regression

			Dependent variables											
			RD			RA			OA			BP		
			OR	95% CI	P values	OR	95% CI	P values	OR	95% CI	P values	OR	95% CI	P values
Independent variables	Socio demographic	Age (years)	1.29	1.25 to 1.34		1.01	1.00 to 1.02	0.006	1.06	1.05 to 1.07	0	0.99	0.99 to 0.98	0.007
		Gender (female)	–	–		3.02	1.71 to 5.32	0	1.21	1.00 to 1.47	0.05	0.74	0.63 to 0.86	0
		Work	1.26	1.09 to 1.45	0.006	–	–		–	–		1.44	1.18 to 1.75	0
		Schooling	1.08	1.06 to 1.09	0	–	–		–	–				
	Ethnicity	Chontal, Mexico	3.35	2.20 to 5.10	0				5.1	3.13 to 8.30	0			
		Mixteco, Mexico	1.37	1.10 to 1.72	0							1.28	1.03 to 1.60	0.02
		Qom, Argentina				2.75	1.30 to 5.83	0.008						
		Kariñas, Venezuela							1.93	1.22 to 3.03	0			
		Chaimas, Venezuela							2.12	1.55 to 2.90	0			
	Comorbidities	DM2	1.15	1.11 to 1.69	0.003	–	–		1.36	1.01 to 1.84	0.04			
		HBP	1.31	1.10 to 1.55	0		–		1.29	1.02 to 1.64	0.034			
		Gastritis	2.06	1.74 to 2.44	0	–	–					1.5	1.22 to 1.83	0
		Heart diseases	1.68	1.29 to 2.19	0	–	–		1.84	1.19 to 2.54	0.001			
	Disability	Disability (HAQ-DI >0.8)	2.58	1.88 to 3.55	0	5.47	3.09 to 9.70	0	1.74	1.19 to 2.54	0.004			

Dependent variables: block 1, rheumatic diseases (RD); block 2, rheumatoid arthritis (RA); block 3, osteoarthritis (OA); block 4, back pain (BP).
DM2, diabetes mellitus type 2; HAQ-DI, Health Assessment Questionnaire-Disability Index; HBP, high blood pressure (hypertension).

a database visualisation network. A MATLAB script was written to analyse the data; the script obtains the number of subjects per variable and grouping them into rheumatic and non-rheumatic subjects; then the prevalence per variable is obtained and we can visualise the variables (nodes) being the biggest difference between the two populations. Node size and colour of the variables were used to calculate OR: the biggest the node, the highest the OR. RA, RRPS and OA nodes had a constant value. The edge relation between the nodes provides a visual tool to analyse which one of the variables had a high degree of relation. For example, the back pain node has a high relation level with gastritis. The HAQ-DI score had the highest OR value; however, it was pushed away from the centre of the network since many subjects do not share other attributes.[33]

A node with a high authority value has a high level of connections with other nodes. Nodes position is calculated according to Fruchterman-Reingold,[40] a method considering graphs as a mass particle system for simulation. The nodes are the masses and the edges with the spring that connects them; the system minimises the energy of the particle system and the resulting network would have the nodes with the highest connection (highest value of the edges between nodes) closing together, while the nodes with the lowest relation are pushed to the outside. In the end, the network visualisation provides three types of relationships: the node size shows the variables that are present with highest frequency in the rheumatic population, the colour shows the authority, and the network structure reveals the connectivity between different variables.[41]

Ethical and biosecurity aspects

This study was first approved by each of the indigenous community authorities and then by the ethics and research committees of the hospitals and institutions where healthcare was offered or where the participating researchers worked. All participants consented to partake in the study after an informed consent process by signing or affixing a fingerprint on consenting forms written in Spanish and in the corresponding indigenous language. Specialised care was provided in the community to all people diagnosed with rheumatic and other diseases and steps were taken to ensure the continuity of their care in their healthcare system.

RESULTS

The study was carried out from January 2011 to December 2015. A total of 6155 indigenous individuals participated in the study; 3757 (61%) were women. Sociodemographic variables by ethnic group are shown in table 1.

Thirty-five per cent of the population surveyed had MSK pain in the past 7 days and 45% had MSK historical pain, or pain at least once at some point in their lifetime (table 2).

Alcohol intake (22.5%), obesity (17.5%), depression (16%), hypertension (high blood pressure, HBP) (13.7%), smoking (13.3%), gastritis (13.3%), peripheral vascular disease (PVD 4.9%) and diabetes mellitus type 2 (DM2) (6.8%) were self-reported conditions reported (online supplementary table S2).

Rheumatologists diagnosed an RD in 2098 individuals (34%) (table 3).

Multivariate analysis showed the following variables associated with RD, RA, OA and back pain: age, being female, being employed, ethnicity, one or more comorbidities (DM2, HBP and heart disease), as well as a HAQ-Di score greater than 0.8 (table 4).

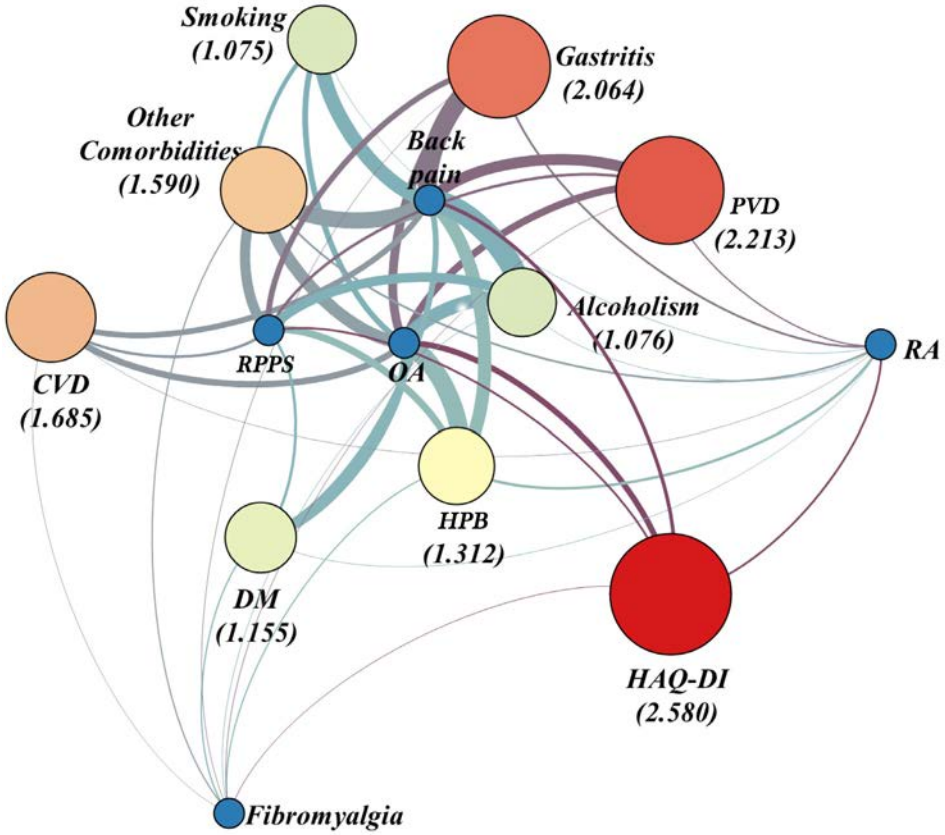


Figure 1 Network representation of the relation between comorbidities and clinical values. The figure shows the results of analysing the relations between the clinical variables used to obtain the OR and the variables for back pain, RA, RRPS, fibromyalgia and OA. In this network, the size and colour of the nodes are given by the OR value, for the variables without OR value a constant value of 1 is given to define their size and colour. The edge size is the intersection of users that have positive values in each pair of variables. DM, diabetes mellitus; HAQ-DI, Health Assessment Questionnaire-Disability Index; OA, osteoarthritis; RA, rheumatoid arthritis; RRPS, regional pain syndrome.

The relation between the clinical variables used to obtain the OR and the variables for back pain, RA, RRPS and OA is shown in figure 1. Such figure shows the network in which variables are used as nodes and the relationship between RD and non-RD. The node size is obtained from the prevalence value; the node colour shows the authority of the node (figure 1).

Network analysis highlights the syndemic relationship between the sociodemographic and epidemiological variables. Figure 2 shows an authority network with the following aspects: (1) Size (density) given by the number of subjects with the assessed condition. (2) Colour, representing a measure of authority or connectivity (the greater the connectivity, the greater the authority). Those with less authority are blue, those with the greatest authority are red. (3) Position, with the most centrally located nodes are those with the greatest authority and high individual connectivity to the nodes closer to them.

Red nodes: Being employed (node 1) was the node with the greatest authority, even higher than disability (node 10); people are able to work despite having a significant disability; male gender (nodes 19 and 20). Alcoholism[8] node was present in individuals with cardiovascular comorbidities (node 4), HBP (node 3) and DM2 (node 2). It was also highly linked to other socioeconomic variables. Disability (node 10) has high connectivity—found in all studied populations—although very few individuals described themselves as disabled (a paradoxical effect discussed elsewhere).

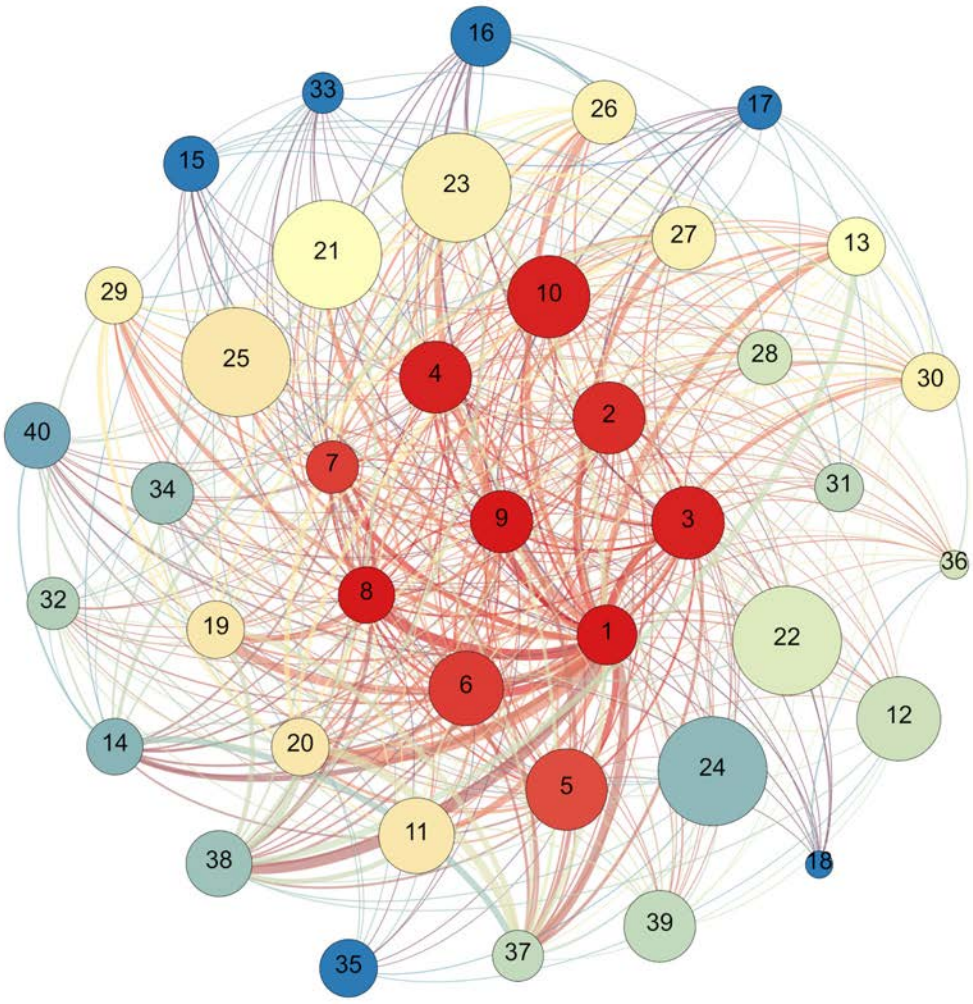
Green nodes: Clinical variables are located in the range of green areas, with node 37 representing the lack of social coverage in healthcare and is connected to all ethnic groups (nodes 11–18), all comorbidities (nodes 2–6), having a disability (node 10), but having most individuals a job (node 1).

Blue nodes: Node 40, which represents private healthcare, has less hierarchy but a stronger relationship with the Mixtecos (node 11) and the Mayas-Yucatecos (node 14) in Mexico. It is also related to comorbidities (nodes 2–8), employment (node 1) and disability (node 10), although this would lead to out-of-pocket expenses on healthcare and an impoverishment of these populations forced to pay for their own healthcare even though there is a healthcare institution available.

DISCUSSION

This study, conducted in eight indigenous communities of Mexico (Mixteco, Chontal, Maya-Yucateco and Rarámuris), Venezuela (Chaima, Kariña and Warao) and Argentina (Qom), demonstrated that MSK disorders and RD are highly prevalent.

RDs are highly prevalent in the indigenous populations we studied, which is related to high physical demands and longer times exposed to their negative impact on functioning. The high RD prevalence observed in this study aligns with findings from other countries such as Canada, Australia, New Zealand and USA, where RDs are more frequent in indigenous than in the non-indigenous populations.[42] In fact, the prevalence of RA in the population studied is one of the highest reported worldwide (1.3%) and is particularly high in the Qom group of Argentina (2.4%).[18] Low back pain and OA were the most common RD identified in



1	Working
2	DM (1.155)
3	HPB (1.312)
4	CVD (1.685)
5	PVD (2.213)
6	Gastritis (2.064)
7	Smoking (1.075)
8	Alcoholism (1.076)
9	Other Comorbidities (1.590)
10	HAQ-DI (2.580)
11	Ethnicity: Mixtecos (1.379)
12	Ethnicity: Chontal (2.356)
13	Ethnicity: Maya (0.730)
14	Ethnicity: Maya (0.730)
15	Ethnicity: Kariña (0.680)
16	Ethnicity: Chaima (1.000)
17	Ethnicity: Warao (0.495)
18	Ethnicity: Raramuri (0.219)
19	Gender: Male (0.94)
20	Gender: Female (1.00)
21	OA
22	RA
23	BACK PAIN
24	Fibromyalgia
25	RPPS
26	Education [0,2) years
27	Education [2,4) years
28	Education [4,6) years
29	Education [6,8) years
30	Education [8,10) years
31	Education [10,12) years
32	Education [12,14) years
33	Education [14,16) years
34	Education [16,18) years
35	Education >18 years
36	Education Not Specify
37	No healthcare
38	Partial public healthcare
39	Public healthcare
40	Private healthcare

Figure 2 Socioeconomical network analysis with respect to clinical variables. The figure shows the network analysis of the clinical variables together with the socioeconomic ones. In the network, the node size is given by the prevalence value normalising the number of rheumatic subjects classified in each variable with respect to the number of healthy subjects in the same variable. The colour shows the authority, measurement of how valuable the information at the node is by counting the number of nodes with edges to the node been measure. A node with a high authority value shows that it is connected to a large number of nodes, relative to the graph size, this serves as a way to show the usefulness of the information at the node. The position of the nodes is calculated using Fruchterman-Reingold: this method considers the graph as a mass particle system for the simulation. The nodes are the masses and the edges with the spring that connects them, the system minimises the energy of the particle system and the resulting network would have the nodes with the highest connection (highest value of the edges between nodes) closing together, while the nodes with the lowest relation are pushed outside. CVD, cardiovascular disease; DM, diabetes mellitus; HAQ-DI, Health Assessment Questionnaire-Disability Index; OA, osteoarthritis; RA, rheumatoid arthritis; RRPS, regional pain syndrome.

this study and this has been reported in other indigenous populations.[43] Both low back pain and OA are related to highly physically demanding lifestyles and the average age of RD onset in the studied populations was relatively young (around 40 years).[44] Considering that MSK disorders have shown to produce a great burden on disability-adjusted life-years,[45] it is fair to conclude that these indigenous peoples are affected by the disabling effects of RD for long times during their lifespan.

Our results also document a case of health inequality for Latin American indigenous groups. Thirty-three per cent of the studied population reported no access to any kind of healthcare and only 3% reported having complete social insurance (ie, insurance that covers all necessary health-related expenses). In addition, only half of participants reported seeking medical care to manage their RD, and 40.6% have never sought medical or traditional treatment despite the high prevalence of severe MSK pain, disability and poor coping. This situation aligns with other findings in the literature about a higher prevalence of RD, especially RA, in contexts of social disadvantage.[42] Not surprising, the participating indigenous communities in this study have the greatest indicators of poverty and disadvantage in their respective countries.[5 9 46]

The disabling effects of these chronic conditions are linked with the presence of comorbidities within a context of health inequities. These inequities are conditioned by social determinants of health that could be secondary to a problem of systematic neglected, including a poor understanding of epidemiological transitions in indigenous populations.

A syndemic approach is necessary to understand why RDs cause negative impact on indigenous communities in order to come up with effective solutions to previously unseen situation.

The concept of 'neglect' can be relevant in infectious diseases. These are referred to as neglected because they impact patients living in poverty and as such are systematically under-reported in

many countries and consequently are not perceived as a public health problem. Because many are not regarded as an epidemiological threat, these diseases largely go unnoticed by the media and the public sector, while the private sector also shows little interest in them as a target market.[7] On careful consideration, the concept of a 'neglected disease' can also be applied to MSK disorders.[7 46]

According to Stuckelberger's theory of the four 'transitions': demographic, epidemiological, sociocultural and technological.[47] Relating these concepts to the results of this study, we can see that our study populations are younger compared with the non-indigenous populations with MSK disorders; that the epidemiological transition is more in line with chronic diseases in adults. All these transitions lead to greater gaps in caring for chronic diseases, particularly MSK diseases.[48 49]

Studying RD in indigenous contexts involves carrying out a relational analysis, such as the network analysis done for this study, which suggests the use of a syndemic approach to RD. Determining the relationship between MSK disorders and other phenomena such as comorbidities, genetics and social factors should be recognised as a first step. Non-inflammatory MSK disorders are associated with high physical demand, overweight and obesity, and pose a risk of accelerating degenerative process of the joints. This is further exacerbated by healthcare inequity, fragmented healthcare systems and high vulnerability of the population, which is true for many indigenous communities in countries lacking public health strategies to prevent or reduce disability.[50 51] On the other hand, inflammatory MSK disorders such as RA are associated with heart disease, mental health conditions such as anxiety and depression, as well as with pain and physical, occupational and social disability. Other aspects are the genetic components, living in stressful environments, cooking with firewood, a predominance of the female gender, associated with discrimination for belonging to the indigenous population or not speaking the mainstream language. We therefore posit that a syndemic approach to the study of RD in a non-indigenous population would allow us to have a more comprehensive picture of how RDs are interacting with other biologic and social factors to create a major syndemic public health problem that requires the design of multidisease and multidimensional interventions on a global scale.[7 52]

The biomedical construction of disease is based on biological and quantitative conceptions, which relegates the social, cultural and subjective experiences of illness to a second level of importance. Nevertheless, our findings showed that sociocultural aspects are important for how RDs manifest and are managed in indigenous contexts. Consequently, our results suggest the importance of implementing culturally sensitive services to improve the health and quality of life of indigenous peoples with RDs as major responsibility of the state.

Limitations

This study has certain limitations arising from its cross-sectional design, enabling us to only describe the prevalence, which was the main objective of the study.

Strengths

The difficulty of carrying out epidemiological studies in indigenous populations stems from complex logistics involved in moving around entire teams of evaluators including rheumatologists, as well as doing laboratory and imaging studies needed to confirm the diagnoses.

It is important to highlight the problems faced by these populations as they have a higher prevalence of RDs, which are not recognised as a public health risk. The problem lies in the concept of indigenous population in different countries and the way they are identified in these censuses.[40 41] A combination of definitions was used in this study, which we consider to be one of its strengths.

CONCLUSIONS

The overall prevalence of MSK disorders in Latin American indigenous communities was 34.5%. Although low back pain and OA were the most prevalent RD, wide variations according to population groups occurred. The prevalence of RA, especially in the indigenous Qom group of Argentina, is among the highest reported worldwide. Understanding the relationship between RDs, comorbidities and socioeconomic conditions in indigenous populations requires a syndemic understanding of RD's negative impacts on health.

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Data sharing statement The data are available but must be requested from the researchers representing the GLADERPO Group (IPB and BAPE) through a specific application request for the use of data, which will be evaluated by the GLADERPO Group.

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EXTENDED REPORT

Biologic refractory disease in rheumatoid arthritis: results from the British Society for Rheumatology Biologics Register for Rheumatoid Arthritis

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ABSTRACT

Objectives Biologic disease-modifying antirheumatic drugs (bDMARDs) have revolutionised treatment and outcomes for rheumatoid arthritis (RA). The expanding repertoire allows the option of switching bDMARD if current treatment is not effective. For some patients, even after switching, disease control remains elusive. This analysis aims to quantify the frequency of, and identify factors associated with, bDMARD refractory disease.

Methods Patients with RA starting first-line tumour necrosis factor inhibitor in the British Society for Rheumatology Biologics Register for RA from 2001 to 2014 were included. We defined patients as bDMARD refractory on the date they started their third class of bDMARD. Follow-up was censored at last follow-up date, 30 November 2016, or death, whichever came first. Switching patterns and stop reasons of bDMARDs were investigated. Cox regression identified baseline clinical factors associated with refractory disease. Multiple imputation of missing baseline data was used.

Results 867 of 13 502 (6%) patients were bDMARD refractory; median time to third bDMARD class of 8 years. In the multivariable analysis, baseline factors associated with bDMARD refractory disease included patients registered more recently, women, younger age, shorter disease duration, higher patient global assessment, higher Health Assessment Questionnaire score, current smokers, obesity and greater social deprivation.

Conclusions This first national study has identified the frequency of bDMARD refractory disease to be at least 6% of patients who have ever received bDMARDs. As the choice of bDMARDs increases, patients are cycling through bDMARDs quicker. The aetiopathogenesis of bDMARD refractory disease requires further investigation. Focusing resources, such as nursing support, on these patients may help them achieve more stable, controlled disease.

INTRODUCTION

Biologic disease-modifying antirheumatic drugs (bDMARDs) have revolutionised treatment pathways for rheumatoid arthritis (RA) management, improving outcomes for patients who do not tolerate or respond to conventional synthetic (cs) DMARDs. However, for some patients, even after multiple bDMARDs, disease control is unachievable with so-called 'difficult-to-treat'[1] or bDMARD refractory disease.[2]

The repertoire of bDMARDs is continually expanding. Tumour necrosis factor inhibitors (TNFi) remain the first-line bDMARD for patients with RA.[3] There are additional cytokine-targeted therapies licensed for RA, including interleukin (IL)-6 pathway inhibitors, IL-1 receptor antagonists, cell-targeted B-cell depleting agents and T-cell costimulation blockers. Some patients may fail their bDMARD due to ineffectiveness, either true lack of effect or non-adherence, adverse effects or intolerance. The National Institute for Health and Care Excellence (NICE) have published guidance recommending rituximab in patients who have failed at least one TNFi unless contraindicated.[4] With increasing treatment options, patients may cycle through several bDMARDs, although the precise extent to which this occurs in clinical practice is unknown. As further bDMARDs are introduced for RA, it also challenges the definition of bDMARD refractory disease, both in clinical and research settings. This is an important area of investigation as there are no current guidelines on optimal bDMARD sequencing beyond a second bDMARD.[4]

The British Society for Rheumatology Biologics Register for RA (BSRBR-RA) is a national ongoing treatment register, capturing bDMARD exposures, treatment response and adverse effects across a large population of patients with RA from the UK. This unique setting may improve understanding of bDMARD refractory disease. The specific analysis objectives were to (1) quantify what proportion of patients starting their first TNFi will subsequently exhibit bDMARD refractory disease, (2) describe bDMARD treatment patterns over time and reasons for sequential use in these patients, and (3) identify clinical predictors of bDMARD refractory disease early in the bDMARD treatment pathway.

METHODS

Study setting

The BSRBR-RA, established in 2001, is a national prospective observational cohort study. It collects data of adults with a physician's diagnosis of RA starting a bDMARD. The overall aim of the register is to monitor long-term safety of bDMARDs in the clinical setting. At start of therapy, baseline data are collected including demographics (age, gender, height, weight, smoking status, comorbidities), disease characteristics (disease duration, rheumatoid factor (RF) status, joint erosions on X-ray),

disease activity (swollen and tender joint count, patient global assessment, erythrocyte sedimentation rate (ESR) and/or C reactive protein) and 28-joint disease activity score (DAS28),[5] Health Assessment Questionnaire (HAQ)[6] for patient function, Medical Outcomes Study 36-item short form health survey (SF-36),[7] and current or previous antirheumatic therapies. Follow-up data on disease activity, disease function and antirheumatic therapies are collected every 6 months for 3 years, with disease activity and antirheumatic therapy data collected annually thereafter. Full details of the BSRBR-RA methodology have been published previously.[8] Ethics approval for the BSRBR-RA was granted by the North West Multicentre Research Ethics Committee in December 2000 (MREC 00/8/53). No additional ethical approval was required for the current analysis.

Exposure to bDMARDs

Patients starting TNFi were recruited from 2001 to 2008, and again from 2011 onwards. This analysis included all patients starting a TNFi as their first bDMARD between 1 October 2001 (study start) until 30 November 2014 (2 years prior to analysis cut-off date to allow sufficient follow-up). NICE allow bDMARD treatment for patients with RA with DAS28 >5.1 despite treatment with at least two csDMARDs.[3] For each patient, the total number of bDMARD treatment courses was identified, irrespective of bDMARD class or whether the bDMARD had been received previously. Subsequently, for each patient, all treatment courses were reviewed and clustered according to bDMARD class: TNFi (adalimumab, certolizumab, etanercept, golimumab, infliximab), B-cell-depleting agent (rituximab or ocrelizumab), IL-1 receptor antagonist (anakinra), IL-6 pathway inhibitor (tocilizumab) and T-cell costimulation blocker (abatacept). Patients who had been exposed to at least three different classes of bDMARD (irrespective of reason for failure to prior bDMARD) were classified as 'bDMARD refractory'. The number of bDMARDs that patients were exposed to, as well as the number of bDMARD classes, are presented in online supplementary table 1.

Statistical analysis

Follow-up started on the date of first TNFi exposure. Patients were defined as bDMARD refractory on the date they started their third class of bDMARD. Patients were censored at their last follow-up date, 30 November 2016 (analysis cut-off), or date of death, whichever came first. Switching patterns and reasons for stopping bDMARDs were presented for all bDMARD refractory patients. Kaplan-Meier analysis was used to quantify bDMARD refractory disease. Body mass index (BMI) was calculated for each patient. Data outside the BMI range of 14 to 50 were assumed incorrect. Obesity was classified if BMI was 30 or greater.[9] Index of multiple deprivation (IMD) quintiles were calculated for England,[10] Scotland[11] and Wales[12] separately, then combined into an overall IMD quintile score. Quintile scores for Northern Ireland were unavailable. Cox regression analysis was used to identify baseline clinical factors associated with bDMARD refractory status. Results were presented as HRs with 95% CI. The SF-36 physical component score was excluded from the multivariable analysis due to the strong association with HAQ (correlation 0.6). A sensitivity analysis including patients recruited from 2011 onwards was completed.

Multiple imputation

Multiple imputation (49 iterations based on proportion of incomplete cases[13]) was used to account for missing baseline covariate data. Complete variables included bDMARD refractory status, registration year, registered TNFi, gender, age at first TNFi, comorbidities and follow-up time until failure or end of study. Imputed values included disease duration at start of first TNFi, tender joint count, swollen joint count, physician global assessment, ESR, DAS28, HAQ, SF-36 physical component score, SF-36 mental component score, RF status, erosions on X-ray, smoking status and BMI. Stata V.13 was used to perform all analyses.[14]

RESULTS

Baseline characteristics

A total of 13 502 patients were registered at start of first TNFi between 2001 and 2014 (table 1), the majority recruited in the first 8 years (86%); 76% women, median age 57 years (IQR 49–65), median disease duration 10 years (IQR 5–18). Disease activity and severity at the start of first TNFi was high; median DAS28 6.5 (IQR 5.8–7.2), median HAQ 2.0 (IQR 1.6–2.4). Over half (53%) reported at least one comorbidity at start of first TNFi, and 22% were current smokers.

bDMARD refractory patients

Over 111 034 person-years of follow-up, 867 (6.4%) patients were classified as bDMARD refractory (exposed to at least three different classes of bDMARD); median time from first TNFi to bDMARD refractory disease 7.9 years (95% CI 5.7 to 10.0) (figure 1). A higher proportion (6.7%) of patients from the earlier recruitment cohort (2001–2008) had bDMARD refractory disease with a longer median time of 8.4 years (95% CI 6.6 to 10.2) to refractory status. In contrast, 4.8% of patients in the 2011–2014 cohort were bDMARD refractory over a shorter median time of 2.0 years (95% CI 1.4 to 2.6). Overall, patients with bDMARD refractory disease remained on their first TNFi for a median of 3.9 years (IQR 1.5–6.6); longer in the earlier recruitment cohort (4.4 years vs 0.8 years, respectively). Reasons for patients stopping their first TNFi were 452 (52%) for ineffectiveness, 205 (24%) following adverse events, 29 (3%) for other reasons (mostly patient choice due to injection-related problems or family planning) and 181 (21%) not recorded. Stop reasons were similar between the recruitment cohorts. Overall, 331 (38%) reported repeated ineffectiveness, 95 (11%) reported repeated adverse events, 383 (44%) reported a mixture of stop reasons, while 58 (7%) had missing stop reasons. Patients with bDMARD refractory disease then spent a median of 1.5 years on their second class (IQR 0.8–2.6) and 1.5 years on their third class of bDMARD (IQR 0.8–2.8), although this was longer in patients recruited 2001–2008 compared with 2011 onwards; 1.5 versus 0.8 years, and 1.6 versus 1.0 years for second and third bDMARD class, respectively. Overall, 5% of the bDMARD refractory patients died over follow-up, lower compared with the remaining population (11%).

bDMARD treatment pathways

The majority of bDMARD refractory patients switched to a B-cell-depleting agent as their second class of bDMARD (n=718; 83%), although the proportion reduced after 2011 (66% vs 85%; p<0.001) (figure 2). The two most common class-switching pathways was from TNFi to B-cell-targeted agent rituximab (aside from use of ocrelizumab in two patients) to either IL-6-targeted agent tocilizumab (n=514; 59%) or T-cell costimulation blocker abatacept (n=204; 24%). Many bDMARD refractory patients had been exposed to multiple bDMARDs within each class. More patients recruited in the earlier years had received at least one more TNFi before switching to their second class of bDMARD (59% vs 19%;

Table 1 Baseline characteristics of all 13 502 patients in the BSRBR-RA starting a first-line TNFi between 2001 and 2014

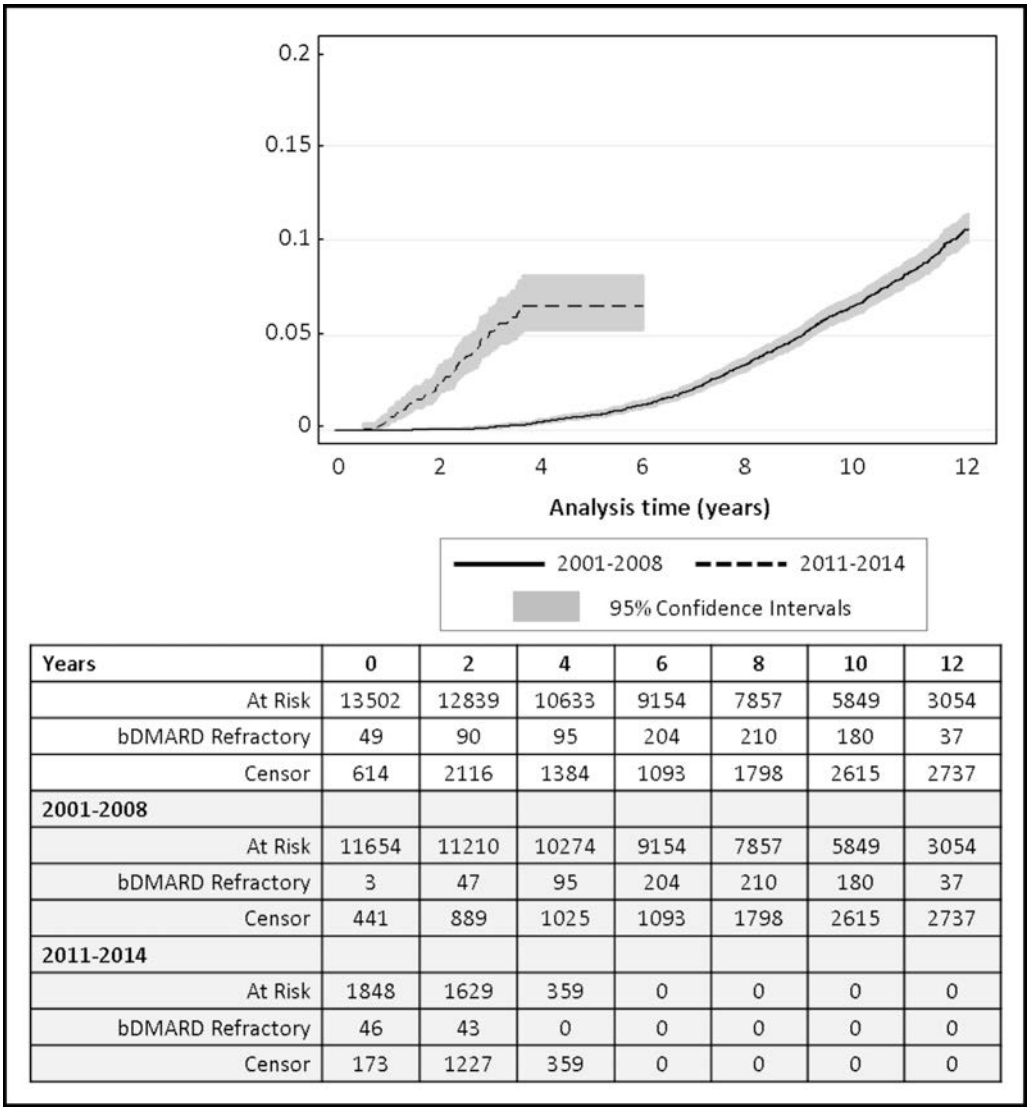
	All patients	bDMARD refractory	Remaining patients
N	13 502	867	12 635
First TNFi (n=13 502)			
Etanercept	4612 (34%)	285 (33%)	4327 (34%)
Infliximab	3794 (28%)	246 (28%)	3548 (28%)
Adalimumab	4322 (32%)	391 (34%)	4031 (32%)
Certolizumab	774 (6%)	45 (5%)	729 (6%)
Registration year (category) (n=13 502)	–	–	–
2001–2008	11 654 (86%)	778 (90%)	10 876 (86%)
2011–2014	1848 (14%)	89 (10%)	1759 (14%)
Women (n=13 502)	10 269 (76%)	705 (81%)	9564 (76%)
Age (years) (n=13 502)	57 (49 to 65)	52 (44 to 59)	58 (49 to 66)
Age (category) (n=13 502)	–	–	–
16–50	3888 (29%)	381 (44%)	3507 (28%)
51–90	9614 (71%)	486 (56%)	9128 (72%)
Disease duration (years) (n=13 360)	10 (5 to 18)	9 (4 to 16)	10 (5 to 18)
Disease duration (category) (n=13 360)	–	–	–
0–10	6835 (51%)	494 (57%)	6341 (51%)
11–72	6514 (49%)	368 (43%)	6157 (49%)
Concurrent methotrexate (n=13 502)	8537 (63%)	578 (67%)	7959 (63%)
Concurrent steroids (n=13 502)	5620 (42%)	364 (42%)	5256 (42%)
Total comorbidities† (n=13 502)	–	–	–
None	6327 (47%)	408 (47%)	5919 (47%)
1 comorbidity	4589 (34%)	294 (34%)	4295 (34%)
2 comorbidities	1894 (14%)	122 (14%)	1772 (14%)
3+ comorbidities	692 (5%)	43 (5%)	649 (5%)
Smoking status (n=13 351)	–	–	–
Current smoker	2899 (22%)	248 (29%)	2651 (21%)
Ex-smoker	5068 (38%)	284 (33%)	4784 (38%)
Never smoked	5384 (40%)	330 (38%)	5054 (40%)
Body mass index (kg/m^2) (n=11 499*)	26 (23 to 30)	26 (23 to 31)	26 (23 to 30)
Obese (body mass index ≥30) (n=11 499*)	2951 (26%)	224 (30%)	2727 (25%)
Disease activity	–	–	–
Tender joint count (range 0–28) (n=13 091)	15 (10 to 22)	16 (11 to 23)	15 (10 to 21)
Swollen joint count (range 0–28) (n=13 083)	10 (6 to 15)	11 (7 to 16)	10 (6 to 15)
Patient global assessment (range 0–10 cm) (n=13 000)	7.5 (6.2 to 8.7)	7.8 (6.6 to 9.0)	7.5 (6.1 to 8.6)
ESR (mm/s) (n=12 084*)	38 (22 to 62)	36 (22 to 60)	38 (22 to 62)
CRP (mm/s) (n=5274*)	27 (12 to 57)	28 (11 to 56)	27 (12 to 57)
DAS28 (range 0–10) (n=13 255)	6.5 (5.8 to 7.2)	6.6 (5.9 to 7.3)	6.5 (5.8 to 7.2)
HAQ (range 0–3) (n=12 364*)	2.0 (1.6 to 2.4)	2.1 (1.8 to 2.5)	2.0 (1.6 to 2.4)
SF-36: Physical Component Score‡ (n=8702*)	15 (10 to 21)	14 (10 to 19)	15 (10 to 21)
SF-36: Mental Component Score‡ (n=8702*)	42 (34 to 51)	40 (32 to 50)	42 (34 to 51)
Index of multiple deprivation (excluding Northern Ireland) (n=12 711*)	–	–	–
Lowest quintile (most deprived)	2082 (16%)	165 (20%)	1917 (16%)
Middle 3 quintiles	8008 (63%)	494 (61%)	7514 (63%)
Highest quintile (least deprived)	2621 (21%)	147 (18%)	2474 (21%)

Results presented as N (%) or median (IQR).
*More than 5% missing data.
†Total comorbidities—hypertension, ischaemic heart disease, stroke, lung disease, renal disease, diabetes, depression, liver disease. ‡SF-36; greater score indicates better health.
bDMARD, biologic disease-modifying antirheumatic drug; BSRBR-RA, British Society for Rheumatology Biologics Register for rheumatoid arthritis; CRP, C reactive protein; DAS28, 28-joint Disease Activity Score; ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; SF-36, 36-item Short Form Survey for quality of life; TNFi, tumour necrosis factor-alpha inhibitor.

p<0.001). Most bDMARD refractory patients reported use of four different bDMARDs (n=328; 38%), 173 (20%) used five and 72 (8%) reported use of at least six different bDMARDs. Twenty per cent of the bDMARD refractory patients reported more than three classes of bDMARDs.

Factors associated with bDMARD refractory disease

In the multivariable analysis (table 2), bDMARD refractory disease was associated with women (HR 1.3; 95% CI 1.1 to 1.5), younger age (HR 0.6 for age >50 years; 95% CI 0.5 to 0.7), shorter disease duration (HR 0.8 for disease duration >10



Years	0	2	4	6	8	10	12
At Risk	13502	12839	10633	9154	7857	5849	3054
bDMARD Refractory	49	90	95	204	210	180	37
Censor	614	2116	1384	1093	1798	2615	2737
2001-2008							
At Risk	11654	11210	10274	9154	7857	5849	3054
bDMARD Refractory	3	47	95	204	210	180	37
Censor	441	889	1025	1093	1798	2615	2737
2011-2014							
At Risk	1848	1629	359	0	0	0	0
bDMARD Refractory	46	43	0	0	0	0	0
Censor	173	1227	359	0	0	0	0

Figure 1 Cumulative incidence plot (95% CIs) of when patients acquire biologic disease-modifying antirheumatic drug (bDMARD) refractory disease (point of starting their third class of bDMARD) (n=13 502). Stratified by recruitment year: prior to 2001–2008 (solid line; n=11 654) and 2011–2014 (dashed line; n=1848).

years; 95% CI 0.7 to 0.95), higher patient global assessment (HR 1.1 per cm; 95% CI 1.0 to 1.1), higher HAQ (HR 1.3 per unit; 95% CI 1.1 to 1.5), current smoking (HR vs never 1.5; 95% CI 1.2 to 1.7) and obesity (HR 1.2 for BMI ≥30; 95% CI 1.0 to 1.4) at the start of first TNFi. Notably, the HR for developing bDMARD refractory disease was 15 times higher (95% CI 10 to 21) among patients recruited from 2011 onwards compared with 2001–2008. A further subanalysis that included social deprivation scores for England, Scotland and Wales also identified that patients in the lowest IMD quintile, representing the highest level of deprivation, were associated with bDMARD refractory disease (HR compared with all remaining patients 1.2; 95% CI 1.0 to 1.4). A sensitivity analysis of the 1848 patients in the 2011 to 2014 recruitment cohort supported the findings that shorter disease duration and worse HAQ were associated with bDMARD refractory RA (see online supplementary table 2).

DISCUSSION

This is the first observational study to evaluate the extent of bDMARD refractory RA, defined as exposed to at least three different classes of bDMARD. Approximately 6% of patients who started TNFi as their first bDMARD were subsequently classified as bDMARD refractory. This important observation provides information that rheumatologists can use to encourage healthcare providers to address refractory patients. Quantifying the frequency of multiple bDMARD class failure is crucial, particularly in an environment where bDMARD choice is largely based on custom and experience rather than by individual biomarkers. As response to subsequent bDMARDs is known to reduce,[15 16] targeted personalised pathways are important to identify. This knowledge can therefore drive clinical guideline development as well as inform cost-effectiveness analyses.

Prior research has suggested that patients achieve a better clinical response when switching from a first-line TNFi to rituximab compared with a second TNFi.[17] However, many patients in the current study were recruited between 2001 and 2008 when other classes of bDMARDs were not readily available (NICE published guidance on the use of rituximab for RA in August 2007[18] and abatacept in April 2008[19]), resulting in TNFi cycling in 59% of patients compared with only 19% in patients recruited from 2011 onwards. The majority of patients switched from TNFi to rituximab, then to either tocilizumab or abatacept,

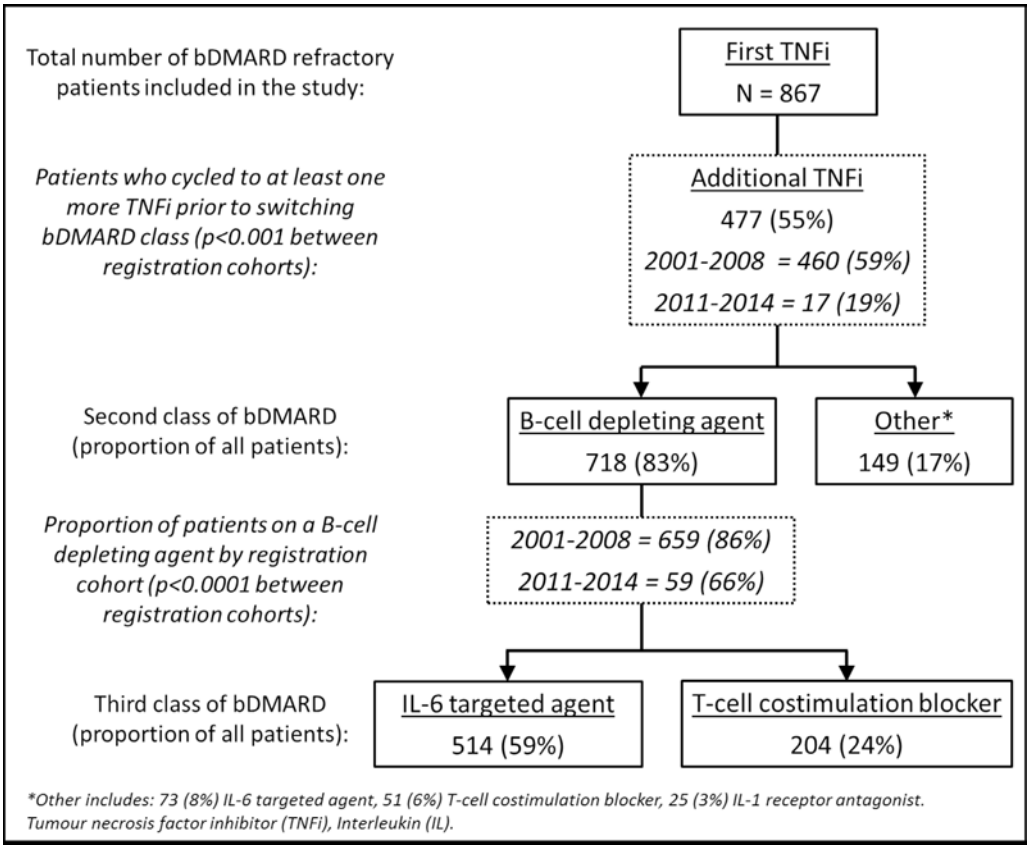


Figure 2 Main pattern of biologic disease-modifying antirheumatic drug (bDMARD) class switching in the 867 bDMARD refractory patients.

largely reflecting the order in which these drugs became available. In more recent years, there appears to have been a move away from rituximab as a second-line bDMARD class (66% compared with 85% in the earlier cohort), although our study was not designed to explore the reasons for temporal trends. Further work comparing effectiveness across different second-line therapies in large datasets is warranted.

As would be expected, the patients recruited earlier within the study (2001–2008) with a longer follow-up were more likely to be classified with bDMARD refractory disease compared with those recruited more recently (2011–2014) with shorter follow-up (6.7% vs 4.8%). However, the patients recruited more recently were 15 times more likely to have bDMARD refractory disease compared with those recruited in the earlier years. This may indicate that if these more recent patients were followed up to the same degree, the proportion classified as bDMARD refractory will likely increase over time. The explanation is likely due to increased class availability and higher expectations of bDMARDs in the more recent cohort, a result of selection bias rather than a true biologic effect. The multivariable analysis aimed to take this temporal change into account. Consequently, the burden of bDMARD refractory disease in patients recruited in the earlier years of this study are very likely an underestimate.

This analysis identified that patients from lower socioeconomic areas were more likely to develop bDMARD refractory disease. It has been previously reported that people from more deprived areas are more likely to smoke and have a higher BMI,[20] known factors associated with drug adverse events and ineffectiveness.[1] All three variables were found to be independent predictors of bDMARD refractory disease in our analysis. Smoking has been previously reported in association with poorer clinical response to TNFi,[21] perhaps due to an association with high concentrations of proinflammatory cytokines.[22 23] The proinflammatory environment associated with adipose tissue may similarly cause obese patients to respond less favourably to treatment compared with patients with a normal BMI.[1] It may also influence the exposure to bDMARDs, especially those with a fixed dose regimen. The basis for social deprivation and association with a higher rate of bDMARD refractory disease is not immediately obvious, but may relate to other unmeasured factors such as comorbidities and/or poor adherence. Poor adherence is strongly associated with lesser DAS28 responses to bDMARDs.[24] Unfortunately, the BSRBR-RA does not currently capture a measure of adherence. Some of the study patients may be refractory due to non-adherence. However, identifying these patients remains important regardless of the reasons for being bDMARD refractory.

Additional factors independently associated with bDMARD refractory disease were female gender, younger age, shorter disease duration, poorer patient global assessment and worse physical function. It has been reported previously that men are more likely to achieve DAS28 remission on bDMARDs.[21 25 26] Younger patients were perhaps treated more aggressively leading to increased switching between bDMARD classes, with more caution practised in the treatment of older patients. The association between shorter disease duration and higher probability of bDMARD refractory disease is interesting, and may reflect current practice of early DMARD introduction and treatment to target, to improve outcomes.[27 28] Function in established RA largely reflects joint damage, which is not reversed with current bDMARDs; it also associates with a poorer patient global assessment. This may drive persistently high DAS28 (in the absence of clear signs of inflammation), and thus bDMARD class switching and refractory disease status. Additionally, in the sensitivity

Table 2 Univariable and multivariable analysis (imputed data, 49 datasets)—HRs for acquiring bDMARD refractory disease

	Univariable HR	Multivariable HR	Multivariable HR (including IMD)*
Registration year (2011–2014 vs 2001–2008)	15 (11 to 20); p<0.001	15 (10 to 21); p<0.001	17 (11 to 24); p<0.001
Women (vs men)	1.3 (1.1 to 1.5); p=0.004	1.3 (1.1 to 1.5); p=0.009	1.2 (1.0 to 1.5); p=0.04
Age, years (>50 vs ≤50)	0.6 (0.5 to 0.7); p<0.001	0.6 (0.5 to 0.7); p<0.001	0.6 (0.5 to 0.7); p<0.001
Disease duration, years (>10 vs ≤10)	0.7 (0.6 to 0.8); p<0.001	0.8 (0.7 to 0.95); p=0.008	0.8 (0.7 to 0.9); p=0.004
RF positive (vs negative)	1.1 (0.9 to 1.3); p=0.3	1.1 (1.0 to 1.3); p=0.1	1.1 (0.9 to 1.3); p=0.2
Erosions on X-ray (vs negative)	0.8 (0.7 to 1.0); p=0.01	1.0 (0.8 to 1.1); p=0.8	1.0 (0.8 to 1.1); p=0.7
Methotrexate at registration (vs none)	1.1 (0.9 to 1.2); p=0.3	1.0 (0.9 to 1.2); p=0.6	1.0 (0.9 to 1.2); p=0.9
On steroids at registration (vs none)	1.0 (0.8 to 1.1); p=0.6	1.0 (0.9 to 1.2); p=0.5	1.1 (0.9 to 1.2); p=0.4
Tender joint count (per joint)	1.02 (1.01 to 1.02); p=0.001	1.0 (1.0 to 1.0); p=0.3	1.0 (1.0 to 1.0); p=0.3
Swollen joint count (per joint)	1.0 (1.0 to 1.0); p=1.0	1.0 (1.0 to 1.0); p=0.9	1.0 (1.0 to 1.0); p=1.0
Patient global assessment (cm)	1.1 (1.1 to 1.1); p<0.001	1.1 (1.0 to 1.1); p=0.007	1.1 (1.0 to 1.1); p=0.02
ESR (mm/h)	0.996 (0.994 to 0.999); p=0.003	1.0 (1.0 to 1.0); p=0.6	1.0 (1.0 to 1.0); p=0.3
DAS28 (whole unit)	1.1 (1.0 to 1.1); p=0.1	1.0 (0.8 to 1.2); p=0.8	1.0 (0.8 to 1.3); p=0.8
HAQ (whole unit)	1.2 (1.1 to 1.3); p=0.005	1.3 (1.1 to 1.5); p=0.001	1.2 (1.1 to 1.4); p=0.003
Total comorbidities† (vs none)			
1 comorbidity	1.0 (0.9 to 1.2); p=0.6	1.1 (0.9 to 1.3); p=0.3	1.1 (0.9 to 1.3); p=0.2
2 comorbidities	1.2 (0.9 to 1.4); p=0.2	1.2 (0.9 to 1.4); p=0.2	1.1 (0.9 to 1.4); p=0.2
3+ comorbidities	1.3 (1.0 to 1.8); p=0.09	1.3 (0.9 to 1.8); p=0.1	1.2 (0.9 to 1.7); p=0.3
Smoke status (vs never smoked)			
Current smoker	1.5 (1.3 to 1.7); p<0.001	1.5 (1.2 to 1.7); p<0.001	1.4 (1.2 to 1.7); p<0.001
Ex-smoker	1.0 (0.8 to 1.1); p=0.7	1.1 (0.9 to 1.3); p=0.4	1.0 (0.8 to 1.2); p=1.0
Obese (body mass index ≥30)	1.3 (1.1 to 1.5); p=0.001	1.2 (1.0 to 1.4); p=0.047	1.2 (1.0 to 1.4); p=0.04
SF-36: Physical Component Score	0.97 (0.95 to 0.99); p=0.01	–	–
SF-36: Mental Component Score	0.98 (0.96 to 1.0); p=0.1	1.0 (1.0 to 1.0); p=0.4	1.0 (1.0 to 1.0); p=0.5
IMD (excluding Northern Ireland) (all other patients as referent)			
Lowest quintile (more deprived)	1.4 (1.2 to 1.7); p<0.001	–	1.2 (1.0 to 1.4); p=0.03

Results are presented as HRs with 95% CIs.
*Patients with IMD data, excluding Northern Ireland (n=12 711).
†Total comorbidities—hypertension, ischaemic heart disease, stroke, lung disease, renal disease, diabetes, depression, liver disease.
bDMARD, biologic disease-modifying antirheumatic drug; DAS28, 28-joint Disease Activity Score (higher score indicates worse health); ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire (higher score indicates worse health); IMD, index of multiple deprivation; RF, rheumatoid factor; SF-36, 36-item Short Form Survey for quality of life (higher score indicates improved health); TNFi, tumour necrosis factor-alpha inhibitor.

analysis of the more recent recruitment cohort, despite reduced power, both disease duration and HAQ remained significant findings.

This analysis was set within one of the largest cohorts of patients with RA starting bDMARDs in the world. However, this analysis should be repeated in other countries where access to bDMARD drugs may differ.[29] Patients starting a non-TNFi as first bDMARD were excluded from this analysis as use is low within the BSRBR-RA and is often due to contraindications to TNFi, providing a less representative patient population. One of the main limitations of this study is that sufficient follow-up time is needed for bDMARD refractory disease to reveal itself and, by definition, multiple bDMARDs must be available for patients to try. The survival method used to calculate the proportion of patients with refractory disease accounted for these variable follow-up times. However, it cannot account for the fact that patients may have died prior to the availability of a second or third class of bDMARD and therefore our calculation of the proportion of patients with bDMARD refractory disease is likely a minimum estimate. Hence, mortality seems lower in bDMARD refractory patients. In addition, these patients could have lower mortality because they are seen more frequently in clinic, thus significant health problems may be identified and treated earlier.

There is currently no accepted definition of bDMARD refractory RA. We defined bDMARD refractory disease as exposure to at least three different classes of bDMARDs to differentiate them from bDMARD non-responders to a single class of drug. As different bDMARDs target different components of the immune system, it may be that disease activity is driven by different pathways between individual patients. Therefore, non-response to a single bDMARD class may not represent true bDMARD refractory disease. While other definitions were considered, development of a specific definition of bDMARD refractory RA was not the remit of this specific analysis. Defining 'difficult-to-treat' RA is one of the current aims of a recently convened European League Against Rheumatism task force.[30] We did not consider only those stopping for ineffectiveness in our analysis, which may be a limitation, but as an initial analysis of this important topic we elected to include all patients in order to describe the full burden of patients requiring multiple bDMARDs. In addition, it was not possible to confirm the response to treatment in those who stopped for adverse events due to missing data. The BSRBR-RA does not capture serological samples, and therefore no measures of drug levels or antidrug antibodies were possible to further delineate reasons for ineffectiveness of therapies. Finally, with the continued introduction of newer targeted (including biologic) DMARD therapies, such as the new kinase inhibitors, it also challenges the definition of when a patient should be classified as being truly bDMARD refractory.

In conclusion, this study has estimated that approximately 6% of patients who start a first-line TNFi will experience bDMARD refractory disease. This is possibly an underestimate as it excludes patients who died, and who persisted with initial therap(ies) or did not start subsequent therapies for reasons such as comorbidity. Overall, our analysis supports recent recommendations for difficult-to-treat patients with RA where evaluation of modifiable lifestyle factors such as obesity and smoking are important.[1] Continued study of these patients is essential, particularly due to the lack of data available from randomised controlled trials of optimal treatment strategies.[31] A better understanding of bDMARD refractory disease should help to better target expensive therapies to those patients who are most likely to respond, developing hand in hand with stratified and personalised medicine approaches.

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Author note The BSR commissioned the BSR Biologics Register in Rheumatoid Arthritis (BSRBR-RA) as a UK-wide national project to investigate the safety of biologic and other targeted therapies in routine medical practice. KH is the principal investigator. The BSR currently receives restricted income from UK pharmaceutical companies, including Abbvie, Celltrion, Eli Lilly, Pfizer, Roche, Samsung Bioepis, Sandoz, Sanofi, UCB and in the past Hospira, MSD and Swedish Orphan Biovitrum (SOBI). This income finances a wholly separate contract between the BSR and The University of Manchester to host the BSRBR-RA. The principal investigator and the BSRBR-RA team at the University of Manchester have full academic freedom and are able to work independently of pharmaceutical industry influence. All decisions concerning analyses, interpretation and publication are made autonomously of any industrial contribution. Members of the BSRBR-RA University of Manchester team, BSR trustees, committee members and staff complete an annual declaration in relation to conflicts of interest. All relevant information regarding serious adverse events outlined in the manuscript have been reported to the appropriate pharmaceutical company as per the contractual agreements/standard operating procedures.

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EXTENDED REPORT

Inequity in access to bDMARD care and how it influences disease outcomes across countries worldwide: results from the METEOR-registry

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ABSTRACT

Objective To establish in a global setting the relationships between countries' socioeconomic status (SES), measured biological disease modifying antirheumatic drug (bDMARD)-usage and disease outcomes. To assess if prescription and reimbursement rules and generic access to medication relates to a countries' bDMARD-usage.

Methods Data on disease activity and drug use from countries that had contributed at least 100 patients were extracted from the METEOR database. Mean disease outcomes of all available patients at the final visit were calculated on a per-country basis. A questionnaire was sent to at least two rheumatologists per country inquiring about DMARD-prices, access to treatment and valid regulations for prescription and reimbursement.

Results Data from 20 379 patients living in 12 different countries showed that countries' SES was positively associated with measured disease activity (meanDAS28), but not always with physical functioning (HAQ-score). A lower country's SES, stricter rules for prescription and reimbursement of bDMARDs as well as worse affordability of bDMARDs were associated with lower bDMARD-usage. bDMARD-usage was negatively associated with disease activity (although not with physical functioning), but the association was moderate at best.

Conclusions Disease activity in patients with rheumatoid arthritis as well as bDMARD-usage varies across countries worldwide. The (negative) relationship between countries' bDMARD-usage and level of disease activity is complex and under the influence of many factors, including—but not limited to—countries' SES, affordability of bDMARDs and valid prescription and reimbursement rules for bDMARDs.

INTRODUCTION

Earlier diagnosis and treatment, the implementation of treat-to-target and new treatment options, including biological disease modifying antirheumatic drugs (bDMARDs), have improved treatment and prognosis of patients with rheumatoid arthritis (RA) tremendously.[1–3] Since many of these treatments are costly, patients across the world may not benefit similarly. Indeed, a lower level of welfare has been associated with higher disease activity in patients with RA in the past.[4]

One of the potentially critical factors is poorer access to bDMARDs.[2 5] Current recommendations advise starting bDMARDs after a first conventional synthetic disease modifying antirheumatic drug (csDMARD) strategy has failed.[5] But such a strategy may not be feasible in greater parts of the world. In many countries, there are various restrictions in the prescription and reimbursement of bDMARD.[6–9] Within Europe, differences in socioeconomic welfare are associated with differences in prescription and reimbursement of bDMARDs.[6 10] Stricter prescription rules and reimbursement criteria of bDMARDs may result in more infrequent use of bDMARDs and in worse health outcomes.[6 9] To date, only one study, limited to European countries, has been performed that has taken into account all currently available bDMARDs.[6]

We have investigated here daily-practice data regarding bDMARD-use in different countries worldwide and have assessed if a lower country's socioeconomic status (SES) is associated with worse clinical outcomes and lower bDMARD-usage. We have also assessed if countries' bDMARD-usage was associated with stricter prescription and reimbursement rules and worse access to medication.

METHODS

Data selection

Disease activity and medication use in patients with RA in various countries on various treatments were extracted from the METEOR registry, an international database capturing data of daily clinical practice of patients with a clinical diagnosis of RA.[11] Visits were unprotocolled and data were gathered retrospectively and anonymously; hence no informed consent was needed. We selected visits after 1-1-2000 from countries that had included at least 100 patients with follow-up data available (supplementary file 1).

Missing data on disease activity and function (HAQ-score) were imputed using multivariate normal imputation (30 imputations).[12] For each country, average DAS28 and HAQ and the proportion of patients in DAS28-remission (DAS28 <2.6) were calculated by taking the average of all patients at the last available visit. Furthermore, the proportion of patients that ever used a biological was calculated per country.

Table 1 Composite scores for the clinical eligibility criteria for the start of bDMARDs and for the access to medication

Composite score clinical criteria start of bDMARDs				
	0	**1**	**2**	
Is there any requirement for disease duration?	Any requirement	No requirement	*NA*	
Number of DMARDs to be failed	>2	2	<2	
Level of DAS28	>3.2	≤3.2	No requirement	
Composite score access to medication				
	0	**1**	**2**	**3**
Number of reimbursed bDMARDs	0	1–5	6–7	8
Average annual price of all reimbursed bDMARDs	Highest quartile	Second quartile	Third quartile	Lowest quartile
Average score on the six acceptability questions	Highest quartile	Second quartile	Third quartile	Lowest quartile

bDMARDs, biological disease modifying antirheumatic drugs; NA, not applicable.

Questionnaire

Per participating country, preferably in the region of data collection, at least two rheumatologists answered a questionnaire, based on questionnaires used by Putrik *et al*.[13] In case of disagreement between rheumatologists, they were contacted by email, and if necessary additional rheumatologists were contacted to also complete the questionnaire. The questionnaire included questions about availability and affordability of DMARDs, acceptability, reimbursement and prescription rules (online supplementary file 2). Drug prices provided in local currency were converted into euros or international dollars at the rate of 10-1-2017. For each DMARD, we took the lowest available price. When all questions were processed, a preliminary report was sent to all collaborators, to check correctness of the data.

Outcome measures

Based on the questionnaire results, two composite scores were calculated: a composite score for clinical eligibility criteria for the start of bDMARDs, based on three questions from the questionnaire and with an optimum score of 5 indicating 'least requirements', and a composite score for access to medication, based on questions on availability, affordability and acceptability, with an optimum score of 9 indicating 'highest level of access' (table 1).[6 13]

In addition, we calculated the average annual national price of the most frequently used csDMARDs and bDMARDs. These included the csDMARDs methotrexate, sulfasalazine, hydroxychloroquine and leflunomide and prednisone and the bDMARDs etanercept, adalimumab, infliximab, rituximab, certolizumab, tocilizumab, abatacept and golimumab. For each DMARD a most common treatment scheme was used to calculate the costs for 1 year usage (the annual national price, averaged over the first two treatment years).[13] Furthermore, an affordability index for bDMARDs was constructed by dividing the average annual national price for all bDMARDs by the gross domestic product.[13] All medication prices reflect official manufacturer's prices per country, not taking into account local or temporary discounts, which fall beyond the scope of this study.

The gross domestic product (GDP) per capita, the household-net-adjusted-disposable-income, the health-expenditure-per-capita in international dollars and the minimum wage per year in US$ were derived from web-based sources.[14–16] Data regarding the minimum wage and the average price for csDMARDs and bDMARDs were used to calculate the days to work at the minimum wage to cover 30 days of treatment with a csDMARD or bDMARD.[13]

Statistical analyses

At a country level, associations between several indicators of SES, clinical outcomes, medication use, access to medication and prescription and reimbursement rules were assessed using univariable linear regression analyses. Since analyses were performed at a country level and the number of included countries was limited, multivariable regression analyses were not performed. Regression results for the GDP per capita, the household-net-adjusted-disposable-income and the health-expenditure-per-capita were assessed per 10 000 Intl$.

To assess whether analysing data at a patient level where possible would change the outcomes, we performed a sensitivity analysis using linear mixed modelling for continuous outcomes and generalised linear mixed modelling for dichotomous outcomes, with patients nested within countries and a random intercept at the country level. All analyses were performed using Stata SE14 (Stata).

RESULTS

Country and database characteristics

Twelve countries with 20 379 patients were analysed: USA (state of Massachusetts), Mexico, South Africa, Japan, Brazil, UK, Spain, Ireland, Portugal, France, India (state of Maharashtra) and the Netherlands. Data from Qatar and Italy were ultimately excluded from the analyses, since only one rheumatologist in Qatar was available to complete the questionnaire and data from Italy were mainly derived from a biologics register. The number of questionnaire responders per country is listed in online supplementary file 3.

Table 2 presents average country and database characteristics. Additional patient characteristics are presented in online supplementary file 4. The number of patients per country ranged from 123 (Spain) to 7749 (India) and the number of patients ever using a bDMARD ranged from 0.9% (South Africa) to 75% (Ireland). There were important differences in DAS28-scores and HAQ-scores across countries. Overall, and expectedly, DAS28 was positively associated with HAQ-score, except in India, where the average DAS28 was highest but the average HAQ-score was among the lowest of all countries. As expected, there were important differences in SES between countries, reflected—for example—by differences in GDP per capita (ranging from Intl$5733 in India to Intl$61 378 in Ireland) and by large differences in the country's number of days required to work at the minimum wage to cover 30 days of treatment with a bDMARD (ranging from 562 days in India to only 19 days in France).

Average annual medication prices also substantially differed between countries (figure 1). For bDMARDs, drug prices (Intl$) in the USA (highest) were 5.9 times higher than in France (lowest) and for csDMARDs, drug prices in the USA (highest) were 14.7 times higher than in the Netherlands (lowest).

Table 2 Baseline characteristics per country

	India	South Africa	Brazil	Mexico	Portugal	Spain
Country characteristics						
Population (×1 000 000)	1311.1	10.4	207.8	127.0	10.4	46.4
GDP per capita (Intl$)	5733	12 393	14 533	16 490	26 549	32 219
Minimum wage per year (US$)	778	2197	3660	1438	8384	10 365
Household net adjusted disposable income (Intl$)	NA	21 481	19 882	33 164	31 649	30 776
Health expenditure per capita (Intl$)	301	1405	1639	1753	3338	3717
Days work at minimum wage to cover 30 days treatment bDMARD	562	160	223	431	53	49
Days work at minimum wage to cover 30 days treatment csDMARD	2.2	4.9	3.3	7.4	0.7	0.7
Mean price bDMARDs year/GDP per capita	8.45	1.93	3.07	3.07	0.84	0.70
Composite score access to medication	3	3	5	1	6	5
Composite score clinical criteria	4	1	1	4	5	4
Database characteristics						
Number of patients	7749	670	189	1191	3874	123
Mean time since diagnosis at last recorded visit (days)	1304	577	4900	2898	5599	1327
% patients bDMARD use	0.95	0.90	19.6	9.0	44.5	16.3
Mean last DAS28	5.1	4.2	4.2	4.0	3.5	3.3
% patients in DAS28-remission	2.3	19.9	17.0	20.9	32.4	42.7
Mean last HAQ	0.67	1.27	1.26	0.71	1.05	0.55

	France	Japan	UK	Netherlands	USA	Ireland
Country characteristics						
Population (×1 000 000)	66.5	127.0	65.1	16.9	321.4	4.64
GDP per capita (Intl$)	37 775	37 872	38 509	46 354	52 704	61 378
Minimum wage per year (US$)	19 886	12 269	21 793	20 673	15 080	20 967
Household net adjusted disposable income (Intl$)	34 092	30 031	31 724	31 685	41 071	25 629
Health expenditure per capita (Intl$)	5681	4070	4678	6499	9403	4730
Days work at minimum wage to cover 30 days treatment bDMARD	19	39	32	26	171	48
Days work at minimum wage to cover 30 days treatment csDMARD	0.3	1.8	0.6	0.2	5.4	0.4
Mean price bDMARDs year/GDP per capita	0.37	0.44	0.69	0.42	1.55	0.62
Composite score access to medication	9	4	5	6	4	4
Composite score clinical criteria	4	5	2	4	5	4
Database characteristics						
Number of patients	161	309	1291	3330	803	689
Mean time between diagnosis and last visit (days)	5375	2503	3256	3181	3513	3921
% patients bDMARD use	60.2	50.5	14.7	28.2	48.6	75.0
Mean last DAS28	2.5	3.2	3.9	3.2	3.6	3.8
% patients in DAS28-remission	61.5	38.2	26.0	39.1	30.5	28.8
Mean last HAQ	0.61	0.59	1.29	0.85	0.67	0.85

'Number of patients' indicates the number of patients with >1 available visit. '% patients bDMARD use' indicates the number of patients using a biological DMARD during at least one visit. 'Mean last DAS28' and 'mean last HAQ' are the DAS28 and HAQ at the last available visit in the database.
bDMARD, biological disease modifying antirheumatic drug; csDMARD, conventional synthetic disease modifying antirheumatic drug; GDP, gross domestic product; Intl$, international dollar; NA, not available.

Countries' SES and clinical outcomes

We first assessed if a lower SES was associated with worse clinical outcomes, by testing associations between GDP per capita and DAS28. Indeed, patients in countries with a higher GDP per capita had a lower average DAS28 and a higher proportion of them were in DAS28-remission (DAS28 lower by β (95% CI) −0.32 (−0.41; −0.021) and an additional 4.2 (0.14; 8.26) per cent of patients in DAS28-remission for every 10 000 Intl$ additional GDP). The effect was less prominent in the USA and Ireland, both countries with the highest GDP per capita (figure 2A,C).

Then, we factored drug-prices into the 'model' by testing the association between the number of days needed to work at the minimum wage in order to afford 30 days of treatment with a bDMARD. Now the association was largely driven by two low-GDP countries (Mexico and India) (figure 2B,D) that yet have among the highest drug prices relative to the income. In most other countries, DAS28 and remission percentages were only slightly higher with each extra working day needed to afford bDMARDs: DAS28 higher by β (95% CI) 0.026 (0.012 to 0.041) and −0.052 (−0.084 to −0.020) less patients in DAS28-remission per additional minimal wage day required to afford 30 days bDMARDs.

Finally, we tested health-expenditures-per-capita as well as household's-net-adjusted-disposable income as proxies for SES and assessed the associations with DAS28. In general, the effects were similar: mean DAS28 was −1.52 (−2.8 to −0.25) points lower for every additional 10 000 IntI$ health-expenditure-per-capita, which culminated into 29.2 (1.91 to 56.6) per cent more patients in DAS28-remission. Such effects were not found for household's net-adjusted disposable income (data not shown).

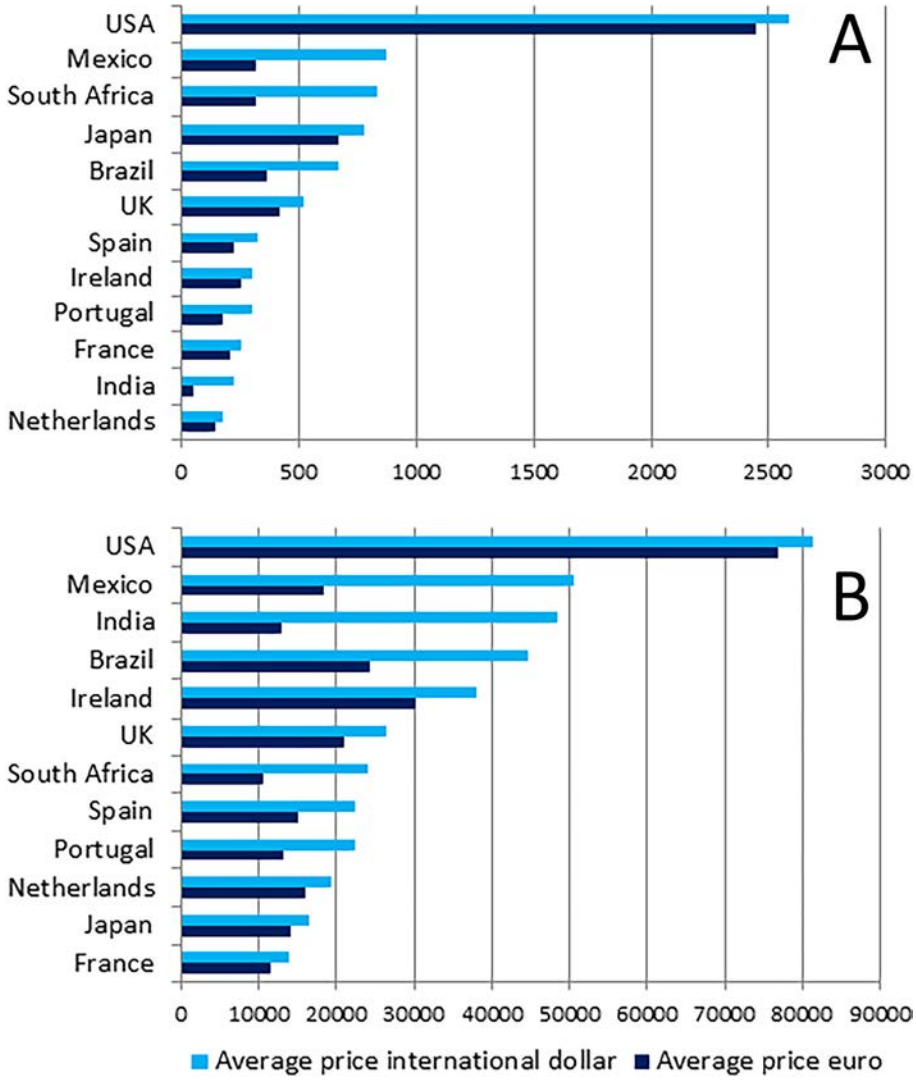

Figure 1 Average annual price for csDMARDs (A) and bDMARDs (B) per country in international dollars (light blue) and in euros (dark blue), prices first quarter 2017. bDMARDs, biological disease modifying antirheumatic drugs.

Overall, patients with RA from low-GDP-countries—on a per-capita basis—appear to have a higher DAS28 than patients from high-GDP-countries, regardless of countries' drug prices. It may be that in some countries drug-prices may mitigate the effects of SES on RA outcomes (drug prices were for instance importantly lower in Brazil and South Africa).

For HAQ-score, however, the associations with all indicators of SES were less clear: for example, −0.031 (−0.13 to 0.064) lower HAQ per 10 000 Intl$ increase in GDP per capita and 0.000034 (−0.00091 to 0.00098) higher HAQ per additional minimal wage day required to afford 30 days bDMARDs.

SES and bDMARD-usage

It is attractive to assume that the inverse association between SES and DAS28 is mediated by the countries' bDMARD use (or: RA care in high-income countries is better since these can afford bDMARDs). We have sought evidence to underscore this assumption. First, we assessed whether SES was associated with bDMARD-usage per country. Indeed, a statistically significant association was found between GDP per capita and the proportion bDMARD-usage (11.2 (4.82 to 17.5), figure 3A), indicating that per additional 10 000 Intl$ GDP per capita an additional 11% of patients used a bDMARD.

When taking drug-prices into account, the picture is more obscure. Although in Mexico and India bDMARD-usage was lowest, in the countries with highest GDP per capita, bDMARD-usage was highly variable (ranging from close to 10% in the UK to 75% in Ireland), (figure 3A, β (95% CI) −0.080 (−0.16 to 0.0021)). This suggests that GDP and drug prices and other mechanisms (such as limitative regulations for reimbursement) determine bDMARD-usage.

bDMARD-usage and clinical outcomes

It is questionable, however, if a higher percentage of bDMARD-usage translates automatically into better disease outcomes. We assessed whether bDMARD-usage across countries are associated with clinical outcomes. Indeed we found a statistically significant relationship between a country's proportion of bDMARD-usage and DAS28 or proportion of patients in DAS28-remission (figure 4A,B). DAS28 was −0.14 (−0.28 to −0.0054) point lower and 2.8% (−0.13 to 5.8) more patients achieved DAS28-remission, for every 10% increase in proportion of patients using a bDMARD. However, bDMARD-usage was not associated with better functional ability (−0.024 (−0.091 to 0.042) lower HAQ-score for every 10% increase in bDMARD-usage).

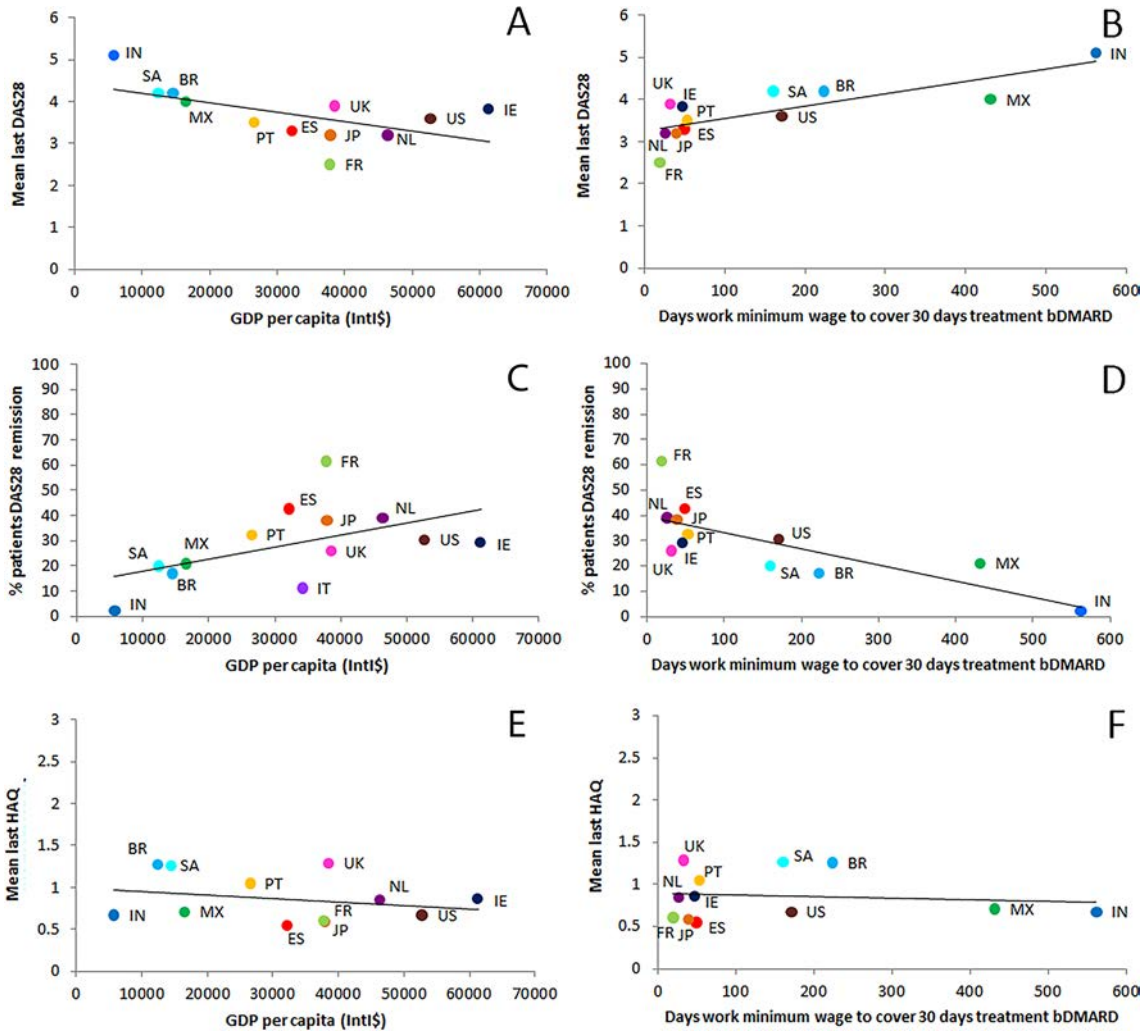

Figure 2 Associations between 'GDP per capita (Intl$)' and 'days to work at the minimum wage to cover 30 days of treatment with a bDMARD' with clinical outcomes per country. bDMARDs, biological disease modifying antirheumatic drugs; BR, Brazil; ES, Spain, FR, France; GDP, gross domestic product; IE, Ireland; IN, India; JP, Japan; MX, Mexico; NL, Netherlands; PT, Portugal; SA, South Africa.

Prescription and reimbursement rules, access to medication and bDMARD-usage

Since bDMARD-usage is not only influenced by a country's SES, it was subsequently assessed whether the stringency of prescription and reimbursement rules and 'access to medication' were associated with proportion of bDMARD-usage.

We found that bDMARD-usage is less if limitative regulations are stricter: 8.5 (–2.7 to 19.8) per cent more bDMARD use per point increase (ie, fewer limitations) in clinical criteria score and a trend (5.9 (–2.0 to 13.8)) that better access to bDMARD-care led to more bDMARD-usage (figure 4D,E).

This shows that the previous relationship found between a country's SES and quality of RA care measured as a country's mean DAS28 is (among others) confounded by regulations. Relatively strict prescription and reimbursement rules in the UK, a high SES country, result in a proportion of bDMARD-usage as

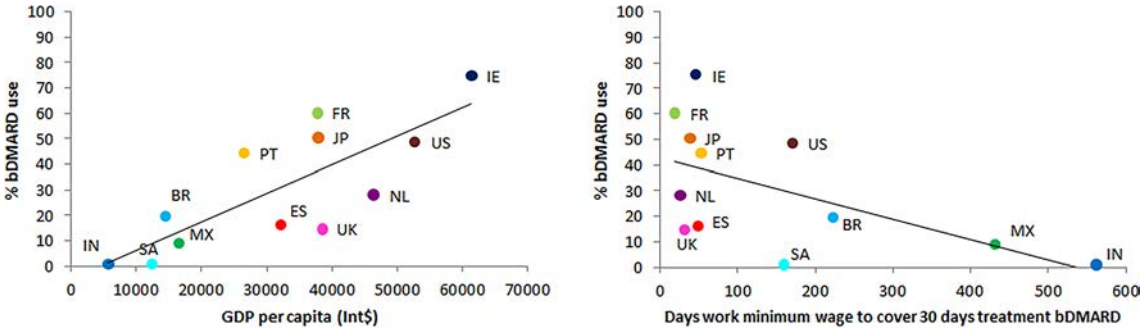

Figure 3 Associations between 'GDP per capita' and 'days to work at the minimum wage to cover 30 days of treatment with a bDMARD' with '% bDMARD use'. bDMARD, biological disease modifying antirheumatic drugs; BR, Brazil; ES, Spain; FR, France; GDP, gross domestic product; IE, Ireland; IN, India; JP, Japan; MX, Mexico; NL, Netherlands; PT, Portugal; SA, South Africa.

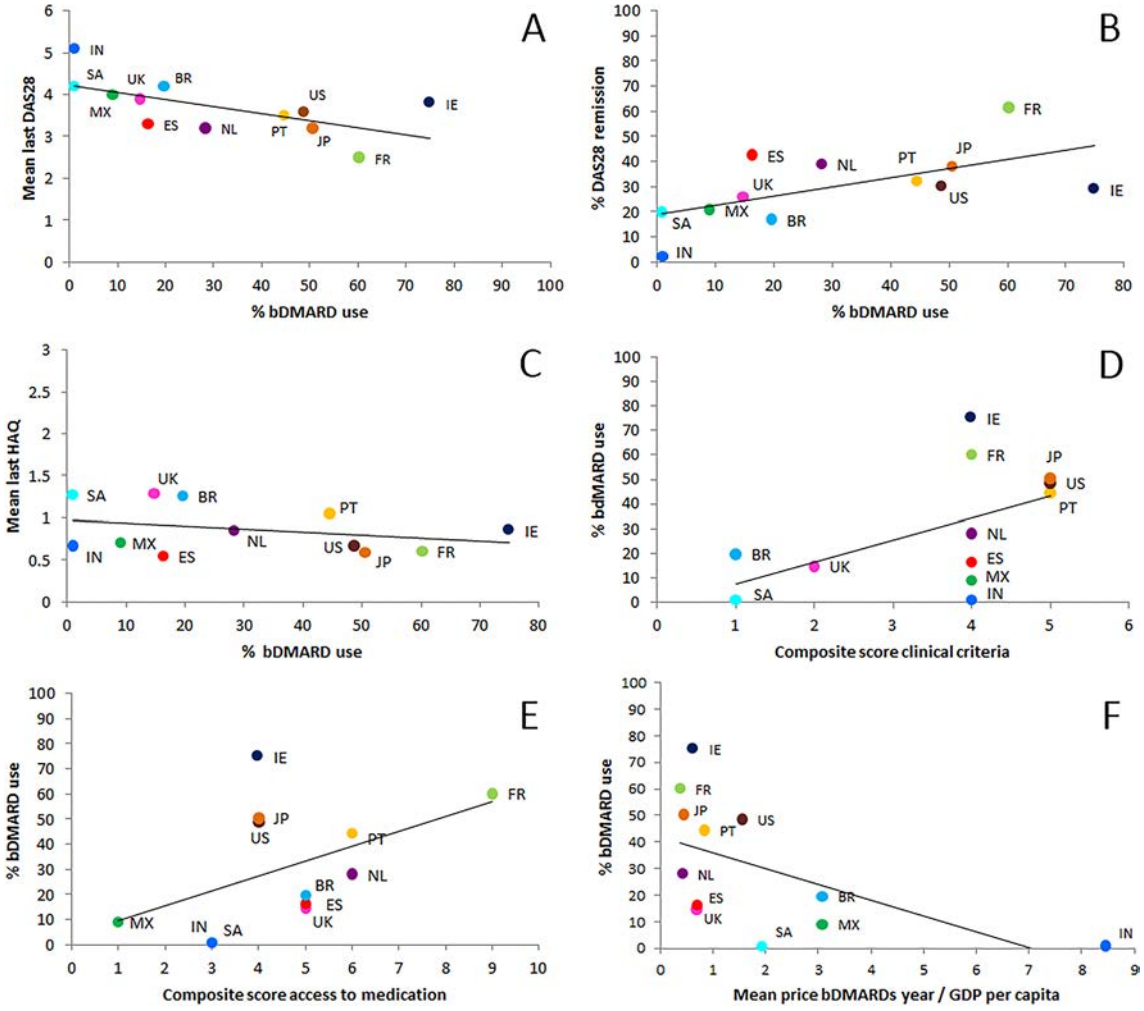

Figure 4 Associations between the '% of patients that ever used a bDMARD' and the 'composite score clinical criteria', 'composite score access to medication' and clinical outcomes. bDMARD, biological disease modifying antirheumatic drugs; BR, Brazil; ES, Spain; FR, France; GDP, gross domestic product; IE, Ireland; IN, India; JP, Japan; MX, Mexico; NL, Netherlands; PT, Portugal; SA, South Africa.

low as in India and Mexico, which both have a low GDP per capita.

Finally, we calculated the quotient of a country's mean drug-price and the GDP per capita (as proxy for affordability, the lower the quotient, the less affordable the drug) and found (1) that even in countries with a same level of affordability (eg, EU countries) significant differences in bDMARD-usage exist, apparently due to other mechanisms than drug-prices alone and (2) that affordability of bDMARDs in some countries is so low that bDMARD-usage is virtually zero.

Sensitivity analyses

Sensitivity analyses using mixed modelling approaches to assess associations between SES (country level), bDMARD-use (country and patient level), disease activity and physical functioning (patient level) showed similar outcomes (online Supplementary file 5).

DISCUSSION

Worldwide, treatment options and clinical outcomes of patients with RA have greatly improved, but not all patients with RA have benefitted similarly. We hypothesised that differences in SES have an impact on bDMARD-usage and on clinical outcomes across countries. Indeed, in this study including a large number of patients from 12 countries, among which several countries that have never been investigated before in this context, we have found substantial differences in DMARD-prices, affordability of these medications and bDMARD-usage across countries. We found that in countries with a lower SES disease activity was generally higher and bDMARD-usage was lower. But a country's proportion of bDMARD-usage was also associated with restrictions through prescription and reimbursement rules, and with affordability of bDMARDs, as defined by us.

It is attractive to assume that higher country's bDMARD-usage will result in a lower country's mean DAS28 and that a lower country's GDP will hinder a sufficiently high proportion of patients with RA getting proper access to care with bDMARDs. But reality is more complicated. The effectiveness of bDMARD-usage in countries' all-day clinical practice may be overstated: previous research estimated that 'only' 7% of the effect of GDP per capita on DAS28 was mediated by the uptake of bDMARDs.[4] We found 'only' 2.8% more patients in DAS28-remission for every additional 10% patients using a bDMARD. A positive effect of bDMARDs on RA treatment effectiveness thus appears to be quite small. Vice versa, this suggests that in low-income countries other factors than 'only' access to bDMARDs determine the success of RA treatment. Nevertheless, a general trend

between countries' proportions of bDMARD-usage and countries' mean-DAS28 remains obvious.

Remarkably, we did not find an association between countries' SES and countries' mean HAQ-score. Here, the effect of outliers is relatively important. In particular, India, the country with lowest GDP, reported a low HAQ-score compared with a high DAS28. Moreover, there may be sociodemographic and cultural differences in the way patients experience or report limitations in function.[17 18] We could not assess the potential contribution of factors such as general access to healthcare and other drug and non-drug therapies, comorbidities and health barriers and support systems.[19]

Previous studies have mentioned associations between access to medication, SES and disease activity.[6 13 20] Such findings point to the negative effects of inequity: budget restrictions, strict regulations as well as limited access to drugs may be a hurdle for starting optimal treatment as recommended in clinical guidelines.[13 21]

But this study also shows that several other factors play a role in determining the success of RA-treatment (here approximated by the countries' mean DAS28). We know several of these factors: countries' SES in general, the presence of a proper functioning healthcare system that may assure access to care to those who are in need, DMARD prices and valid national regulations that are in place to constrain the expenses for bDMARD-usage.[4 6–9 13 21] It appears obvious that the country's mean level of DAS28 is the resultant of a complicated interplay of a country's SES, drug prices and regulations. In addition, it is difficult to argue that unlimited access to expensive effective treatments makes the difference between 'good and bad care' for patients with RA, nor can we claim that countries with similar GDP per capita or similar levels of access to care have similar proportions of patients on expensive bDMARDs; there is huge variation. Nevertheless, penetration of bDMARDs in low GDP-countries stays behind and it is to be expected that this—among others—may go at the cost of effectiveness of RA care. It is impossible to conclude from this study whether this is due to drug prices, failing healthcare systems or simply worse access to optimal care. We can only conclude that there are substantial differences in mean DAS28 (as a proxy for quality of RA care) across countries.

This study has some strengths and many limitations. A strength of this study is that it captures real life clinical data from 12 countries worldwide with large differences in wealth, totally different (if any) healthcare and health-insurance systems and many patients with RA. As such, this study can be considered a 'big-data study' allowing subtle differences across countries to be elucidated.

But the strengths of our study (real life observational, size and international diversity) also carry limitations: case-ascertainment (cases cannot be verified), completeness of data (we had to statistically impute missing data) and reliability of data-points (we had to rely on the report of the participating physicians) are among them. Other epidemiological limitations are that only few centres per country participated and we had to assume that these centres were to some extent representative of the country. In addition, we had to make certain assumptions to facilitate computations, such as declaring bDMARD-reimbursement as 'absent', if according to the rheumatologist's questionnaires less than 20% of patients in a country had health insurance coverage. Furthermore, we used retail prices of bDMARDs, but final drug prices may be influenced by local and/or temporary discounts. Such assumptions—if flawed—may influence the reported associations. In a few cases, we relied on regional health-economical information rather than on country-specific data, in the appreciation that within a big country access to healthcare and regulations can be very different.

Moreover, differences between the patient samples from the included countries could potentially influence the associations that are investigated in this study. However, it is very unlikely that the available variables—which were all measured at a patient level—act as a confounder for the investigated associations. Therefore, we presented unadjusted models.

A final limitation of this database is that it will only include patients with RA that have come to the attention of the rheumatologist. If countries differ with regard to access to a rheumatologist, patients per country cannot be assumed to be comparable. Consequently, associations may be spurious. With regard to this latter argument, it can be postulated that the associations in this study are conservative and will likely be more exaggerated in real life.

Epidemiological limitations of 'big-data studies' restrict their interpretability. As such, causal interpretations will never be possible and should always be mistrusted. We have taken care to not exaggerate our conclusions that all remain at the level of associations and allow the possibility of bias and confounding as explanatory factors. In addition, we were interested in associations at the country level, but at the patient level results may be different. Still, 'big data studies' make sense in that they can point to relevant differences between countries, that may help policymakers to guide necessary change, pharmaceutical industry to direct market access and drug-prices and rheumatologists and healthcare workers to help improving access to rheumatology care.

In conclusion, we have documented using a registry of patients with RA spanning 12 countries worldwide that mean DAS28 as well as bDMARD-usage varies across countries. While we suggest an inverse relationship between the countries' bDMARD-usage and mean DAS28, this relationship is influenced by many other factors, including countries' GDP per capita, strictness of prescription and reimbursement rules and affordability of bDMARDs. Altogether these findings point to the existence of worldwide inequity with regard to optimal (access to) RA healthcare.

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Data sharing statement The dataset used and analysed during the current study are available from the corresponding author on reasonable request.

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CONCISE REPORT

Clinical and ultrasound remission after 6 months of treat-to-target therapy in early rheumatoid arthritis: associations to future good radiographic and physical outcomes

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ABSTRACT

Objective To explore associations between remission, based on clinical and ultrasound definitions, and future good radiographic and physical outcome in early rheumatoid arthritis (RA).

Methods Newly diagnosed patients with RA followed a treat-to-target strategy incorporating ultrasound information in the Aiming for Remission in rheumatoid arthritis: a randomised trial examining the benefit of ultrasound in a Clinical TIght Control regimen (ARCTIC) trial. We defined 6-month remission according to Disease Activity Score, Disease Activity Score in 28 joints-erythrocyte sedimentation rate, American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) Boolean criteria, Simplified Disease Activity Index, Clinical Disease Activity Index and two ultrasound definitions (no power Doppler signal, grey scale score ≤2). Two outcomes were defined: no radiographic progression and good outcome (no radiographic progression+physical function≥general population median), both sustained 12–24 months. We calculated the ORs of these outcomes for the remission definitions.

Results Of 103 patients, 42%–82% reached remission at 6 months, dependent on definition. Seventy-one per cent of patients had no radiographic progression and 37% had good outcome. An association between 6-month remission and no radiographic progression was observed for ACR/EULAR Boolean remission (44 joints, OR 3.2, 95% CI 1.2 to 8.4), ultrasound power Doppler (OR 3.6, 95% CI 1.3 to 10.0) and grey scale remission (OR 3.2, 95% CI 1.2 to 8.0). All clinical, but not ultrasound remission criteria were associated with achievement of a good outcome.

Conclusions Our data support ACR/EULAR Boolean remission based on 44 joints as the preferred treatment target in early RA. Absence of ultrasound inflammation was associated with no radiographic progression.

Trial registration number NCT01205854; Post-results.

INTRODUCTION

Early initiation of disease-modifying antirheumatic drug (DMARD) therapy with a defined treatment target within 6 months has become a keystone in the management of patients with rheumatoid arthritis (RA).[1 2] Prevention of joint damage and disability are now achievable outcomes for a large proportion of newly diagnosed patients with RA.[3]

Composite scores such as the Disease Activity Score (DAS), Disease Activity Score in 28 joints (DAS28), Simplified Disease Activity Index (SDAI) and Clinical Disease Activity Index (CDAI) are used to measure disease activity and guide therapeutic decisions.[1 4–6] Additionally, the Boolean-based American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) remission criterion was developed to optimise radiographic and functional outcomes.[7] The ACR/EULAR task force recommended inclusion of ankles and forefeet in the assessment of remission, although formally not required.[7]

Remission according to composite scores and Boolean-based criteria is associated with less radiographic joint damage,[7–9] and remission should be sustained as radiographic progression is a consequence of cumulative inflammation.[10–12] However, not all patients fulfilling clinical remission criteria show absence of radiographic progression, and ongoing subclinical inflammation detected by ultrasonography and MRI may explain this discrepancy.[13]

The aim of this study was to explore the association between remission at 6 months and two outcomes of importance for evaluation of treatment success: (1) future no radiographic progression and (2) a combined good outcome of no radiographic progression and physical function comparable to the general population. In particular, we wanted to assess how potential ultrasound definitions of remission performed in comparison to clinical definitions.

METHODS

Patients and study design

DMARD-naïve patients with early RA fulfilling the 2010 ACR/EULAR criteria were enrolled in the Aiming for Remission in rheumatoid arthritis: a randomised trial examining the benefit of ultrasound in a Clinical TIght Control regimen (ARCTIC) trial, randomising patients to a conventional or ultrasound tight control strategy.[14] Only patients with ultrasound examinations at all visits (ultrasound strategy, n=118) were included in the current analyses to allow

Table 1 The performance of various remission criteria at 6 months for identifying patients without radiographic progression 12–24 months and patients with a good combined outcome* 12–24 months. Statistically significant findings are shown in bold. n=103

	No radiographic progression						Good combined outcome*					
	Prevalence of no radiographic progression						Prevalence of good combined outcome					
	Patients in remission n/N (%)	Patients not in remission n/N (%)	Sensitivity	Specificity	LR+ (95% CI)	LR– (95% CI)	Patients in remission n/N (%)	Patients not in remission n/N (%)	Sensitivity	Specificity	LR+ (95% CI)	LR– (95% CI)
Clinical outcomes												
DAS	47/61 (77)	26/42 (62)	0.64	0.53	1.38 (0.91 to 2.10)	0.67 (0.42 to 1.05)	30/61 (49)	8/42 (19)	0.79	0.52	**1.66 (1.22 to 2.24)**	**0.40 (0.21 to 0.78)**
DAS28-ESR	49/64 (77)	24/39 (62)	0.67	0.50	1.34 (0.91 to 1.99)	0.66 (0.40 to 1.07)	31/64 (48)	7/39 (18)	0.82	0.49	**1.61 (1.21 to 2.13)**	**0.37 (0.18 to 0.76)**
SDAI	39/50 (78)	34/53 (64)	0.53	0.63	1.46 (0.87 to 2.44)	0.74 (0.51 to 1.06)	29/50 (58)	9/53 (17)	0.76	0.68	**2.36 (1.59 to 3.50)**	**0.35 (0.19 to 0.63)**
CDAI	36/48 (75)	37/55 (67)	0.49	0.60	1.23 (0.75 to 2.02)	0.84 (0.58 to 1.22)	27/48 (56)	11/55 (20)	0.71	0.68	**2.20 (1.47 to 3.30)**	**0.43 (0.25 to 0.72)**
ACR/EULAR Boolean (44 joints)	36/43 (84)	37/60 (62)	0.49	0.77	**2.11 (1.06 to 4.21)**	**0.66 (0.49 to 0.89)**	27/43 (63)	11/60 (18)	0.71	0.75	**2.89 (1.80 to 4.62)**	**0.38 (0.23 to 0.64)**
ACR/EULAR Boolean (28 joints)	36/47 (77)	37/56 (66)	0.49	0.63	1.34 (0.80 to 2.27)	0.80 (0.56 to 1.14)	28/47 (60)	10/56 (18)	0.74	0.71	**2.52 (1.65 to 3.85)**	**0.37 (0.2 to 0.65)**
No swollen joints (44 joints)	50/67 (75)	23/36 (64)	0.69	0.43	1.21 (0.85 to 1.71)	0.73 (0.43 to 1.24)	28/67 (42)	10/36 (28)	0.74	0.40	1.23 (0.93 to 1.62)	0.66 (0.36 to 1.21)
Ultrasound												
Power Doppler=0	64/84 (76)	9/19 (47)	0.88	0.33	**1.32 (1.01 to 1.72)**	**0.37 (0.17 to 0.82)**	32/84 (38)	6/19 (32)	0.84	0.20	1.05 (0.88 to 1.26)	0.79 (0.33 to 1.90)
Grey scale score=<2	39/47 (83)	34/56 (61)	0.53	0.73	**2.00 (1.07 to 3.76)**	**0.64 (0.46 to 0.88)**	20/47 (43)	18/56 (32)	0.53	0.59	1.27 (0.83 to 1.92)	0.81 (0.55 to 1.20)

*Good combined outcome: a combination of no radiographic progression and stable physical function assessed by the Patient-Reported Outcome Measurement Information System (PROMIS) ≥ the median of the general population between 12 and 24 months.
ACR, American College of Rheumatology; CDAI, Clinical Disease Activity Index; DAS, Disease Activity Score; DAS28, Disease Activity Score in 28 joints; ESR, erythrocyte sedimentation rate; EULAR, European League Against Rheumatism; LR, likelihood ratio; SDAI, Simplified Disease Activity Index.

for assessment of potential ultrasound definitions of remission. Patients without two radiographs during the second year of the study were excluded (n=15). Patients attended 13 visits in 2 years with treatment adjustments according to an algorithm targeting clinical remission (DAS <1.6), no swollen joints and absence of ultrasound power Doppler signal (online supplementary table S1). Ultrasound examination of 32 joints was performed by trained physicians with semiquantitative 0–3 scoring of synovitis for grey scale and power Doppler.[14 15] Patients were started on methotrexate with prednisolone bridging (online supplementary table S1). Therapy was escalated if the target was not reached, patients with high disease activity and risk factors for progressive joint destruction could start biologics more rapidly (online supplementary table S1). The study was conducted in compliance with the Declaration of Helsinki.

Definitions of remission

Four clinical composite remission criteria were assessed: DAS, DAS28-erythrocyte sedimentation rate, CDAI and SDAI. Additionally, we evaluated the ACR/EULAR Boolean criteria, based on 28 and 44 joints, and three alternative definitions of remission: no swollen joints (of 44), no ultrasound power Doppler signal and minimal grey scale synovitis (sum score ≤2 of 0–96).[14–16] For secondary analyses, we defined sustained remission as remission at all of the 6, 8, 10 and 12-month visits.

Radiographs and outcomes

Radiographs (12, 16 and 24 months) were scored by two trained readers, blinded for clinical data, in chronological order using the van der Heijde modified Sharp method.[17] We defined no radiographic progression as <1 unit change 12–24 months (average score of the readers). Good outcome was defined as a combination of no radiographic progression and stable physical function assessed by the Patient-Reported Outcome Measurement Information System ≥ the median of the general population between 12 and 24 months,[18] in line with the good outcome definition used in the development of the ACR/EULAR remission criteria.[7]

Statistical analysis

Baseline characteristics were described as proportions (%), means (SD) and medians (25th, 75th percentiles). Associations between remission status at 6 months and outcomes were assessed using logistic regression, with similar analyses for sustained remission. Additionally, we calculated sensitivities and specificities, and positive and negative likelihood ratios. The potential effect modification of biologic therapy on radiographic outcome was assessed by including remission status, biologic treatment and interaction terms in separate logistic regression models for the two main outcomes.

In secondary analyses, we calculated the ORs of no radiographic progression according to state of clinical disease activity (remission, low disease activity, moderate/high disease activity) at the 6-month visit, using moderate/high disease activity as reference category.

Missing radiographs were imputed by interpolation or extrapolation if a minimum of two radiographs were available, whereas missing clinical, laboratory or ultrasound variables at the follow-up visits were imputed by interpolation. Statistical analyses were performed using STATA V.14.

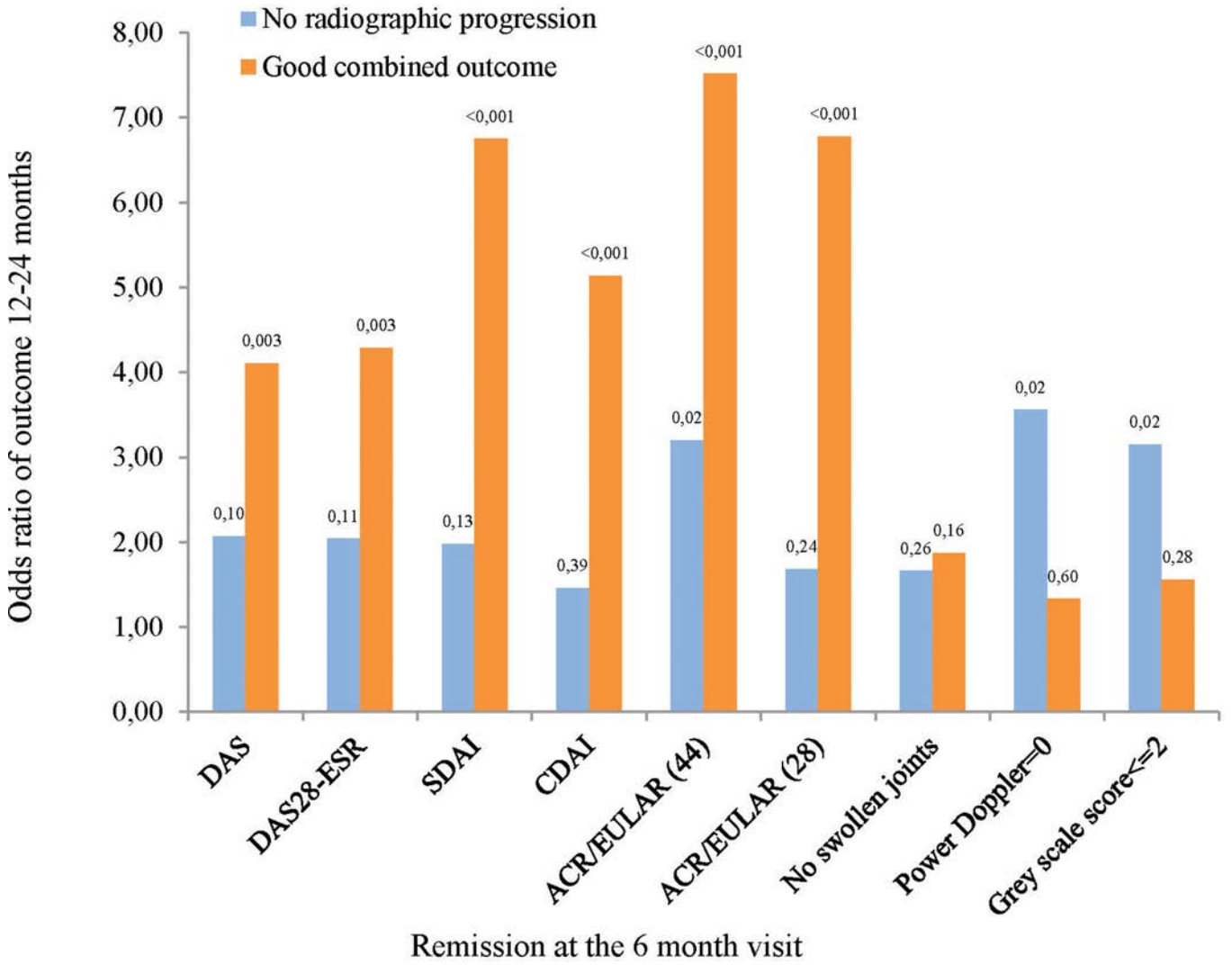

Figure 1 The association between remission at 6 months and no radiographic progression (blue bars) and good combined outcome (orange bars, no radiographic progression+physical function ≥ median in the general population), both 12–24 months. ACR, American College of Rheumatology; CDAI, Clinical Disease Activity Index; DAS, Disease Activity Score; DAS28, Disease Activity Score in 28 joints; ESR, erythrocyte sedimentation rate; EULAR, European League Against Rheumatism; SDAI, Simplified Disease Activity Index.

RESULTS

Patient characteristics

Of 103 patients, 74% were female, mean (SD) age was 51.4 (12.9) years, disease duration 6.7 (5.3) months and DAS 3.5 (1.1) (online supplementary table S2).

Remission and radiographic progression

ACR/EULAR Boolean remission based on 44 joints was achieved by 42% of patients at the 6-month visit, while 59% were in DAS remission and 49% in SDAI remission (table 1). Mean/median radiographic progression 12–24 months was 0.8 (1.3)/0.5 (0.0, 1.0), 71% had no progression. Patients in ACR/EULAR Boolean remission (44 joints) had higher odds of no radiographic progression from 12 to 24 months than patients not in remission, as had patients in ultrasound remission versus not being in ultrasound remission (figure 1, table 1). Patients in remission according to the composite indices at 6 months, except for CDAI, had a significantly higher odds of no radiographic progression compared with patients with moderate/high disease activity, and this was not significant for patients in low disease activity by any of the definitions (table 2). Adjustment for biologic treatment at the 6-month visit (n=12) did not show any effect on the association between remission and radiographic progression. Results for patient in sustained remission are presented in online supplementary table S3.

Remission at 6 months and good outcome

A good outcome was achieved by 37%. Being in remission at 6 months according to any established clinical remission criteria predicted a good outcome, while the ultrasound definitions and no swollen joints did not (figure 1, table 1). Similar results were found for patients in sustained remission (online supplementary table S3).

DISCUSSION

We found that clinical remission by all established definitions increased the odds of reaching a good combined radiographic and physical outcome in early RA, while achieving ultrasound remission as well as ACR/EULAR Boolean remission was associated with no radiographic progression during the subsequent year. To our knowledge, this is the first study assessing both

Table 2 ORs of no radiographic progression 12–24 months according to state of clinical disease activity composite measures at 6 months. Moderate/high disease activity as reference category. Statistically significant findings are shown in bold. n=103

	Classification at 6 months n/N (%)	No radiographic progression 12–24 months	
		OR (95% CI)	P values
DAS			
Moderate/high disease activity	15/103 (15)	Ref	Ref
Low disease activity	27/103 (26)	2.71 (0.73 to 10.04)	0.14
Remission	61/103 (59)	**3.84 (1.18 to 12.45)**	**0.03**
DAS28-ESR			
Moderate/high disease activity	19/103 (18)	Ref	Ref
Low disease activity	20/103 (19)	3.33 (0.86 to 12.92)	0.08
Remission	64/103 (62)	**3.63 (1.24 to 10.58)**	**0.02**
SDAI			
Moderate/high disease activity	17/103 (17)	Ref	Ref
Low disease activity	36/103 (35)	2.02 (0.62 to 6.62)	0.25
Remission	50/103 (49)	**3.15 (0.98 to 10.09)**	**0.05**
CDAI			
Moderate/high disease activity	17/103 (17)	Ref	Ref
Low disease activity	38/103 (37)	2.49 (0.75 to 8.22)	0.14
Remission	48/103 (47)	2.67 (0.84 to 8.46)	0.10

CDAI, Clinical Disease Activity Index; DAS, Disease Activity Score; DAS28, Disease Activity Score in 28 joints; ESR, erythrocyte sedimentation rate; SDAI, Simplified Disease Activity Index.

clinical remission and ultrasound remission with regard to future joint damage and good physical function in patients treated according to current recommendations.[1 2]

EULAR recommends achievement of remission within 6 months in early RA.[1 2] In our study, a good combined outcome was predicted by remission according to any assessed clinical composite score. In addition to the two ultrasound remission definitions, only ACR/EULAR Boolean remission at 6 months, with assessment of 44 joints, predicted no radiographic progression when comparing patients in remission with all patients not in remission. These findings support ACR/EULAR Boolean remission as the preferred definition of remission in early RA,[1] but also underline previous publications recommending inclusion of the feet when assessing remission.[7 19] When assessing categories of disease activity, low disease activity at 6 months was less associated with no radiographic progression than achievement of remission by this point. This adds validity to the choice of remission as the preferred treatment target in early RA.[1 2]

Good physical function is important to patients. We found that being in ultrasound remission did not capture the functional aspects of the disease as well as the clinical criteria. Thus, our data support clinical definitions of remission when aiming for a good combined outcome, although the data suggest limited specificity and sensitivity for all remission definitions. This is in line with the recent findings that targeting ultrasound remission is not superior to targeting clinical remission or low disease activity.[14 20] However, the importance of being in ultrasound remission on other patient-related outcomes, such as pain, needs to be further explored. In some cases, components of the clinical disease activity measures might be influenced by non-RA-related factors,[2] and in such settings ultrasonography might be suitable to help guide treatment decisions to prevent radiographic progression.

A limitation of our study is the overall low radiographic progression, which makes it difficult to study the association between remission and future joint damage. Thus, the absence of significant associations between sustained clinical remission and radiographic progression may be attributed to the low overall radiographic progression. This has also been proposed as a possible explanation in the Combination Therapy for Rheumatoid Arthritis (COBRA) light trial which demonstrated that remission was associated with a good functional outcome, but not predictive of absent radiographic progression.[21] The low rate of radiographic progression reflects RA management when applying modern treatment strategies. As the results presented in this report are based on secondary or exploratory analyses, the possibility of lack of power cannot be excluded. Another limitation is the use of 44-joint count that might not be feasible in clinical practice. The results are strengthened by the broad inclusion criteria compared with industry-sponsored pharmaceutical trials, capturing a broad range of patients with early RA, and the opportunity to assess ultrasound remission. However, the generalisability of the findings to other clinical settings, with different treat-to-target strategies, and to other populations such as established RA, is unknown.

In conclusion, absence of ultrasound inflammation was associated with no subsequent radiographic progression, while being in ACR/EULAR Boolean remission after 6 months of targeted therapy increases both the odds of no radiographic progression and a good outcome. Our results support current recommendations stating that ACR/EULAR remission including assessment of the feet should be the preferred treatment target in early RA, and that low disease activity is a less preferred target.

Correction notice This article has been corrected since it published Online First. The shared authorship statement has been added.

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Contributors Conception and design of the study: ABA, ICO, HBH, TU, DvdH, TKK, SL and EAH. Acquisition of data: ABA, HBH, TU and EAH. Analysis and interpretation of data: NPS, ABA, ICO, HBH, TU, DvdH, TKK, SL and EAH.

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Competing interests ICO has received consultancy honorarium from Pfizer. ABA has sat on advisory boards for UCB, AbbVie and Pfizer and received honorariums for development of educational material for UCB. HBH has received honorariums as a speaker from AbbVie, Bristol-Myers Squibb, Roche, UCB Pharma, Novartis and Pfizer. TU has received honorariums as a speaker from AbbVie, Bristol-Myers Squibb, Lilly, Roche, Novartis, UCB Pharma and Pfizer. DvdH has received consultancy honorariums from AbbVie, Amgen, Astellas, AstraZeneca, Bristol-Myers Squibb, Celgene, Daiichi, Eli Lilly, Galapagos, Merck, Novartis, Pfizer, Roche, Sanofi Aventis, Janssen and UCB and is the owner of Imaging Rheumatology. TKK has received fees for speaking and/or consulting from AbbVie, Biogen, BMS, Boehringer Ingelheim, Celgene, Celltrion, Eli Lilly, Epirus, Hospira, Merck-Serono, MSD, Mundipharma, Novartis, Oktal, Orion Pharma, Hospira/Pfizer, Roche, Sandoz and UCB. EAH has received research funding from Pfizer, UCB, Roche, MSD and AbbVie for the submitted work, honorariums as a speaker from Pfizer, UCB, Roche and AbbVie, and honorariums for development of educational material from Pfizer and has sat on advisory boards for Pfizer, Eli Lilly and Celgene.

Patient consent Not required.

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EXTENDED REPORT

Baseline ultrasound examination as possible predictor of relapse in patients affected by juvenile idiopathic arthritis (JIA)

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ABSTRACT

Objectives To define the correlation between joint ultrasonography and clinical examination in patients with juvenile idiopathic arthritis (JIA) and to assess whether synovitis detected by ultrasonography in clinically inactive patients predicts arthritis flares.

Methods 88 consecutive patients with JIA—46 (52%) with persistent oligoarthritis, 15 (17%) with extended oligoarthritis, 15 (17%) with rheumatoid factor-negative polyarthritis and 12 (14%) with other forms of JIA, all clinically inactive for a minimum of 3 months—underwent ultrasound (US) assessment of 44 joints. Joints were scanned at study entry for synovial hyperplasia, joint effusion and power Doppler (PD) signal. Patients were followed clinically for 4 years.

Results US was abnormal in 20/88 (22.7%) patients and in 38/3872 (0.98%) joints. Extended oligoarthritis and rheumatoid factor-negative polyarthritis were more frequent in US-positive than in US-negative patients (35.0% vs 11.8% and 30.0% vs 13.2%, respectively; P=0.005). During 4 years of follow-up, 41/88 (46.6%) patients displayed a flare; 26/68 (38.2%) were US-negative and 15/20 (75%) were US-positive at baseline. Abnormality on US examination, after correction for therapy modification, significantly increased the risk of flare (OR=3.8, 95% CI 1.2 to 11.5). The combination of grey scale and PD abnormalities displayed a much higher predictive value of relapse (65%, 13/20) than grey scale alone (33%, 6/18).

Conclusions US abnormalities are a strong predictor of relapse at individual patient level. Irrespective of treatment, the risk of flare in US-positive versus US-negative patients was almost four times higher. In case of US abnormalities, patients should be carefully followed regardless of both the International League of Associations for Rheumatology and Wallace categories.

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INTRODUCTION

Achieving remission is the target in treating children affected by juvenile idiopathic arthritis (JIA). Preliminary clinical and laboratory criteria to define remission and clinically inactive disease have been published.[1 2] Unfortunately, the risk of flare after discontinuation of methotrexate or biological therapy is high,[1–9] and tools predicting relapse are urgently needed. A recent paper by Guzman *et al*[10] showed that there are no clinical features reliably associated with the risk of flare except the JIA subtype.

In rheumatoid arthritis, the role of ultrasonography (US) to predict disease relapse and structural damage in rheumatoid arthritis is well established.[11–14] Studies on US of joints in healthy children and in JIA have already been published.[15–21] It has been suggested that US may be more sensitive in detecting joint inflammation in patients with JIA than the clinical evaluation.[22–27] However, the role of US-detected synovitis when clinical manifestations in JIA are absent is unknown. Furthermore, the only published study on the role of US in predicting JIA relapse showed no predictability.[28]

We, therefore, performed a prospective study with the aim of determining the value of US in clinical remission and its power to predict flare.

MATERIALS AND METHODS

Population and study design

We enrolled the following groups of subjects (see online supplementary table 1): (1) 88 consecutive patients with inactive JIA to study the association between US and synovitis flare; (2) 30 consecutive patients with active JIA and (3) 30 healthy subjects for US interpretation setting and calibration; and (4) 30 patients with active JIA recruited during an educational event with the aim to determine the intraobserver and interobserver reliability.

All patients in group 1 were classified according to the International League of Associations for Rheumatology (ILAR) criteria[29] with the disease being inactive for a minimum of 3 months. The recruitment occurred between January 2011 and December 2012. At study entry, all patients in groups 1 and 2 and the healthy subjects (group 3) underwent a clinical assessment. The following data were recorded: sex, birth date and, in case of patients with JIA, ILAR category, age and medications at baseline, and disease activity status (ie, clinical remission off medication—CR; clinical remission on medication—CRM; inactive disease—ID). Within the same session, each subject (groups 1, 2 and 3) underwent a US assessment, which was performed separately, blindly and immediately after the clinical evaluation, by one of two rheumatologists (ODL, VR) skilled in paediatric musculoskeletal US (MSUS). All inactive patients were followed for 4 years, every 3–6 months, without repeating US examination, according to our usual

clinical practice. We recorded the flares during the follow-up, the time to flare from baseline, the joints affected by flare, the therapy administered at baseline and its modification during the follow-up. A flare of synovitis was defined as a recurrence of clinically overt active arthritis, in one or more joints.

Group 4 was included only to assess the reliability between the machines and among the examiners and did not take part in the main study. Informed consent was obtained.

Clinical assessment

Clinical remission was defined according to Wallace criteria as CR, CRM and ID.[1] Joint assessment was performed by experienced paediatric rheumatologists blinded to US findings for groups 1, 2 and 3. Joints were assessed for swelling, tenderness/pain on motion and restricted motion. A joint with active arthritis was defined as a joint with swelling or, in the absence of swelling, with tenderness/pain on motion and restricted range of motion.

Ultrasonographic evaluation

The US examination technique and the standard scans were based on published Outcome Measures in Rheumatology (OMERACT) guidelines and on the studies on paediatric MSUS available at study entry.[15 30–33]

US setting and calibration

To standardise evaluation, the two examiners shared two consensus sessions: on active patients (group 4) and on stored images of groups 2 and 3, discussing all issues related to evaluation. Definite guidelines on paediatric US evaluation were not available at the study entry. However, the adopted criteria fit very well with the recent OMERACT preliminary definition of synovitis in children.[34] In each joint, three elements were assessed by MSUS: synovial hyperplasia, synovial effusion and power Doppler (PD) signal.

Synovial hyperplasia was defined as an abnormal hypoechoic joint space, distinct from the intra-articular fat pad and non-displaceable with the transducer. Synovial effusion was detected as the presence of an abnormal hypoechoic or anechoic space within the joint, which was displaceable. Synovial hyperplasia and synovial effusion were considered indifferently as a single abnormality in grey scale examination. PD signal was considered pathological in the presence of vessel dots only inside an area of synovial hyperplasia. In each joint, synovial hyperplasia, effusion and PD signal were graded semiquantitatively on a 0–3 scale.[32] For statistical calculations, however, we used the categories present (if ≥ 1) or absent (if <1) only. The possible presence of a physiological small amount of synovial fluid in the joints of healthy children was considered.

In the presence of synovial fluid, the joint recess was measured and the effusion scored ≥ 1, if greater than the average value plus 2 SD of the normal joint space, for each joint according to published data.[15 18] As for some joints published data are missing, we referred to data collected in our cohort of healthy children (online supplementary table 2).

A total of 44 joints for each patient (2 shoulders, 2 elbows, 2 wrists, 10 metacarpophalangeal, 10 proximal interphalangeal of hands, 2 hips, 2 knees, 2 subtalar, 2 tibiotalar and 10 metatarsophalangeal) were scanned for the presence of synovial hyperplasia/joint effusion and PD signal. To perform the examination, an Esaote MyLab 70 with a 6–18 MHz linear probe and a GE Logiq S8 with an 8–18 MHz linear probe were used. The grey scale frequency was adjusted according to the depth of the joint (18 MHz for small superficial joints, 1412 MHz for medium size and 10 or less for deeper joints); the highest frequencies were used for younger children. The PD frequency was set to 9–10 MHz for superficial joints and 8–6 MHz for medium and deep joints. The pulse repetition frequency was 750 Hz and the gain was set at a level just below the disappearance of the noise under the cortical bone.

Intraobserver and interobserver reproducibility

Given the problem of various Doppler sensitivities between different US machines,[35] a comparison of the two devices in their ability to detect blood flow in the volar aspect of the finger pulp and the feeding vessel of the capitate bone in five subjects of group 4 was made prior to the inter-rater assessment to ensure their comparability. A good agreement was found (Cohen's $k=0.80$).

The interobserver reproducibility was determined in group 4. On each patient 44 joints were assessed by the two operators (ODL and VR) independently and on the same day. The intraobserver reproducibility was calculated using saved images of the same group of patients 3 months after the day of data acquisition. Cohen's kappa coefficient of agreement was calculated to evaluate the intraobserver and interobserver reliability.

Statistical analysis

Sample size calculation of group 1 was based on the main hypothesis and on the feasibility of this study considering the number of patients usually attending our outpatient clinic (see online supplementary statistical analysis paragraph for details).

The dimension of groups 2, 3 and 4 was determined in 30 subjects for each category requiring an absolute precision of 0.2 in Cohen's k calculation with $\alpha=0.05$.

Descriptive statistics were used to calculate mean and SD for continuous variables and percentage for categorical variables. Difference in mean age between US-positive and US-negative patients was analysed by unpaired t-test. Association between categorical variables was assessed by χ^2 and Fisher's exact tests when appropriate.

Diagnostic test performance was defined by accuracy, specificity, sensitivity and positive/negative predictive values (PPV and NPV, respectively). Wilson Score[36] was used to calculate CI on binomial probability ('binom' R package). McNemar's test without continuity correction was applied to determine the significance of the disagreement in group 2.

Risk was measured in terms of OR, and Baptista-Pike mid-p[37] was used to obtain CI on OR (ORCI R package). The probability of flare-free condition was calculated with Kaplan-Meier method, and log-rank test was applied to compare stratified curves. To assess the role of treatment in the ability of US to predict flare, a stratified analysis was performed and the stratum-specific ORs were calculated. Breslow-Day test was applied to test the null hypothesis of homogeneity between stratified OR estimates, and Cochran-Mantel-Haenszel method was performed to compute a weighted average of the ORs across the strata. A P value <0.05 was considered as significant.

Statistical analysis was performed using R V.3.0.2 for Windows.

RESULTS

Harmonisation of US evaluation between examiners

Demographic and clinical characteristics of group 4 are included in online supplementary table 3. Cohen's k value was 0.89 for intraobserver reliability and 0.82 for interobserver reliability.

The site-by-site inter-reader agreement is displayed in online supplementary table 4.

Setting and calibration of US evaluation

Demographic and clinical characteristics of groups 2 and 3 are summarised in online supplementary table 3.

All 30 healthy children (17 female and 13 male, mean age 8.6±2.9 years) were attending our outpatient clinics but without any clinical and laboratory evidence of inflammatory musculoskeletal disorders. They were negative both at physical and US examination (none of the elementary lesion reached a score grading ≥1, in particular no sign of synovial hyperplasia was found). As expected, all the healthy children showed physiological Doppler signal, typical of the immature joints (the feeding vessels of physis, epiphyses, fat pads, carpal and tarsal bones). Sixty-three per cent (19/30) of healthy subjects had a minimal amount of synovial fluid detectable in at least one joint.

Group 2 with clinically active disease (21 female and 9 male, mean age 7.9±4.2 years) included 16 patients with persistent oligoarthritis, 12 with extended polyarthritis, 1 with rheumatoid factor-positive polyarthritis and 1 with enthesitis-related arthritis. There were 173 joints positive at US examination and 144 positive at clinical examination; the clinical and US examinations were comparable (McNemar's test P value >0.05).

Association between baseline US and subsequent clinical flares in the follow-up of patients with inactive disease at study entry

Characteristics of patients with inactive JIA and risk of flare

The main features of the 88 clinically inactive patients are summarised in table 1. We scanned by US 44 joints per patient for a total of 3872 joints. In 20 (22.7%) out of 88 patients and in 38 out of 3872 joints (0.98%), the US examination was abnormal for at least one of grey scale elementary lesions (synovial hyperplasia/joint effusion), with or without abnormal PD signal.

Comparing clinical examination and US, the most discordant joints were 15 knees, 8 wrists, 7 tibiotalar, 4 subtalar, 3 metatarsophalangeal and 1 elbow.

We compared the demographic and clinical characteristics of the patients with (20/88) and without (68/88) US abnormalities (defined as US-positive and US-negative patients, respectively).

As shown in table 1, extended oligoarthritis and rheumatoid factor-negative polyarthritis were almost three times more represented in US-positive than in US-negative patients (35.0% vs 11.8% and 30.0% vs 13.2%, respectively). Regarding Wallace criteria, the ID category was three times more represented in US-positive (30.0%) than in US-negative patients (10.3%), whereas CRM was only slightly more frequent in US-positive than in US-negative ones (60.0% vs 51.5%). In other words, US-positive patients compared with US-negative ones showed a statistically different distribution of both ILAR categories and Wallace criteria (P=0.005 and P=0.015, respectively). Noteworthy, US-positive patients showed a remission duration significantly (P<0.001) shorter than US-negative ones (0.99 vs 1.96 years). This difference was confirmed in relapsed patients (1.11 vs 1.81 years; P=0.019).

Out of a total of 88 patients, 41 (46.6%) had a flare of disease during follow-up: 38.2% (26/68) of US-negative patients and 75% (15/20) of US-positive patients (table 2).

This means that the presence of an abnormal US examination increases the risk of flare by almost five times (OR=4.9, 95% CI 1.6 to 14.9; P=0.005). The PPV of US is equal to 75% (95% CI 56.0 to 94.0). Performance measures of US are shown in table 3.

Table 1 Main features of the 88 inactive patients stratified into US-positive and US-negative groups

	US-positive (n=20)	US-negative (n=68)	P value
Sex (female, %)	16 (80.0)	52 (76.5)	1.000
Age (mean±SD)	10±4.3	9.9±4.4	0.949
ILAR category			
Persistent oligoarticular JIA, n (%)	7 (35.0)	39 (57.4)	0.005
Extended oligoarticular JIA, n (%)	7 (35.0)	8 (11.8)	
Rheumatoid factor-negative JIA, n (%)	6 (30.0)	9 (13.2)	
Other	0 (0.0)	12 (17.6)	
Wallace criteria			0.015
CR, n (%)	2 (10.0)	26 (38.2)	
CRM, n (%)	12 (60.0)	35 (51.5)	
ID, n (%)	6 (30.0)	7 (10.3)	
Disease duration at baseline (years) (mean±SD)	4.52 (3.17)	7.70 (4.51)	0.095
Remission duration at baseline (years) (mean±SD)	0.99 (0.68)	1.96 (0.87)	<0.001
Therapy discontinuation			
Yes, n (%)	6 (30.0)	22 (32.4)	0.047
No, n (%)	11 (55.0)	19 (27.9)	
NA, n (%)	3 (15.0)	27 (39.7)	
Treatment*			
Intra-articular steroids, n (%)	10 (50.0)	47 (69.1)	0.528
Oral steroids, n (%)	2 (10.0)	4 (5.9)	
Methotrexate, n (%)	13 (65.0)	40 (58.8)	
Biological drugs, n (%)	2 (10.0)	3 (4.4)	

*The sum is not equal to the number of US-positive or US-negative patients because each patient can have more than one treatment at a time.
CR, complete remission off medication; CRM, complete remission on medication; ID, inactive disease; ILAR, International League of Associations for Rheumatology; JIA, juvenile idiopathic arthritis; NA, not applicable since they were not on therapy at study onset; US, ultrasound.

With regard to medical treatment, 30/88 patients (34.1%) were not on Disease modifying anti rheumatic drugs (DMARDs)/biological therapy at study entry, while 58 out of 88 were on such therapy (65.9%). Twenty-eight of fifty-eight (48.3%) discontinued, while 30 (51.7%) continued the therapy during the follow-up.

More than 50% of subjects on DMARDs/biological therapy (17/30 and 15/28 of those who continued and discontinued therapy, respectively) and 30% (9/30) of untreated patients had a flare. Although the association between treatment discontinuation and flare was not significant (χ^2=5.09, P=0.08), a stratified analysis was performed to better evaluate whether and how the therapy could affect the ability of US examinations to predict a flare (Breslow-Day test). Medical treatment did not result to have a modifier effect (χ^2=2.98, P=0.224), and an adjusted OR was calculated across strata. Irrespective of treatment status, the risk of flare in US-positive patients versus US-negative ones was 3.8 (95% CI 1.2 to 11.5). As the proportional difference between the unadjusted (4.9) and adjusted (3.8) ORs is more than 10%, the contribution of therapy as confounder effect cannot be ruled out.

Time to flare

On average, the US-negative patient group showed a significantly greater probability of remission than the US-positive group (log-rank test: χ^2=15.1, P<0.001). At 1 year, the

Table 2 Main clinical features of the 88 inactive patients stratified by the outcome at the end of follow-up and by the US abnormalities

	Overall patients (n=88)	Patients with inactive disease (n=47)		Patients with synovitis flare (n=41)	
		US-negative (n=42)	US-positive (n=5)	US-negative (n=26)	US-positive (n=15)
ILAR category					
PO JIA, n (%)	46 (52.3)	24 (57.1)	1 (20.0)	15 (57.7)	6 (40.0)
EO JIA, n (%)	15 (17.0)	4 (9.5)	2 (40.0)	4 (15.4)	5 (33.3)
RF-negative JIA, n (%)	15 (17.0)	7 (16.7)	2 (40.0)	2 (7.7)	4 (26.7)
Other	12 (13.6)	7 (16.7)	0 (0.0)	5 (19.2)	0 (0.0)
Wallace criteria					
CR, n (%)	28 (31.8)	20 (47.6)	0 (0.0)	6 (23.1)	2 (13.3)
CRM, n (%)	47 (53.4)	17 (40.5)	2 (40.0)	18 (69.2)	10 (66.7)
ID, n (%)	13 (14.8)	5 (11.9)	3 (60.0)	2 (7.7)	3 (20.0)

CR, clinical remission off medication; CRM, clinical remission on medication; EO, extended oligoarthritis; ID, inactive disease; ILAR, International League of Associations for Rheumatology; PO, persistent oligoarthritis; RF, rheumatoid factor-negative polyarthritis; US, ultrasound.

remission probability was 94.1% (95% CI 88.7 to 99.9) and 55.0% (95% CI 37.0 to 81.8) in US-negative and US-positive patients, respectively (figure 1). The difference remained statistically significant even after stratification by therapy (log-rank test: χ^2=8.9, P=0.0029) (online supplementary table 5).

Analysis of specific joints in US-positive patients at baseline

US examination at study entry was positive in 38 joints (from one up to six joints per patient): 20/38 were positive in both grey scale and PD signal; 18 only in grey scale (tables 4 and 5). Flares occurred in 34 joints during the follow-up. Nineteen of thirty-four (55.9%) were predicted by US examination (positive at baseline), while 15/34 (44.1%) were previously US-negative. Among joints that were initially US-positive and flared in the follow-up, 13/19 (68%) were positive in both grey scale and PD signal, while 6/19 (31.6%) only in grey scale. The combination of grey scale and PD abnormalities displays a much higher predictive value of relapse (65%) than grey scale alone (33%) (tables 4 and 5).

DISCUSSION

Our study shows that US signs of inflammation are detectable in joints of children with JIA in full clinical remission. Furthermore, they are predictive of relapses, notably in the largest series of patients reported in the literature regarding this issue.

We show that up to 22.6% of children with inactive disease display US signs of inflammation in one or more joints. The incidence of US-detected abnormalities in clinically inactive patients is lower than reported in another study (about 70% in grey scale)[28] but close to that of the Spanish cohort,[38] where 38% of children showed US abnormalities.

A reason for the lower percentage of subclinical synovitis could be the more stringent criteria for defining the synovitis by US, both in grey scale (small amount of physiological fluid measured and judged according to standard reference values[15]) and in Doppler mode (which was scored only inside synovial hyperplasia[18 38]).

In addition, the preliminary consensus sessions and the examination of healthy children and patients with active disease, respectively, could have improved the reliability of the US in clinically inactive children.

Stringent criteria to distinguish normal from pathology are mandatory; the definition of minimal disease activity in children is still unclear. In the report by Magni-Manzoni *et al*,[28] this might have precluded the ability of US to predict flares given the high number of grade 1 abnormalities both in grey scale and PD mode.

US in paediatrics has some additional pitfalls.[39] For instance, the epiphysial cartilage is anechoic as the synovial fluid. A grey scale alteration (particularly a small amount of synovial fluid) may not be associated with an inflammatory state. Moreover, PD may be easily misinterpreted.[39] We tried to minimise these limitations by enrolling healthy subjects and active patients and by harmonising the US interpretation.

An important point is the prospective nature of our study, which assessed 88 inactive patients, the largest series reported in literature.

Forty-one out of 88 inactive patients had a flare (46.5%), similar to a recent study.[10] The patients with US abnormality showed a higher percentage of flares compared with those without abnormalities. US abnormalities increased the risk of flare by almost four times after considering the therapy as a confounder. As expected, the vascularisation of the synovial membrane, detected by the combination of the PD signal in an area of synovial hyperplasia, is the most reliable US finding for synovial inflammation. This finding is consistent with the preliminary definition of synovitis in children.[34]

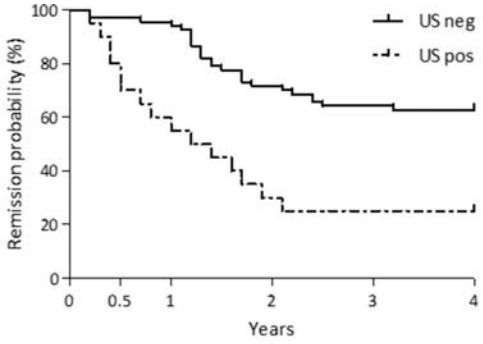
In a previous large study, physician global assessment (PGA) >30, active joint count >4, positive rheumatoid factor polyarthritis, antinuclear antibody (ANA) positivity and DMARDs or biological therapy before attaining inactive disease were associated with increased risk of flare, but US examination was not available.[10] Considering only significant flares and the results of a multivariate analysis, active joint count >4 and joint injections were still associated with the risk of relapse, with the maximum value of HR equal to 1.29. Our study focuses on US and if confirmed could support US examination as a useful tool for clinical management.

There are no differences in the probability of developing a relapse with regard to ILAR categories (persistent oligoarthritis=21/46, extended oligoarthritis=9/15, rheumatoid factor-negative=6/15; P=0.560). However, the association between US-positive and relapse is significant only for patients with persistent oligoarthritis (P=0.037). In particular, the probability of having a synovitis flare

Table 3 Estimates and relative 95% CI of accuracy, sensitivity, specificity and predictive values of ultrasound examination in juvenile idiopathic arthritis in clinical remission

	Estimate	Lower 95% CI	Upper 95% CI
Accuracy	64.8 (57/88)	54.4	73.9
Sensitivity	36.6 (15/41)	23.6	51.9
Specificity	89.4 (42/47)	77.4	95.4
Positive predictive value	75.0 (15/20)	53.1	88.8
Negative predictive value	61.8 (42/68)	49.9	72.4

CI was calculated using the Wilson Score method.



	Number of patients at risk (N events) Remission probability [95%CI]					
Time (years)	**0.5**	**1.0**	**1.5**	**2.0**	**3.0**	**4.0**
US negative	66 (2) 0.971 [0.931 to 1.000]	65 (2) 0.941 [0.887 to 0.999]	54 (11) 0.779 [0.687 to 0.884]	49 (5) 0.706 [0.605 to 0.823]	43 (5) 0.632 [0.528 to 0.758]	42 (1) 0.618 [0.512 to 0.745]
US positive	16 (6) 0.700 [0.525 to 0.933]	12 (3) 0.550 [0.370 to 0.818]	9 (2) 0.450 [0.277 to 0.731]	6 (3) 0.300 [0.154 to 0.586]	5 (1) 0.250 [0.117 to 0.534]	5 (0) 0.250 [0.117 to 0.534]

Figure 1 Kaplan-Meier curves showing 4-year remission probability by ultrasound (US) result. A US-positive result is significantly (log-rank test: χ^2 =15.1, P<0.001) related to a greater probability of flare than a US-negative result.

is about six times greater than the probability of not having the event (6/7 vs 1/7) in US-positive persistent oligoarthritis, while the probability of having a synovitis flare is about half of the probability of not having the event (15/39 vs 24/39) in US-negative persistent oligoarthritis (table 2).

Considering the Wallace criteria, although no statistical significance was achieved, the likelihood of developing a relapse in the presence of US positivity appears to be more associated with CR (PPV=2/2; P=0.074) and CRM (PPV=10/12; P=0.087). On the contrary, the number of false-positives in ID is far greater (37.5%, 3/8) than in the other two groups (0% (0/20) and 10.5% (2/19) for CR and CRM, respectively). This would support the hypothesis that the false-positives might be persistent US findings in the early stages of clinical remission that will diminish over time in patients with stable remission, a finding that has been documented in rheumatoid arthritis as well.[40]

Considering US as a potential predictor tool, false-positives are relatively few (5/47; specificity of 89.4%) and false-negatives are relatively many (26/41; sensitivity of 36.6%). In conclusion, in case of positive US, a follow-up should be carried out regardless of both the ILAR and Wallace categories because of the high probability of relapse (75%), while a negative result does not rule out the risk. Furthermore, patients with a clinical remission period around or less than a year should be followed more carefully since a US-positive result can confirm the flare. This finding is in line with the study showing that a deeper clinical remission was associated with a milder subclinical synovitis and a lower risk to relapse in adult patients with RA.[41]

A weakness of this study is that the discontinuation of DMARDs therapy seems not to influence disease relapse, in contrast to what was previously published.[4–8 10] However, a P value of 0.08 between those who stopped and those who maintained the therapy is very close to significance; the lower OR after adjusting for therapy confirms this statement. We may speculate that with a larger cohort

Table 4 Frequency of US abnormalities at study entry in specific joints of the 88 patients with clinically defined inactive disease

	n	Grey scale abnormalities, n (%)	Power Doppler signal, n (%)	Any US abnormalities, n (%)
All patients	88	20 (22.7)	13 (14.8)	20 (22.7)
Scanned joints	3872	38 (0.98)	20 (5.2)	38 (0.98)
Shoulder	176	0 (0.0)	0 (0.0)	0 (0.0)
Elbow	176	1 (5.7)	1 (5.7)	1 (5.7)
Wrist	176	8 (4.5)	4 (2.3)	8 (4.5)
MCP	880	0 (0.0)	0 (0.0)	0 (0.0)
PIP	880	0 (0.0)	0 (0.0)	0 (0.0)
Hip	176	0 (0.0)	0 (0.0)	0 (0.0)
Knee	176	15 (8.5)	9 (5.1)	15 (8.5)
Ankle	352	11 (3.1)	6 (1.7)	11 (3.1)
MTP	880	3 (0.34)	3 (0.34)	3 (0.34)

MCP, metacarpophalangeal; MTP, metatarsophalangeal; PIP, proximal interphalangeal of hands; US, ultrasound.

Table 5 Frequency of US abnormalities at study entry in the 44 joints which had a relapse of synovitis

		Joints with US abnormalities at study entry (n)		
Joint	**Joints with synovitis relapse (n=75)**	**Total* (n=19)**	**Grey scale abnormalities (n=19)**	**Power Doppler signal (n=13)**
Elbow	5	1 (20.0)	1	1
Wrist	11	4 (36.4)	4	2
MCP	6	0 (0.0)	0	0
PIP	2	0 (0.0)	0	0
Knee	28	8 (28.6)	8	6
Ankle	20	6 (30.0)	6	4
MTP	3	0 (0.0)	0	0

The total number of joints with synovitis relapse includes joints which were US-positive (19), US-negative (41) or belonging to US-positive patients, but with synovitis flare found in a different joint with respect to the US examination at study entry (15).
*Data are expressed as numbers (percentage).
MCP, metacarpophalangeal; MTP, metatarsophalangeal; PIP, proximal interphalangeal of hands; US, ultrasound.

of patients, we could have shown an effect of therapy discontinuation on relapses.

Another point to consider is that joints where JIA relapsed were different from those detected by US at study entry. We can hypothesize that US likely shows synovitis in patients with a higher systemic inflammation that can emerge over time in joints different from the ones detected at study entry.

In conclusion, US could be a predictor of relapse at the patient level since a subclinical synovitis increased by about four times the risk of flare. If confirmed by further studies, US may offer a useful tool in stratifying the risk of flare in patients with JIA.

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EXTENDED REPORT

Prediction of autoimmune connective tissue disease in an at-risk cohort: prognostic value of a novel two-score system for interferon status

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ABSTRACT

Objective To evaluate clinical, interferon and imaging predictors of progression from 'At Risk' to autoimmune connective tissue diseases (AI-CTDs).

Methods A prospective observational study was conducted in At-Risk of AI-CTD (defined as antinuclear antibody (ANA) positive; ≤1 clinical systemic lupus erythematosus (SLE) criterion; symptom duration <12 months and treatment-naïve). Bloods and skin biopsy (non-lesional) were analysed for two interferon-stimulated gene expression scores previously described (IFN-Score-A and IFN-Score-B). Forty-nine healthy controls (HCs) and 114 SLE were used as negative and positive controls. Musculoskeletal ultrasound was performed. Progression was defined by meeting classification criteria for AI-CTDs at 12 months.

Results 118 individuals with 12-month follow-up were included. Of these, 19/118 (16%) progressed to AI-CTD (SLE=14, primary Sjogren's=5). At baseline, both IFN scores differed among At-Risk, HCs and SLE groups (p<0.001) and both were elevated in At-Risk who progressed to AI-CTD at 12 months versus non-progressors, to a greater extent for IFN-Score-B (fold difference (95% CI) 3.22 (1.74 to 5.95), p<0.001) than IFN-Score-A (2.94 (1.14 to 7.54); p=0.018). Progressors did not have significantly greater baseline clinical characteristics or ultrasound findings. Fold difference between At-Risk and HCs for IFN-Score-A was markedly greater in skin than blood. In multivariable logistic regression, only family history of autoimmune rheumatic disease, OR 8.2 (95% CI 1.58 to 42.53) and IFN-Score-B, 3.79 (1.50–9.58) increased the odds of progression.

Conclusion A two-factor interferon score and family history predict progression from ANA positivity to AI-CTD. These interferon scores may allow stratification of individuals At-Risk of AI-CTD permitting early intervention for disease prevention and avoid irreversible organ damage.

INTRODUCTION

Autoimmune connective tissue diseases (AI-CTDs) include systemic lupus erythematosus (SLE), primary Sjogren's syndrome (pSS), systemic sclerosis, inflammatory myopathies, mixed and undifferentiated CTDs. A hallmark of their pathogenesis is loss of self-tolerance leading to autoreactivity and production of antibodies against self-nuclear antigens (ANAs). ANA can be detected in serum up to 10 years before clinical features, representing a phase of subclinical autoimmunity.[1] However, ANA is present in up to 25% of the general population, of whom less than 1% develop clinical autoimmunity.[2 3] Individuals with ANA therefore constitute At-Risk population of whom a minority will progress to AI-CTD.[4 5] The factors that dictate whether this autoreactivity develops into autoimmune disease are unknown. But if these were understood and predictable, then effective intervention might be possible, preventing the severe disease and heavy glucocorticoid use for remission induction of a newly diagnosed AI-CTD.

Variants in type I interferon (IFN-I) pathway are prominent in the genetic susceptibility to AI-CTDs and therefore a focus for investigation.[6–8] However, their role in disease initiation is currently unclear. IFN activity is usually quantified using expression of interferon-stimulated genes (ISGs). Interpretation of ISG expression is complex with multiple IFN subtypes produced by different cell types and tissues, as well as a transcriptional response in all nucleated cells with variation between cell types. Previously used IFN signatures have a categorical high/low classification[9 10] or may have been affected by the ISGs selected.[11–13] We recently described two continuous ISG expression scores (IFN-Score-A and IFN-Score-B) that in combination better identify clinically meaningful differences in IFN status between and within autoimmune diseases.[14]

In other autoimmune diseases such as rheumatoid arthritis (RA), early evidence of progression to disease may be found at a target tissue level.[15] The tissues most commonly affected in AI-CTDs are the joints and skin. Musculoskeletal ultrasound can detect subclinical synovitis in SLE[16] but has not been assessed in At-Risk individuals. In skin, specialised local immune processes are found in SLE. Previous studies comparing keratinocytes or skin biopsies isolated from patients with cutaneous lupus and healthy controls (HCs) found marked differences in IL-18R responsiveness,[17] IFN-λ expression,[18] as well as a role of IFN-κ in initiating a feed-forward loop, which promoted exaggerated ISG activation in cutaneous lupus.[19] IFN-I status in the skin has not been assessed in At-Risk individuals.

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The aims of this study were to evaluate clinical, blood and tissue interferon and imaging biomarkers of progression from At-Risk to AI-CTD with a view to establish a strategy for disease prevention.

METHODS

Patients and design

A prospective observational study was undertaken in individuals who were referred from primary care to Leeds Teaching Hospitals NHS Trust due to suspected AI-CTD between November 2014 and May 2017. Inclusion criteria were (1) ANA-positive of at least 1:80 titre on indirect immunofluorescence and using multiplex immunoassays (excluding those with scleroderma (centromere, Scl-70) or myositis-specific (PL-12, OJ, PL-7, Mi-2, Ku, Jo-1, PM-Scl75, PM-Scl100, SRP and EJ) antibodies only); (2) ≤1 clinical criterion based on 2012 Systemic Lupus International Collaborating Clinics Classification Criteria (SLICC)[20] and not meeting classification criteria for other AI-CTD[21–23] or RA[24]; (3) symptom duration <12 months; (4) glucocorticoid, antimalarial and immunosuppressive treatment-naïve. Forty-nine HCs and 114 patients with SLE were used as negative and positive controls.

Assessment schedule and outcome

Comprehensive assessments including clinical, laboratory, imaging, bloods and skin biomarkers were performed at baseline, 12 months and annually for 3 years. Participants were given a helpline number for an additional flare visit if they had new or worsening inflammatory symptoms. Progression was defined by meeting the 2012 SLICC criteria for SLE,[20] 2016 ACR/EULAR criteria for pSS[21] or other relevant classification criteria for AI-CTD[22 23] at 12 months as assessed by rheumatologists.

Clinical and laboratory assessment

Age, gender, ethnicity, history of first-degree or second-degree relative(s) with autoimmune rheumatic diseases (ARDs), smoking history, SLICC criteria for SLE,[20] signs or glandular symptoms criteria for pSS,[21] patient and physician global health assessment using 100 mm Visual Analogue Scale were recorded.

ANA was tested using indirect immunofluorescence and a panel of nuclear autoantibodies including anti-dsDNA, extractable nuclear antigens (including Ro52, Ro60, La, Sm, Chromatin, RNP, Sm/RNP and Ribosomal P) and antiphospholipid antibodies (Cardiolipin and B2-Glycoprotein IgGs) using Bioplex 2200 Immunoassay. Lupus anticoagulant tests including activated partial thromboplastin time (APTT) (Actin FS), APTT-synthetic phospholipid (with correction) and dilute Russell's viper venom time (with correction) were deemed positive if persistent when repeated at 12 weeks. Full blood count was processed at a single accredited diagnostic laboratory. Complement levels (C3 and C4) were measured by nephelometry.

Musculoskeletal ultrasound

Ultrasound examination of wrists, metacarpophalangeal and proximal interphalangeal joints were performed by two rheumatologists, using General Electric S7 machine with a 6–15 MHz transducer. Outcome Measures in RA Clinical Trials (OMERACT) criteria[25] were used to define synovitis, that is, the presence of grey-scale (GS) ≥grade 2 and/or power Doppler (PD) ≥grade 1.

Blood and skin IFN scores

A two-score system of ISGs, as previously described,[14] was calculated without the knowledge of participant's clinical status. See online supplementary file for details. Briefly, peripheral blood mononuclear cells (PBMCs) were separated using density gradient method (Lymphoprep; Alere Technologies, Norway) from EDTA-anticoagulated blood. Total RNA purification kit (Norgen Biotek, Canada) was used followed by quantitative real-time reverse transcriptase-PCR (qRT-PCR) using TaqMan assays (Applied Biosystems, Invitrogen) for the selected 30 ISGs.[7] These assays were performed using the BioMark HD System with appropriate cycling protocols for the 96.96 chip. Data were normalised using Peptidylprolyl isomerase A as a reference gene to calculate ΔCt.

Factor analysis was used to reduce the 30 ISGs into a smaller number of factors.[26] Two factors, IFN-Score-A and IFN-Score-B, explained 84% of the variance with limited cross-loading. Factor scores were calculated as the median level of expression of the genes loaded by each factor.

Skin biopsy

One 4 mm biopsy was obtained from non-lesional non-sun-exposed areas (upper back or upper arms) of At-Risk individuals (n=10) and HCs (n=6), and from active lesions of patients with SLE (n=10). Biopsies were snap frozen in optimum cutting temperature (OCT) compound and sectioned at a thickness of 5 µm ensuring no remaining OCT material contaminating subsequent RNA extraction/RT procedures. Gene expression analysis and calculation of factor scores were conducted as for PBMCs.

Statistical analyses

Associations between categorical variables were tested by Fisher's exact and Stuart-Maxwell tests for independent and paired samples, respectively. Continuous variables were compared using either Student's t-tests or analysis of variance (ANOVA) followed by pairwise Tukey tests. For associations, Kendall's tau-b correlation was used if ties were present, otherwise using Pearson's correlation. Receiver operator curves (ROCs) were used to assess predictive strength and identify optimal thresholds for predicting progression to AI-CTD. For 13 At-Risk patients, gene expression data were missing at random due to samples not being processed on the day. For comparisons with HC and SLE groups, only At-Risk patients with complete data were presented. For prediction of progression, multiple imputation by chained equations was used to create 20 complete datasets, results of which were combined according to Rubin's rules. Multivariable analyses were performed using penalised logistic regression by Lasso method.[27] Leave-one-out cross-validation (R package cv.glmnet)[28] identified the largest penalty coefficient lambda within 1 SE of the value that minimised deviance in each imputed dataset; average coefficients from the best models were calculated. All analyses of IFN Scores were conducted using ΔCt scaling; results were then converted to relative expression ($2^{-\Delta Ct}$) or fold difference (FD) ($2^{-\Delta\Delta Ct}$).

Statistical analyses were performed using Stata V.13.1 (StataCorp, College Station, Texas, USA), R V.3.3.3[29] and GraphPad Prism V.7.03 (GraphPad, La Jolla, California, USA) for Windows.

RESULTS

Patient characteristics

The flowchart of participants is presented in figure 1. A total of 135 At-Risk individuals were recruited. Of these, 118 had at least 12 months of follow-up and were analysed. Baseline characteristics are described in table 1.

Table 2 Clinical characteristics of At-Risk progressors at 12 months

Clinical criteria	Baseline (n=19)	12 months (n=19)
Mucocutaneous		
ACLE or SCLE	5/19 (26%)	13/19 (68%)
Mucosal ulcers	2/19 (11%)	8/19 (42%)
Alopecia	0	4/19 (21%)
Musculoskeletal		
Synovitis	9/19 (47%)	18/19 (95%)
Haematological		
Leucopaenia or lymphopenia	3/19 (16%)	7/19 (37%)
Thrombocytopenia	0	1/19 (5%)
Glandular signs	0	6/19 (32%)
Serositis		
Pleural effusion	0	1/19 (5%)
Renal		
Class III nephritis	0	1/19 (5%)

ACLE, acute cutaneous lupus erythematosus; SCLE, sub-acute cutaneous lupus erythematosus.

Clinical outcomes at 12 months

At 12 months, 19/118 (16 %) At-Risk individuals progressed to a diagnosis of AI-CTD. These were SLE (n=14; 74%) and pSS (n=5; 26%). In those who progressed, all had one clinical criterion at baseline. The number of clinical SLE criteria increased to 2 in 4/19 (21%), 3 in 9/19 (47%) and 4 in 6/19 (32%) (Stuart-Maxwell χ^2=20.0, p<0.001) at 12 months. These details are presented in table 2 and online supplementary figure S1. Two patients developed internal organ involvement; pleural effusion and class III lupus nephritis.

In contrast, 19/99 (19%) of the non-progressors had no clinical SLE criteria at both baseline and 12 months, 1/99 (1%) increased from 0 to 1, 41/99 (42%) decreased from 1 to 0 indicating a remission of autoimmunity and 38/99 (38%) had one criterion at both time points (Stuart-Maxwell χ^2=38.1, p<0.001).

Notably, 1/99 (1%) of non-progressors had ankylosing spondylitis while 4/99 (4%) of had cancers (lung=1, hepatocellular=1, prostate=1 and leiomyosarcoma=1).

Interferon status in At-Risk differs from SLE

At baseline, IFN-Score-A differed between groups (ANOVA F=40.26; p<0.001). It was increased relative to HC (n=49) in both At-Risk (n=105; FD (95% CI) 2.21 (1.22 to 4.00), p=0.005) and SLE (n=114; 7.81 (4.33 to 14.04), p<0.001), and was increased in SLE relative to At-Risk (3.54 (2.22 to 5.63), p<0.001) (figure 2A). In contrast, although IFN-Score-B differed between groups overall (F=63.35; p<0.001), it did not differ between At-Risk and HC (0.98 (0.66 to 1.46), p=0.993), but was increased in SLE to both HC (3.85 (2.60 to 5.72), p<0.001) and At-Risk (3.93 (2.87 to 5.37), p<0.001) (figure 2B).

Relationships of interferon scores with autoantibodies, complement and lymphopaenia

Correlations between routine immunology markers and IFN Scores were performed in observed data using reflected ΔCt so that higher IFN Scores represented greater expression. At baseline, there was no association between number of positive ANA specificities (ie, anti-dsDNA, Ro, RNP etc.) and IFN-Score-A (n=105, Kendall's tau-b 0.13, p=0.084) or IFN-Score-B (tau-b 0.09, p=0.234) (figure 2C,D).

The titres of two antibodies that were mostly prevalent using Bioplex, anti-dsDNA and anti-Ro, were divided into three and two groups, respectively. There were no differences in both IFN Scores among the three anti-dsDNA groups (online supplementary figure S2A,B). Elevated levels of IFN-Score-A (FD 2.41 (95% CI 1.10 to 5.26)) but not Score-B were found in the high titre, that is, ≥8 AI anti-Ro antibody positive group (online supplementary figure S2C, D).

There was a weak negative correlation between C4 levels and IFN-Score-A (n=97, Pearson's r=−0.221, p=0.029) (figure 2E) but not IFN-Score-B (r=−0.089, p=0.385). There was a weak negative correlation between lymphocyte count and IFN-Score-A (n=105, r=−0.230, p=0.018) (figure 2F) but not IFN-Score-B (r=−0.127, p=0.195).

Baseline interferon status in skin

In parallel to results obtained for PBMC, at baseline only IFN-Score-A was increased in non-lesional skin biopsies in At-Risk (n=10) versus HC (n=6); FD 28.74 (1.29 to 639.48), p=0.036. There was no difference in IFN-Score-B; FD 1.82 (0.86 to 3.86), p=0.100. As expected, both IFN Scores were higher in SLE (active lesions) compared with either At-Risk or HC; all p<0.05.

Comparison of baseline interferon status between blood and skin

Expression of both IFN Scores was higher in At-Risk versus HC in both skin and PBMC, but FDs were greater in skin (figure 3C). This might have been due to the small sample size for skin samples (paired skin–PBMC samples were not available).

Prediction of AI-CTD using baseline interferon scores in blood

When At-Risk were divided according to AI-CTD progression status at 12 months, both IFN Scores differed among the groups overall (p<0.001) and both were elevated in At-Risk progressors (n=19) versus non-progressors (n=86), to a greater extent for IFN-Score-B (FD 3.22 (1.74 to 5.95), p<0.001) than IFN-Score-A (2.94 (1.14, 7.54), p=0.018) (figure 3A,B). Non-progressors did not differ from HC (n=49) for both scores; IFN-Score-B (0.79 (0.51 to 1.23), p=0.520) and IFN-Score-A (1.82 (0.93 to 3.53), p=0.096). Neither IFN Score differed between At-Risk progressors and SLE (both p>0.1).

Since the number of skin biopsies obtained in At-Risk was small (n=10), no formal association between IFN Scores and progression could be determined.

Baseline IFN-Score-B threshold of progression to AI-CTD

Prognostic ability of baseline IFN Scores to predict progression to AI-CTD at 12 months was assessed using ROC curve analysis. The area under the ROC curve was greater for IFN-Score-B (0.82 (95% CI 0.73 to 0.92)) than IFN-Score-A (0.70 (0.57 to 0.83)); χ^2=4.19, p=0.041. A cut-off of ≤5.01 ΔCt for IFN-Score-B maximised the Youden's index (sensitivity+specificity−1) yielding 95% (95% CI 75% to 99%) sensitivity, 60% (50% to 70%) specificity, 35% (23% to 48%) positive predictive value (PPV) and 98% (90% to >99%) negative predictive value (NPV). However, for a rule-in biomarker for future prevention studies, a high specificity is required to exclude individuals with the lowest risk. For this purpose, we propose a cut-off of ≤3.90 ΔCt that resulted in 68% (46% to 85%) sensitivity, 80% (70% to 88%) specificity, 43% (27% to 61%) PPV and 92% (84% to 96%) NPV (figure 3D).

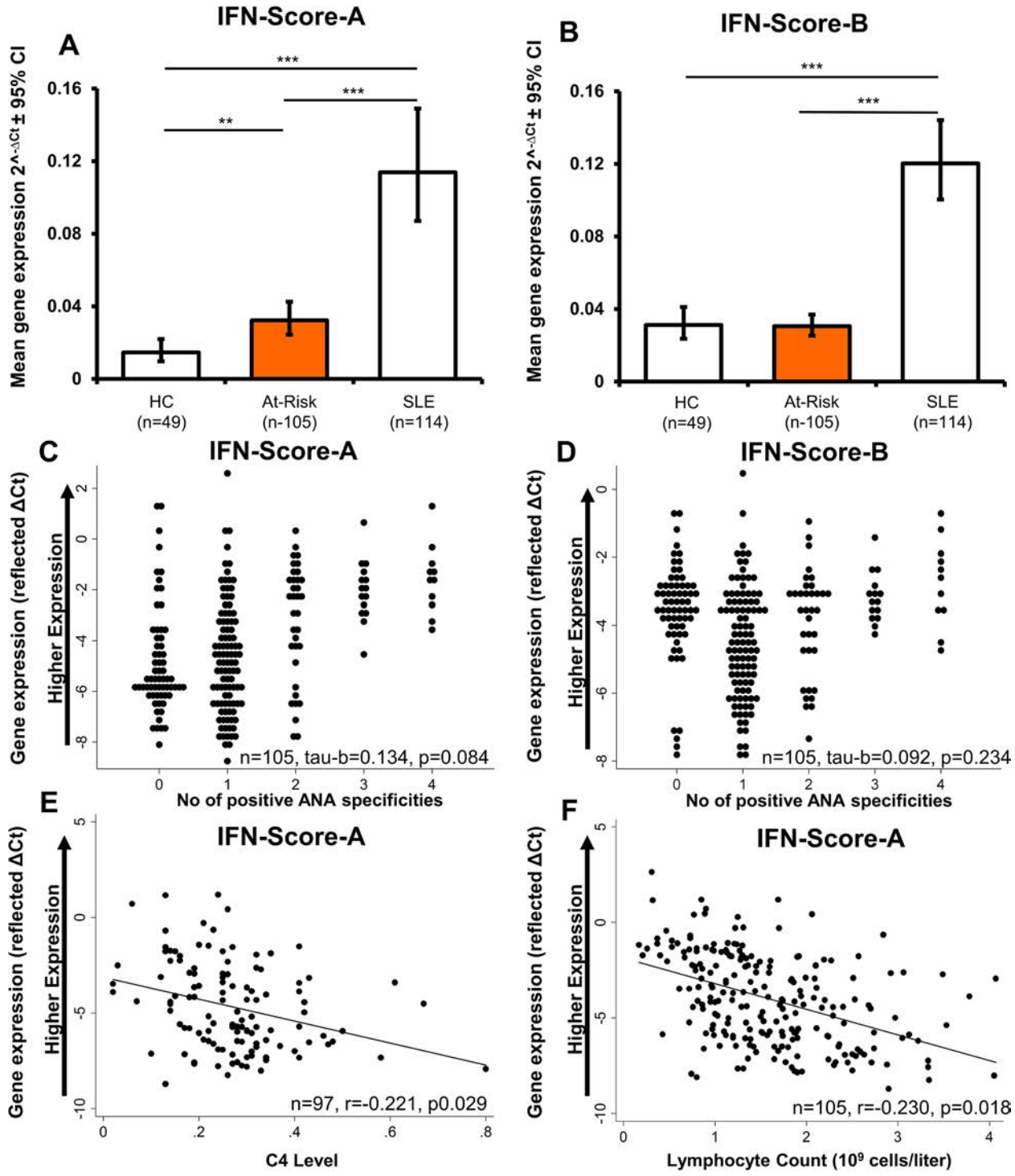

Figure 2 Pattern of baseline interferon scores and their relationships with clinical immunology markers. (A) Baseline expression of IFN-Score-A was higher in At-Risk individuals compared with healthy controls. (B) However, there was no difference in IFN-Score-B between both groups. ***Highly significant (p<0.001), **moderate significant (0.001<p<0.01), *significant (0.01<p<0.05). (C, D) Both IFN scores were not correlated with the number of positive antinuclear antibody (ANA) specificities (ie, sum of anti-dsDNA, Ro, La, Sm, Chromatin, RNP, Sm/RNP and Ribosomal P) and (E, F) there were only weak correlations between IFN-Score-A and complement and lymphocyte count. Data for gene expression were expressed as reflected values for ΔCt so that higher IFN Scores represented greater expression.

Baseline IFN Scores were lower in At-Risk without versus with one clinical criterion

All 20/118 (17%) At-Risk individuals who had no SLE clinical criterion at baseline did not progress to AI-CTD at 12 months. At baseline, FDs for both IFN scores differed among the groups overall (p<0.001) and both were lower in At-Risk with no criterion (n=17) versus with one criterion (n=88); all p<0.05 (online supplementary figure S3 in the online supplementary file).

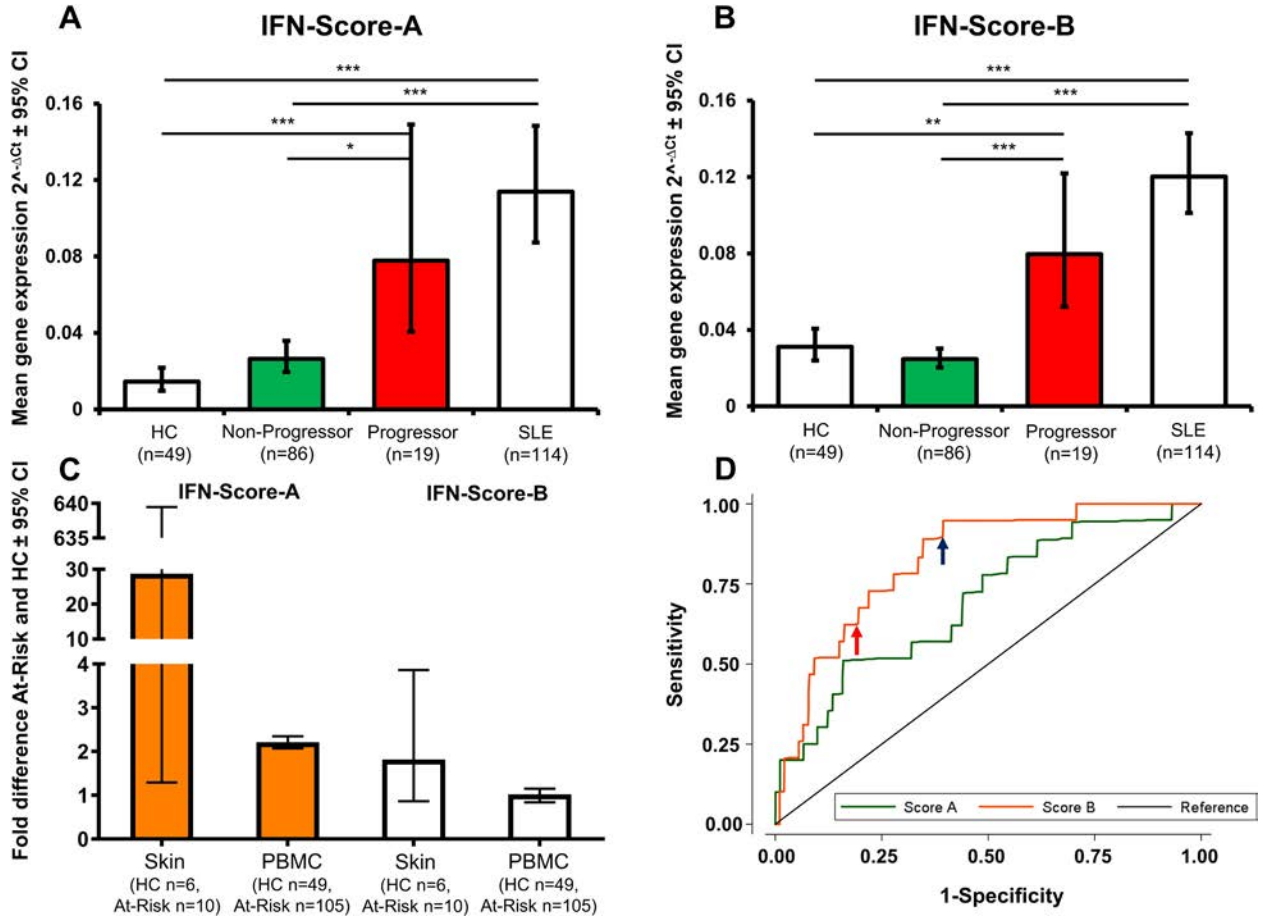

Figure 3 Baseline interferon (IFN) scores in bloods as prognostic biomarkers. (A–B) Baseline expression of both IFN-Score-A and IFN-Score-B were higher in At-Risk individuals who progressed to autoimmune-related connective tissue disease compared with the non-progressors, but to a greater fold difference in the latter. ***Highly significant (p<0.001), **moderately significant (0.001<p<0.01), *significant (0.01<p<0.05). (C) Fold differences for both IFN scores between At-Risk and healthy controls (HCs) were greater in skin than bloods. (D) The area under the receiver operating characteristic curve was significantly greater for IFN-Score-B than IFN-Score-A. The blue arrow denotes the optimal cut-off using Youden's index while the red arrow denotes the proposed cut-off for prevention study. PBMC, peripheral blood mononuclear cell; SLE, systemic lupus erythematosus.

Musculoskeletal ultrasound

Of the 117 At-Risk individuals with ultrasound available, 21 (18%) had ultrasound-defined synovitis at baseline (GS ≥2 only=13, PD ≥1 with or without GS ≥2=8). Of the 20 individuals who progressed, 7 (35%) had positive ultrasound at baseline versus 14% of non-progressors; p=0.050, PPV (95% CI)=33% (17% to 55%), NPV 86% (78% to 92%).

Furthermore, 43/118 of At-Risk individuals had clinical arthritis based on SLICC[20] (8/43 (19%) had ≥2 joints with swelling or effusion while 35/43 (81%) had ≥2 joints with tenderness and early morning stiffness of ≥30 min) while 75/118 had no arthritis. In those without arthritis, ultrasound-defined synovitis was detected in 10/75 (13%) and 4/10 (40%) progressed to AI-CTD. Conversely, in those with arthritis, only 11/42 (26%) had ultrasound-defined synovitis and 3/11 (27%) progressed to AI-CTD at 12 months. Sensitivity and specificity of physician-judged arthritis with ultrasound-defined synovitis were 52% and 68%, respectively.

Multivariable analysis of baseline predictors of progression to AI-CTD

In imputed univariable analyses, all putative predictors were associated with progression to AI-CTD at 12 months at the 10% level of significance except for complement level and lymphocyte count (both p>0.1), which were excluded from multivariable analysis (table 3). In multivariable logistic regression, family history of ARDs (OR 8.20, p=0.012) and IFN-Score-B (OR=3.79, p=0.005) were independently associated with progression. Penalised ORs remained substantive for these variables when all other variables were removed from the model. Results in complete data (n=100) were similar (data not shown).

DISCUSSION

In this study, we report a unique cohort of At-Risk of AI-CTD individuals with longitudinal follow-up until progression to clinical autoimmunity. We demonstrate that IFN activity is strongly associated with progression independent of baseline clinical status, with measurement according to a two-score system we described being crucial. These results provide a rationale for diagnostic and preventative treatment pathways as well as assert the importance of interferons in disease initiation.

Referrals of ANA-positive individuals to rheumatologists has increased over the last decade.[30] Concerns are that these At-Risk individuals may be discharged prematurely or be observed in an inefficient 'watch and wait' fashion until the diagnosis is clear, by which time the potential to prevent disease and confer the most benefit may be lost. Thus, by undertaking the largest prospective study of At-Risk individuals, which is the first to integrate clinical, imaging and immunological assessments (including skin),

Table 3 Penalised logistic regression for predictors of progression to autoimmune-related connective tissue disease at 12 months

Baseline predictors	No progression n=99	Progression n=19	Univariable OR (95% CI), p values	Multivariable OR (95% CI), p values	Penalised coefficient to OR
Age, mean (SD)	49.0 (15.8)	39.6 (11.9)	0.96 (0.93 to 0.99), 0.016	0.97 (0.92 to 1.02), 0.232	0.000 to 1.000
Ever smoked, (%)	41.8%	20.0%	0.35 (0.11 to 1.12), 0.076	0.34 (0.06 to 1.91), 0.222	0.000 to 1.000
Family history of ARDs (%)	30.6%	65.0%	4.21 (1.53 to 11.61), 0.005	**8.20 (1.58 to 42.53), 0.012**	0.243 to 1.275
No of positive ANA specificities, median (IQR)	1 (1–1)	1 (1–2)	2.07 (0.97 to 4.40), 0.060	2.41 (0.71 to 8.20), 0.161	0.000 to 1.000
Complement C4 level, mean (SD)	0.29 (0.12)	0.26 (0.08)	0.06 (0.00 to 8.05), 0.264	Excluded	Excluded
Lymphocyte count, mean (SD)	2.04 (0.77)	1.83 (0.67)	0.67 (0.34 to 1.34), 0.257	Excluded	Excluded
No of joints with positive ultrasound for synovitis, median (IQR)	0 (0–0)	0 (0–2)	1.20 (0.97 to 1.47), 0.086	1.44 (0.98 to 2.11), 0.061	0.002 to 1.002
Patient VAS, median (IQR)	36 (16–61)	47 (26–75)	1.02 (1.00 to 1.04), 0.079	1.01 (0.98 to 1.04), 0.484	0.000 to 1.000
Physician VAS, median (IQR)	11 (3–31)	31 (15–47)	1.04 (1.01 to 1.06), 0.008	1.01 (0.97 to 1.06), 0.618	0.000 to 1.000
IFN-Score-A (−ΔCt), mean (SD)*	−5.3 (1.9)	−3.8 (2.26)	1.43 (1.11 to 1.84), 0.005	0.87 (0.54 to 1.39), 0.560	0.000 to 1.000
IFN-Score-B (−ΔCt), mean (SD)*	−5.3 (1.4)	−3.7 (1.0)	2.55 (1.60 to 4.08), <0.001	**3.79 (1.50 to 9.58), 0.005**	0.319 to 1.376

*Analysis was made based on reflected ΔCt. Thus, the higher the number, the higher the gene expression to give positive values for ORs.
ANA, antinuclear antibody; ARD, autoimmune rheumatic disease; IFN, interferon; VAS, Visual Analogue Score.

our findings offer a novel approach, biomarkers and have implications for future development of targeted therapies for this group of patients.

Within ANA-positive individuals, different immune phenotypes could be defined. At baseline, IFN-Score-A was elevated but not IFN-Score-B compared with HC. However, IFN-Score-B (and to a lesser degree, IFN-Score-A) were mostly elevated in those who progressed to AI-CTD. IFN-Score-A comprises many well-known ISGs that respond to IFN-I (IFN-α, IFN-β, IFN-κ, IFN-ω). In contrast, IFN-Score-B comprises ISGs that coincide with M3.4 and M5.12 modules of a previous microarray study.[7] These ISGs were suggested to be responsive to IFN-II (IFN-γ), IFN-III (IFN-λ) as well as IFN-I. However, we cannot exclude the influence of other inflammatory mediators on this pattern of gene expression.[14] Some studies suggested that IFN-I contributes to priming cells to secrete IFN-II.[31 32] Conversely, a study that measured IFN activity from serum postulated a sequential role of IFN-II augmentation that led to autoantibody accumulation and subsequent elevations in IFN-α prior to SLE.[33] Although we could not confirm which IFN pathways predominate, our findings suggest that progression to AI-CTD may not be exclusively driven by IFN-I but by a synergistic activation of ISGs induced by a range of IFNs and IFN-Score-B could act as a biomarker for more diverse immune activation.

At the tissue level, this is the first study that quantifies IFN activity in non-lesional skin of At-Risk individuals. Interestingly, similar patterns of immune dysregulation were shown between skin and PBMC. However, markedly greater FDs in both IFN scores were found in the former compared with the latter, thus highlighting skin as a potential site of AI-CTD initiation.

Only a third of the At-Risk individuals who had ultrasound-defined synovitis progressed to AI-CTD within 12 months. Additionally, small numbers of asymptomatic patients with ultrasound-detected synovitis were identified, so further work is required to determine the role of ultrasound in assessing At-Risk individuals.

Together with a family history of ARD, IFN-Score-B from blood is independently predictive of progression and is convenient as a biomarker. We have defined a cut-off level of IFN-Score-B with a moderate diagnostic accuracy in order to design a prevention study.

This study has some limitations. First, the cohort was recruited from secondary care as well as positive ANA detected by both Bioplex and indirect immunofluorescence, which might contribute to moderate-to-high pre-test probabilities for AI-CTD. Thus, our results might not be generalised to all ANA-positive cases in primary care setting. However, our cohort was quite heterogenous in terms of ethnicity and 17% of the patients had no SLE criterion at baseline. Second, we excluded individuals with scleroderma or myositis-specific only autoantibodies, which might lead to preponderance of progression to SLE or pSS. Surprisingly, one patient had a severe ankylosing spondylitis and required biological therapy. Moreover, 4% of non-progressors had cancers thus highlighting the need to be vigilant of paraneoplastic manifestation in ANA-positive individuals as well as diverse alternative diagnoses in general. Lastly, sample size was still relatively small for multivariable analysis. However, we used penalised logistic regression to minimise overfitting of data.

In conclusion, a novel ISG score, IFN-Score-B and family history of ARD predict progression from ANA positivity to AI-CTD. After validation, the predictive value of IFN scores may allow us to identify patients with imminent AI-CTD for earlier intervention using therapies that block IFNs or conventional immunosuppressants to avoid irreversible organ damage and glucocorticoid exposure. Additionally, patients with benign autoreactivity can be better identified.

Correction notice This article has been corrected since it published Online First. The affiliations have been updated.

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Patient consent Not required.

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EXTENDED REPORT

Trimethoprim–sulfamethoxazole prophylaxis prevents severe/life-threatening infections following rituximab in antineutrophil cytoplasm antibody-associated vasculitis

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ABSTRACT

Objective We aimed to assess risk factors for the development of severe infection in patients with antineutrophil cytoplasm antibody-associated vasculitis (AAV) receiving rituximab.

Methods 192 patients with AAV were identified. Univariate and multivariate analyses were performed to identify risk factors for severe infection following rituximab. Severe infections were classified as grade ≥3 as proposed by the Common Terminology Criteria for Adverse Events V.4.0.

Results 95 severe infections were recorded in 49 (25.52%) patients, corresponding to an event rate of 26.06 per 100 person-years. The prophylactic use of trimethoprim–sulfamethoxazole was associated with a lower frequency of severe infections (HR 0.30, 95% CI 0.13 to 0.69), while older age (HR 1.03, 95% CI 1.01 to 1.05), endobronchial involvement (HR 2.21, 95% CI 1.14 to 4.26), presence of chronic obstructive pulmonary disease (HR 6.30, 95% CI 1.08 to 36.75) and previous alemtuzumab use (HR 3.97, 95% CI 1.50 to 10.54) increased the risk. When analysis was restricted to respiratory tract infections (66.3% of all infections), endobronchial involvement (HR 4.27, 95% CI 1.81 to 10.06), severe bronchiectasis (HR 6.14, 95% CI 1.18 to 31.91), higher neutrophil count (HR 1.19, 95% CI 1.06 to 1.33) and major relapse (HR 3.07, 95% CI 1.30 to 7.23) as indication for rituximab use conferred a higher risk, while refractory disease (HR 0.25, 95% CI 0.07 to 0.90) as indication had a lower frequency of severe infections.

Conclusions We found severe infections in one quarter of patients with AAV receiving rituximab. Trimethoprim–sulfamethoxazole prophylaxis reduced the risk, while especially bronchiectasis and endobronchial involvement are risk factors for severe respiratory infections.

INTRODUCTION

Antineutrophil cytoplasm antibody (ANCA)-associated vasculitis (AAV) encompasses three entities, namely granulomatosis with polyangiitis (GPA, previously Wegener's granulomatosis), microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (EGPA, previously Churg-Strauss Syndrome). The availability of ANCA facilitates diagnosis and treatment strategies, and has led to a better prognosis over recent decades.[1] Nevertheless, comorbidities attributable to the persistence of the disease or side effects of treatment remain a challenge. Forty-eight per cent of deaths occurring during the first year are caused by infections and remain a major cause of mortality thereafter.[2] Infectious complications have been studied especially in cyclophosphamide-treated patients. Several risk factors have been identified, including treatment intensity (cumulative steroid and cyclophosphamide dose), reduced creatinine clearance (estimated glomerular filtration rate (eGFR) of ≤30 mL/min) or dialysis dependency, older age and pulmonary involvement.[3] Rituximab showed similar efficacy compared with a cyclophosphamide-based treatment in the induction of remission in two randomised controlled trials. However, rituximab did not show a reduced rate of severe infections compared with cyclophosphamide.[4 5] Patients recruited into trials may have a lower adverse event rate due to rigorous monitoring and selection of patients according to exclusion criteria,[6] and the rate of side effects might be even higher in routine practice. Several observational studies have reported severe/life-threatening infectious complications following rituximab, including cases with *Pneumocystis jirovecii*, *Pseudomonas aeruginosa*, pulmonary aspergillosis and progressive multifocal leukoencephalopathy.[7–9] While *P. jirovecii* prophylaxis is widely accepted in patients receiving cyclophosphamide (CYC), no such recommendations exist for patients receiving rituximab.

This study investigated the frequency of severe/life-threatening infections in 192 patients with AAV treated with rituximab. It also aimed to identify risk factors for severe infection in this patient population.

METHODS

Study population

This study included patients with AAV older than 18 years who were referred for rituximab to two tertiary care specialist centres, Addenbrooke's Hospital (Cambridge, UK) and the Medical University Innsbruck (Innsbruck, Austria), between 2004 and 2014. Diagnosis of AAV was established according to the European Medicines Agency (EMA) algorithm.[10] Follow-up of patients began at the time of rituximab administration and ended on the date of death, the date patients were lost to follow-up, 2 years after first rituximab

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administration or on 1 January 2015, whichever occurred first. This study was conducted in accordance with the ethical principles stated in the Declaration of Helsinki. The Institutional Review Board of both university hospitals approved the use of anonymised patient data for research purposes.

Clinical data

The following data were obtained from the respective electronic medical records of the patients: demography (age, gender), diagnosis, date of diagnosis, time to rituximab, ANCA serotype, disease phenotype, organ involvement, prior immunosuppressive therapies, cumulative cyclophosphamide exposure (in grams), immunosuppression during the year before rituximab, concomitant treatment, laboratory values (serum creatinine, C reactive protein (CRP), erythrocyte sedimentation rate (ESR), neutrophils, white blood count (WBC), lymphocytes, CD3/CD4/CD8/CD19/CD56 counts, immunoglobulins), indication for the use of rituximab (see online supplementary appendix), comorbidities (including chronic obstructive pulmonary disease, diabetes mellitus, hypertension, chronic heart failure), smoking history, antibiotic prophylaxis (trimethoprim–sulfamethoxazole or others) and the occurrence of severe/life-threatening infections (grade ≥3), as classified by the Common Terminology Criteria for Adverse Events (CTCAE) V.4.0 (see online supplementary appendix).[11] Hypogammaglobulinaemia was defined as a IgG level of below 7 g/L. Patients with incomplete or missing medical records were excluded from further analyses. The cumulative doses of rituximab during follow-up were determined.

Statistical analysis

Categorical variables were compared using the χ^2 test (or Fisher's exact test, when appropriate), and metric variables were compared using the Mann-Whitney U test. Metric variables are shown as median (and minimum to maximum), and nominal variables are shown as per cent (%). Both univariate and multivariate Cox regression analyses were performed to determine significant risk factors for severe/life-threatening and respiratory infections. The occurrence of at least one episode of severe/life-threatening infection during the follow-up period of 24 months was the outcome of interest. Kaplan-Meier plots and log-rank test were performed to assess univariate associations. All variables showing significant association with the dependent variable in the univariate Cox regression analysis were entered into a multivariate Cox regression model. A backward selection procedure was then used (with p values greater than 0.100 as the removal criterion, using Wald's test). Neutrophils correlated with WBC, CRP and ESR and sinusitis correlated with ear, nose and throat (ENT) involvement, thus only neutrophils at baseline and sinusitis were included in multivariate analysis. Results are expressed as HRs with 95% CIs. All statistical analyses were performed with SPSS Statistics V.21.0 (IBM).

RESULTS

Patient characteristics

The total number of patients included in the analysis was 192 (134 with GPA, 28 with MPA and 30 with EGPA). Mean duration of initial diagnosis to initiation of rituximab was 4.33 years. Patients were followed for a mean time of 22.67 months from the time of rituximab initiation (mean rituximab dose 4.75 g). Forty-nine patients presented with 95 infectious complications classified as CTCAE V.4.0 ≥3. In detail, 71 episodes were CTCAE V.4.0 grade 3, 23 as grade 4 and 1 as grade 5 (multiorgan failure as a consequence of sepsis related to an urinary tract infection). The overall event rate was 26.06 per 100 person-years. Twenty-five per cent of the observed infections occurred during the first 4 months of follow-up, while 50% and 80% were observed after 12 and 18 months, respectively. Antibiotic prophylaxis with trimethoprim–sulfamethoxazole was administered in 73 out of 192 (38.02%). During the follow-up period, seven fatalities were recorded. Baseline characteristics of patients with severe infections and those without are depicted in table 1.

Infections

Respiratory tract infection was the most common infectious complication (n=63), followed by urinary tract (n=12), gastrointestinal tract (n=8), mastoiditis/otitis externa (n=4), skin (n=3), sepsis/septicaemia with unidentified site of infection (n=1), catheter-associated exit site infections (n=1), orbital mass infection (n=1), lacrimal gland abscess (n=1) and eye (n=1) (online supplementary table S1). Moreover, in cases with a positive microbial result, opportunistic pathogens were seen, including *P. aeruginosa* (n=4), *Staphylococcus aureus* including methicillin-resistant strains (n=4), *Escherichia coli* (n=3), *Clostridium difficile* (n=2), *P. jirovecii* (n=1), *Legionella pneumophila* (n=1) and invasive aspergillosis (n=1). In addition, one case of *Campylobacter jejuni* gastroenteritis was observed (online supplementary table S1 and online supplementary table S2).

Rituximab treatment and risk of infections

To identify specific risk factors associated with the development of infectious complications, univariate analysis was performed. Older patients (HR 1.02, 95% CI 1.00 to 1.04), patients with endobronchial involvement (HR 2.44, 95% CI 1.38 to 4.32) and severe bronchiectasis (HR 4.79, 95% CI 1.47 to 15.59) were at increased risk for severe infections. Patients presenting with sinusitis (HR 0.48, 95% CI 0.27 to 0.84) or in general ENT involvement (HR 0.46, 95% CI 0.26 to 0.82) had fewer severe infections. While there was no correlation with serum creatinine, higher eGFR (HR 0.99, 95% CI 0.98 to 1.00) emerged as a protective factor. Higher ESR (HR 1.11, 95% CI 1.03 to 1.20), WBC (HR 1.06, 95% CI 1.01 to 1.10), higher steroid doses (HR 1.02, 95% CI 1.01 to 1.04) and an IgG decline ≥30% (HR 1.88, 95% CI 1.04 to 3.39) at baseline were predictors of severe infections. Concomitant comorbidities, such as chronic obstructive pulmonary disease (COPD, HR 16.07, 95% CI 4.41 to 58.49), diabetes (HR 2.35, 95% CI 1.14 to 4.85) and reduced left ventricular ejection fraction/previous myocardial infarction (HR 2.21, 95% CI 1.07 to 4.56) emerged as risk factors. Treatment with alemtuzumab (ALM) ever before rituximab was associated with an increased risk (HR 2.49, 95% CI 1.05 to 5.91). Antibiotic prophylaxis to prevent *P. jirovecii* infections with trimethoprim–sulfamethoxazole reduced the risk of severe infections (HR 0.45, 95% CI 0.23 to 0.88). A multivariate logistic regression analysis revealed that the use of trimethoprim–sulfamethoxazole as prophylactic antibiotic measure had an impact on reduction of severe infections (HR 0.30, 95% CI 0.13 to 0.69). Moreover, the use of trimethoprim–sulfamethoxazole significantly reduced the time to first significant infection (p=0.016) (table 2 and figure 1). Moreover, older age (HR 1.03, 95% CI 1.01 to 1.05), endobronchial involvement (HR 2.21, 95% CI 1.15 to 4.26), COPD (HR 6.30, 95% CI 1.08 to 36.75) and ALM treatment before rituximab (HR 3.97, 95% CI 1.50 to 10.54) emerged as independent risk factors to develop severe infections following rituximab (table 2).

Table 1 Baseline characteristics of patients having severe infections versus those without severe infections

	No severe infection (n=143)	Severe infection (n=49)	P values
Demographics			
Age (years)	56 (16–85)	60 (22–82)	**0.023**
Gender (male, %)	45	41	0.573
Type of vasculitis (%)			0.407
GPA	71	65	
MPA	13	20	
EGPA	16	14	
Symptoms (%)			
B-symptoms (night sweat, fever, unintentional weight loss)	21	14	0.353
Neuropathy	27	24	0.774
Sinusitis	72	53	**0.015**
Deafness/mastoiditis/otitis media	31	22	0.266
Arthralgia	45	33	0.117
Organ involvement (%)			
CNS	7	7	1
Subglottic/tracheal stenosis	12	14	0.661
Skin	18	14	0.533
Kidney	44	51	0.398
Eye	29	17	0.112
Others	7	8	1
ENT	79	61	**0.014**
Lung	54	65	0.162
Imaging findings (%)			
Pulmonary cavities	24	26	0.75
Endobronchial	20	41	**0.004**
Severe bronchiectasis	1	8	0.054
Disease activity measures			
BVAS	6 (0–28)	6 (0–18)	0.602
DEI	6 (2–12)	6 (2–10)	0.848
Laboratory values			
Creatinine (µmol/L)	86 (45–1451)	98 (49–879)	**0.027**
eGFR (MDRD/Modification of Diet in Renal Disease equation) mL/min/1.73 m^2	75 (3–163)	60 (5–155)	**0.002**
CRP (0–6 mg/L)	5.0 (0.7–215.0)	14.0 (1.0–215.0)	**0.001**
ESR (5–15 in the 1st hour)	16 (2–116)	22 (1–109)	**0.006**
Neutrophils (2–8×10^9/L)	7.1 (2.0–18.6)	8.3 (2.4–21.4)	**0.025**
WBC (4–11×10^9/L)	9.4 (3.6–42.0)	10.7 (3.3–24.4)	**0.006**
Lymphocytes (1–4.5×10^9/L)	1.0 (0.1–3.7)	1.0 (0.4–4.5)	0.145
CD19 (0.1–0.5)	0.04 (0.00–0.80)	0.03 (0.00–0.77)	0.781
CD3 (0.7–2.1)	0.82 (0.05–7.20)	0.70 (0.21–3.32)	0.246
CD4 (0.3–1.4)	0.48 (0.03–1.98)	0.38 (0.11–2.80)	0.303
CD8 (0.2–0.9)	0.29 (0.02–1.93)	0.20 (0.07–0.95)	0.414
CD56 (0.12–0.88)	0.11 (0.00–0.70)	0.15 (0.00–0.80)	0.09
IgG (6–13 g/L)	9.0 (2.8–22.6)	8.8 (3.0–18.9)	0.823
IgG decline ≥30% (%)	20	35	**0.041**
Hypogammaglobulinaemia (%)	13	16	0.593
IgM (0.4–2.2 g/L)	0.7 (0.3–2.6)	0.7 (0.3–2.0)	0.398
IgA (0.8–3.7 g/L)	1.8 (0.4–5.3)	2.1 (0.5–4.3)	0.715
ANCA-positive (%)	73	76	0.703
Comorbidities (%)			
COPD	1	6	0.053
Diabetes	6	18	**0.021**
Hypertension	37	33	0.557
Myocardial infarction/reduced LVEF	8	18	**0.036**
Indication (%)			
Minor relapse	41	29	0.114
Major relapse	27	39	0.13

Continued

Table 1 Continued

	No severe infection (n=143)	Severe infection (n=49)	P values
Maintenance	78	82	0.622
Refractory disease	31	27	0.516
Steroid sparing	17	22	0.375
1st line	5	10	0.187
Premedication (last 12 months)			
CYC (g)	0 (0–45)	0 (0–22)	0.632
MMF (g)	0 (0–1080)	15 (0–1080)	0.798
AZA (g)	0 (0–81)	0 (0–72)	0.036
MTX (mg)	0 (0–1286)	0 (0–1286)	0.739
IVIG (ever) (%)	4	12	0.128
Anti-TNF (ever) (%)	3	5	0.65
PLEX (ever) (%)	9	7	1
ALM (ever) (%)	5	14	0.079
Medication used concurrently with RTX			
Steroids (mg)	15 (0–60)	15 (5–60)	0.087
Trimethoprim–sulfamethoxazole (%)	43	22	**0.009**
Other antibiotic prophylaxis (%)	9	16	0.172

Metric variables are shown as median and (minimum–maximum), nominal variables are shown as %. Statistics tests are χ quadrate test/Fisher's exact test and Mann-Whitney U test where appropriate. The respective reference ranges, if applicable, are given in parentheses. P values indicating significant changes are highlighted in bold font.
ALM, alemtuzumab; ANCA, antineutrophil cytoplasm antibody; AZA, azathioprine; BVAS, Birmingham Vasculitis Activity Score; CD, cluster of differentiation; CNS, central nervous system; COPD, chronic obstructive pulmonary disease; CRP, C reactive protein; CYC, cyclophosphamide; DEI, Disease Extent Index; eGFR, estimated glomerular filtration rate; EGPA, eosinophilic granulomatosis with polyangiitis; ENT, ear, nose and throat; ESR, erythrocyte sedimentation rate; GPA, granulomatosis with polyangiitis; IVIG, intravenous immunoglobulins; LVEF, left ventricular ejection fraction; MMF, mycophenolate mofetil; MPA, microscopic polyangiitis; MTX, methotrexate; PLEX, plasma exchange; RTX, rituximab; TNF, tumour necrosis factor; WBC, white blood count.

Risk for lower respiratory tract infections after rituximab

Since respiratory tract infections were the leading cause of infectious complications (n=63), we aimed to identify factors predicting the risk. Nine patients underwent bronchoscopy and most of them had at least two respiratory tract infections (7/9). Patients with preserved eGFR (HR 0.99, 95% CI 0.98 to 1.00), presenting with sinusitis (HR 0.47, 95% CI 0.23 to 0.98) and ENT involvement (HR 0.43, 95% CI 0.20 to 0.87) as well as receiving rituximab for refractory disease (HR 0.35, 95% CI 0.12 to 0.99), had a lower likelihood to develop severe pulmonary infections. In contrast, lung involvement (HR 2.53, 95% CI 1.08 to 5.93) and in particular endobronchial involvement (HR 4.30, 95% CI 2.06 to 8.94) and severe bronchiectasis (HR 7.48, 95% CI 2.22 to 25.16) emerged as risk factors. Higher CRP (HR 1.01, 95% CI 1.00 to 1.01), ESR (HR 1.02, 95% CI 1.00 to 1.03), neutrophils (HR 1.15, 95% CI 1.15) and WBC (HR 1.07, 95% CI 1.01 to 1.12) at baseline were associated with severe pulmonary infections. Moreover, those with concomitant COPD (HR 19.75, 95% CI 5.23 to 74.63), major relapse as indication (HR 2.65, 95% CI 1.28 to 5.49) and higher steroid doses (HR 1.02, 95% CI 1.00 to 1.04) had more pulmonary infections. Multivariate analysis retained endobronchial involvement (HR 4.30, 95% CI 2.06 to 8.94), severe bronchiectasis (HR 7.48, 95% CI 2.22 to 25.16), neutrophil count at baseline (HR 1.19, 95% CI 1.06 to 1.33) and major relapse (HR 2.65, 95% CI 1.28 to 5.49) as independent risk factors, while rituximab use in the setting of refractory disease was negatively associated with severe pulmonary infections (HR 0.35, 95% CI 0.12 to 0.99) (online supplementary table S3).

Prescription pattern and side effects of trimethoprim–sulfamethoxazole

The dose of trimethoprim–sulfamethoxazole used as a prophylaxis was not consistent. Most patients received 480 mg on alternate days (38.36%), followed by 960 mg on alternate days (21.92%) and 960 mg twice daily (12.33%, further details see online supplementary table S4). Among differences in the prescription pattern, a diagnosis of GPA, ENT involvement including sinusitis and deafness, mastoiditis and otitis media were associated with a more frequent prescription. Lower CD4 T-cell count as well as cyclophosphamide in the year before and a higher concomitant steroid use led to trimethoprim–sulfamethoxazole prescription (online supplementary table S5). Next, we assessed side effects of trimethoprim–sulfamethoxazole focusing on recently reported adverse events in rheumatological indications.[12] Trimethoprim–sulfamethoxazole was stopped in five patients due to haematopoietic complications in three (lymphopenia, pancytopenia, neutropenia), sore mouth in one and abnormal liver function test in the remainder. In general, trimethoprim–sulfamethoxazole prophylaxis was maintained for 14.67 months.

DISCUSSION

Comorbidities, either attributable to active disease or immunosuppression, remain a major issue in the management of AAV. An analysis of the early EUVAS trials revealed that infections contributed to the majority (28/59, 48%) of deaths within the first year of trial inclusion, whereas it is among the three leading causes thereafter (15/74, 20%). A direct effect of induction treatment was proposed to be causative of severe infections within the first year.[2] A recent study analysing the Chapel Hill cohort highlighted that infections were responsible for a high proportion of deaths within the first year (4/31, 13%), while active disease (29%) was the leading cause in a large cohort comprising 421 patients with a follow-up of at least 1 year.[13] Differences in the treatment modalities may have accounted for the differences leading to fatal infections in diverse cohorts. The methylprednisolone versus plasma exchange (MEPEX) trial (one of the early European Vasculitis Society (EUVAS) trials) randomised patients

Table 2 Univariate and multivariate analysis of risk factors for severe or life-threatening infection following rituximab treatment during 24 months of follow-up

	Univariate analysis			Multivariate analysis		
	HR	95 % CI	P values	HR	95 % CI	P values
Demographics						
Age (years)	1.02	1.00 to 1.04	**0.031**	1.03	1.01 to 1.05	0.012
Gender (male)	0.88	0.50 to 1.55	0.647			
Type of vasculitis						
GPA	Reference	–	–			
MPA	1.59	0.78 to 3.23	0.203			
EGPA	0.95	0.42 to 2.15	0.899			
Symptoms/manifestations						
B-symptoms (night sweat, fever, unintentional weight loss)	0.67	0.28 to 1.58	0.355			
Neuropathy	0.89	0.46 to 1.70	0.72			
Sinusitis	0.48	0.27 to 0.84	**0.01**			
Deafness/mastoiditis/otitis media	0.69	0.35 to 1.35	0.275			
Arthralgia	0.63	0.35 to 1.14	0.127			
Organ involvement						
CNS	1.12	0.35 to 3.63	0.85			
Subglottic/tracheal stenosis	1.14	0.51 to 2.54	0.746			
Skin	0.74	0.33 to 1.64	0.45			
Kidney	1.27	0.72 to 2.21	0.411			
Eye	0.52	0.23 to 1.17	0.113			
Others	0.82	0.26 to 2.65	0.745			
ENT	0.46	0.26 to 0.82	**0.008**			
Lung	1.57	0.87 to 2.82	0.136			
Imaging findings						
Pulmonary cavities	1.11	0.56 to 2.21	0.765			
Endobronchial	2.44	1.38 to 4.32	**0.002**	2.21	1.14 to 4.26	0.018
Severe bronchiectasis	4.79	1.47 to 15.59	**0.009**			
Disease activity measures						
BVAS	1.01	0.95 to 1.07	0.811			
DEI	0.98	0.84 to 1.15	0.840			
Laboratory values						
Creatinine	1	1.00 to 1.00	0.141			
eGFR (MDRD equation) mL/min/1.73 m^2	0.99	0.98 to 1.00	**0.011**			
CRP	1.01	1.00 to 1.01	0.061			
ESR	1.01	1.00 to 1.02	**0.014**			
Neutrophils	1.11	1.03 to 1.20	**0.005**			
WBC	1.06	1.01 to 1.10	**0.013**			
Lymphocytes	0.73	0.48 to 1.11	0.142			
CD19	1.17	0.11 to 12.51	0.896			
CD3	0.75	0.45 to 1.25	0.27			
CD4	0.74	0.34 to 1.61	0.44			
CD8	0.58	0.16 to 2.09	0.407			
CD56	2.75	0.34 to 22.10	0.341			
IgG	1.02	0.93 to 1.12	0.663			
IgG decline ≥30 %	1.88	1.04 to 3.39	**0.036**			
Hypogammaglobulinaemia	1.22	0.54 to 2.74	0.633			
IgM	0.72	0.38 to 1.35	0.304			
IgA	1.11	0.80 to 1.54	0.535			
ANCA positive	1.11	0.58 to 2.14	0.744			
Comorbidities						
COPD	16.07	4.41 to 58.49	**<0.001**	6.3	1.08 to 36.75	0.041
Diabetes	2.35	1.14 to 4.85	**0.021**			
Hypertension	0.79	0.44 to 1.44	0.445			
Myocardial infarction/reduced LVEF	2.21	1.07 to 4.56	**0.032**			
Indication						

Continued

Table 2 Continued

	Univariate analysis			Multivariate analysis		
	HR	95% CI	P values	HR	95% CI	P values
Minor relapse	0.6	0.32 to 1.11	0.102			
Major relapse	1.63	0.92 to 2.90	0.097			
Maintenance	1.15	0.56 to 2.37	0.708			
Refractory disease	0.8	0.42 to 1.51	0.491			
Steroid sparing	1.37	0.70 to 2.68	0.36			
1st line	1.95	0.77 to 4.91	0.159			
Premedication (last 12 months)						
CYC (g)	0.97	0.90 to 1.04	0.389			
MMF (g)	1	1.00 to 1.00	0.273			
AZA (g)	0.97	0.95 to 1.00	0.066			
MTX (mg)	1	1.00 to 1.00	0.979			
IVIG (ever)	2.4	0.94 to 6.12	0.067			
Anti-TNF (ever)	1.41	0.34 to 5.84	0.636			
PLEX (ever)	0.75	0.23 to 2.42	0.629			
ALM (ever)	2.49	1.05 to 5.91	**0.039**	3.97	1.50 to 10.54	0.006
Medication used concurrently with RTX						
Steroids (mg)	1.02	1.01 to 1.04	**0.006**			
Trimethoprim–sulfamethoxazole	0.45	0.23 to 0.88	**0.02**	0.3	0.13 to 0.69	0.005
Other antibiotic prophylaxis	1.63	0.76 to 3.47	0.209			

Demographics of the respective patients, the form of ANCA-associated vasculitis, symptoms, laboratory values, comorbidities, indication for rituximab use, the premedication and the concomitant therapy are given. P values indicating significant changes are highlighted in bold font.
ALM, alemtuzumab; ANCA, antineutrophil cytoplasm antibody; AZA, azathioprine; BVAS, Birmingham Vasculitis Activity Score; CD, cluster of differentiation; CNS, central nervous system; COPD, chronic obstructive pulmonary disease; CRP, C reactive protein; CYC, cyclophosphamide; DEI, Disease Extent Index; eGFR, estimated glomerular filtration rate; EGPA, eosinophilic granulomatosis with polyangiitis; ENT, ear, nose and throat; ESR, erythrocyte sedimentation rate; GPA, granulomatosis with polyangiitis; IVIG, intravenous immunoglobulins; LVEF, left ventricular ejection fraction; MMF, mycophenolate mofetil; MPA, microscopic polyangiitis; MTX, methotrexate; PLEX, plasma exchange; RTX, rituximab; TNF, tumour necrosis factor; WBC, white blood count.

either to plasma exchange or high-dose methylprednisolone alongside standard induction therapy reported 19 deaths (out of 137 patients) related to infections within the first year.[14]

Little is known about infections in patients with AAV treated with rituximab. In the first 6 months, the rate of severe infections (defined as grade ≥3 CTCAE V.3.0 event) was 7% in the group of patients receiving either rituximab or standard of care in the RAVE trial.[4] Over 18 months, 12% in the rituximab and 11% of participants in the standard of care group had at least one episode of grade ≥3 infections.[6] In the RITUXVAS trial, a higher occurrence of severe infectious complications was observed in both treatment arms. While the rate of severe infections was 18% in both arms, the number of patients presenting with non-severe infections was higher (18% vs 9%) in the rituximab group.[5] In general, patients with vasculitis may carry an increased risk to develop severe infections following rituximab administration. In patients with rheumatoid arthritis (RA), long-term follow-up of a global clinical trial programme revealed a serious infection event rate of 3.76 per 100 person-years. In contrast to our findings, opportunistic infections remained rare during follow-up with an event rate of 0.05 events per 100 patient-years in the RA cohort.[15]

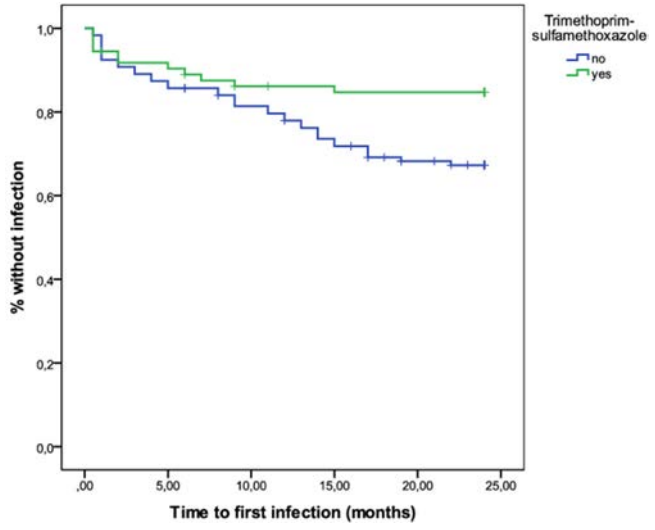

Figure 1 Kaplan-Meier curve of patients presenting with severe infections and either receiving trimethoprim–sulfamethoxazole or prophylaxis or not.

The current European League Against Rheumatism/European Renal Association - European Dialysis and Transplant Association (EULAR/ERA-EDTA) recommendations for the management of AAV encourage *P. jirovecii* prophylaxis in patients receiving cyclophosphamide.[16] However, no concrete recommendation concerning rituximab is given. In the updated EMA label, prophylaxis is recommended during and following rituximab, as appropriate.[17] In this study, the frequency of *P. jirovecii* infection was low (n=1). This frequency is in line with a study reporting one case of *P. jirovecii* in patients receiving mainly cyclophosphamide as induction treatment.[13] Currently, it is uncertain if patients with AAV receiving rituximab benefit from *P. jirovecii* prophylaxis since the reported frequency of severe adverse events attributable to trimethoprim–sulfamethoxazole is high in patients with systemic autoimmune diseases, with some fatalities.[12 18] A randomised controlled trial investigating the role of trimethoprim–sulfamethoxazole in therapeutic dosage (960 mg twice a day for 2 years) found a reduction in respiratory

tract infections and a trend towards fewer non-respiratory tract infections (p=0.05) compared with placebo.[19] This is in line with our study confirming a protective effect of prophylactic trimethoprim–sulfamethoxazole use on the risk to develop severe infections. Thus, it may be appropriate to conclude that trimethoprim–sulfamethoxazole may reduce *P. jirovecii* pneumonia and also reduces overall infective risk and prophylaxis should be initiated in patients with AAV receiving rituximab. In our cohort, patients tolerating trimethoprim–sulfamethoxazole remained on prophylaxis during the 2-year period (mean 14.67 months). Five patients stopped trimethoprim–sulfamethoxazole due to adverse events.

The reported occurrence of severe infections in observational studies of AAV varies (frequency 20%–60%)[3] influenced by follow-up times, prophylactic measures and the impact of different criteria for infections. In our study, 26.06% patients presented with at least one severe infection. The observed frequency is higher compared with both Rituximab versus Cyclophosphamide for ANCA-Associated Vasculitis (RAVE) and Rituximab versus Cyclophosphamide in ANCA-Associated Renal Vasculitis (RITUXVAS) trials. This may be explained by the scheduled rigorous study visits, allowing for early detection of infection and prescription of antimicrobials, or the selection of a lower risk cohort for the clinical trials. However, the frequency of observed severe infections is similar to other observational studies reported to date.[7 8 20] Older age was an independent risk factor for infections in the pre-rituximab era.[3] We observed an association between age and severe infections in our cohort. Patients with lung involvement and concomitant COPD may be particularly vulnerable to severe infections. Endobronchial involvement and COPD were risk factors for infections and endobronchial involvement alongside severe bronchiectasis predictors of severe pulmonary infections. In patients with AAV on immunosuppressive treatment, most severe infections are located in the respiratory tract.[13 21 22] In rituximab-treated patients, 20 out of 30 infectious complications were restricted to the upper and lower respiratory tract during a follow-up period of 230.4 patient-years.[8] Respiratory tract infections were the leading cause of severe infections in our cohort as well.

Compared with a matched background population, patients with AAV are at an increased risk of severe infections, including non-specific (HR 4.55), Gram-negative (HR 3.49) and *S. aureus* septicaemia (HR 3.40), pneumonia (HR 3.27), acute upper respiratory tract infections (HR 8.88), *C. difficile* infection (HR 5.35) and skin infections (HR 5.35).[23] Interestingly, no difference related to infectious complications was observed when an early cohort was compared with a recent cohort.[23] Another study corroborated an impact of *S. aureus* in patients with AAV, being the most prevalent causative organism (34% of 249 positive cultures). Among 85 positive cultures, 18 (21%) of *S. aureus* isolates were grown despite trimethoprim–sulfamethoxazole prophylaxis. Moreover, 14% of infections caused by *S. aureus* were severe.[13] In contrast, our study found a broad spectrum of opportunistic pathogens and *P. aeruginosa* as well as *S. aureus* (four severe infections, each) were the leading causative organism, followed by *E. coli* (three severe infections). The spectrum of isolates is in line with a recent study reporting the efficacy and safety profile of rituximab in induction and maintenance of remission. Out of 12 severe infections, four led to fatality in four subjects with either coma (meningitis) or respiratory failure (pneumonitis with detection of *P. aeruginosa* or *P. jirovecii*).[7] Both *S. aureus* and Gram-negative bacteria may have a direct impact on disease onset or relapse,[24] which is a potential explanation for the high number of infections caused by these pathogens.

Most infections occur within the first months of treatment. McGregor *et al* showed the highest risk of infections during the first 3 months of follow-up and in general severe infections within the first 12 months were associated with death (19% vs 4%).[13] A recent registry analysis highlighted that a high proportion of severe infections occurred during the first 6 months of follow-up (38.4%).[23] In contrast, severe infections occurred during the whole observational period in our cohort of rituximab-treated patients. In retrospective studies, hypogammaglobulinaemia was a frequently observed complication of rituximab with the need of IgG replacement due to recurrent infections in 4.2% of the patients.[25] Univariate analysis revealed an association between IgG decline of at least 30% from baseline in patients with severe infections. This may indicate that this subgroup of patients with a drop in IgG levels may be specifically prone towards infections.

In conclusion, we found severe infections occurring in approximately one quarter of patients in a 2-year observation period after rituximab therapy for AAV. There was a reduction of severe infections when trimethoprim–sulfamethoxazole prophylaxis was used. Respiratory tract infections were the leading cause of severe infections. We found an association of endobronchial involvement, bronchiectasis and rituximab use for major relapses with severe respiratory tract infections. While these results require confirmation, they support routine use of trimethoprim–sulfamethoxazole in rituximab-treated patients.

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EXTENDED REPORT

Characterisation of the nasal microbiota in granulomatosis with polyangiitis

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ABSTRACT

Objectives Prior studies have suggested a potential link between nasal microbes and granulomatosis with polyangiitis (GPA; Wegener's), but these studies relied on culture-dependent methods. This study comprehensively examined the entire community of nasal microbiota (bacteria and fungi) in participants with GPA compared with healthy controls using deep sequencing methods.

Methods 16S rRNA and internal transcribed spacer gene sequencing were performed on nasal microbial DNA isolated from nasal swabs of 60 participants with GPA and 41 healthy controls. Alpha and beta diversity were assessed as well as the relative abundance of the most abundant bacterial and fungal taxa. The effects of covariates including disease activity and immunosuppressive therapies on microbial composition were evaluated.

Results Compared with controls, participants with GPA had a significantly different microbial composition (weighted UniFrac p=0.04) and lower relative abundance of *Propionibacterium acnes* and *Staphylococcus epidermidis* (for both, false discovery rate-corrected p=0.02). Disease activity in GPA was associated with a lower abundance of fungal order *Malasseziales* compared with participants with GPA in remission (p=0.04) and controls (p=0.01). Use of non-glucocorticoid immunosuppressive therapy was associated with 'healthy' nasal microbiota while participants with GPA who were off immunosuppressive therapy had more dysbiosis (weighted UniFrac p=0.01). No difference in the relative abundance of *Staphylococcus aureus* was observed between GPA and controls.

Conclusions GPA is associated with an altered nasal microbial composition, at both the bacterial and fungal levels. Use of immunosuppressive therapies and disease remission are associated with healthy microbial communities.

INTRODUCTION

Granulomatosis with polyangiitis (GPA; Wegener's) is a systemic vasculitis characterised by granulomatous inflammation and up to 90% of participants will develop sinonasal inflammation during the course of the disease.[1] Despite the many therapeutic advances in the management of GPA, relapses remain a significant issue and, in particular, disease activity in the sinuses and nose often persists when a patient is otherwise in clinical remission.[2] It has been speculated that microbes may be involved in the pathogenesis of GPA. A study using culture data found that chronic nasal carriage of *Staphylococcus aureus* is associated with a higher risk of relapse[3] and two randomised clinical trials showed trimethoprim–sulfamethoxazole prevents relapse in GPA.[4 5]

Advances in high-throughput genomic sequencing and the development of new tools for analysing metagenomic data allow for a better understanding of the dynamic community of microbes (bacteria, fungi, viruses) that inhabit the human body (human microbiome) in a relatively inexpensive and accessible manner. The human microbiota has a large potential to impact many human physiological functions including immune homeostasis.[6] Dysbiosis, the imbalance or disruption of microbial communities, has been proposed to contribute to the pathophysiology of several autoimmune diseases including inflammatory bowel disease, rheumatoid arthritis, multiple sclerosis and type 1 diabetes mellitus.[7–11] Furthermore, microbial composition varies across different body sites and, while much attention has been given to the gut microbiome, microbial communities in other body habitats, including the nose, have also been implicated in human health and disease.[12–15]

This is the first study to comprehensively examine the entire community of nasal bacteria and fungi in participants with GPA compared with healthy controls using culture-independent sequencing methods. We chose to focus on the nose due to its distinctive involvement in the natural history of GPA and its role as an active component of the immune system.[16]

METHODS

Study participants

Participants were recruited through the Penn Vasculitis Centre at the University of Pennsylvania. Participants with GPA were eligible if they met the modified American College of Rheumatology classification criteria for GPA.[17–19] Healthy controls were participants without a systemic vasculitis or other inflammatory disorder. Both participants with GPA and controls were excluded if they had another systemic inflammatory disorder, history of intranasal cocaine use within the prior 3 years, known history of HIV or primary immunodeficiency, lymphoma or other malignancy that mimics AAV. Antibiotic use was not an exclusionary criteria but was incorporated into the analysis.

Nasal mucosa was sampled by swabbing the middle meatus with a sterile flocked specimen collection swab (Copan Diagnostics), which was transferred to a −80°C freezer. To control for environmental contamination, negative controls (swab

exposed to ambient air) were obtained with each participant sampling and processed in parallel.

Clinical data were collected at time of sampling and included demographics and medication use within prior 6 months (antibiotics, systemic glucocorticoids and non-glucocorticoid immunosuppressive therapy such as cyclophosphamide, rituximab, azathioprine, methotrexate, etc). For participants with GPA, a detailed disease history was obtained including antineutrophil cytoplasmic antibody (ANCA) type by ELISA (proteinase-3, myeloperoxidase or negative), disease status determined by the Birmingham Vasculitis Activity Score for Wegener's Granulomatosis[20] (BVAS/WG; BVAS/WG>0 indicates active disease and BVAS/WG=0 indicates disease remission) and sinonasal damage according to the ear, nose and throat items on the Vasculitis Damage Index.[21]

Microbiota profiling using DNA sequencing

DNA was extracted using QIAamp ultraclean production pathogen mini kit. For bacterial profiling, PCR amplification targeting the V1–V2 region of the 16S rRNA gene was performed. For analyses of fungal communities (mycobiome), the internal transcribed spacer (ITS1) region was targeted, using a fungal-specific primer set.[22] Amplicons were purified and sequenced by the sequencing core of the Penn-CHOP Microbiome programme on an Illumina MiSeq instrument, yielding 250 bp paired-end sequence reads. Environmental and reagent control samples consisting of air-exposed swabs, DNA-free water and empty wells were processed alongside participant samples.[23]

Sequencing data analysis was performed by QIIME V.1.9.1.[24] Paired-end reads were quality filtered and joined, clustered into operational taxonomic units (OTUs) with 97% sequence similarity using UCLUST.[25] Taxonomic assignments were generated by alignment to the Greengenes r13_8 reference databases.[26] Taking into consideration the various lengths of the ITS1 regions, we used high-quality forward reads (R1) for analysis, clustered into OTUs with 95% sequence similarity, and taxonomy assignments generated by comparison with the nt reference database using the consensus method implemented in BROCC software.[27]

16S reads assigned as archaea, mitochondria or chloroplast were removed. Potential contaminant OTUs showing a strong negative correlation with the amplicon concentration (false discovery rate (FDR)<0.05) were also removed[28] (online supplementary table 1). Samples with more than 10 000 read counts for 16S analysis and 4000 read counts for ITS analysis were retained. To reduce the influence of low-abundant fungi, which are potential contaminants, a PicoGreen-corrected OTU abundance was determined by multiplying the relative abundance of fungal taxa by the post-PCR ITS DNA concentration as previously described and validated.[29]

Taxonomic heatmap was generated at the genus level to depict all taxa with a relative abundance of over 1% in at least one sample. The most abundant OTUs within taxa of interest were also identified for secondary analyses.

Analytical approach

The main comparison of interest was between GPA and controls. Outcome measures included alpha and beta diversity and relative abundance of individual taxa. We measured alpha diversity, or within-sample diversity, using the Shannon diversity index, which accounts for evenness and abundance of OTUs within a sample. Beta diversity, which compares dissimilarity of microbial composition between samples, was measured by UniFrac distance that estimates the fraction of a sample's phylogenetic tree that differs from another sample, with (weighted) or without (unweighted) accounting for the relative abundance of OTUs.[30 31] UniFrac distances were visualised using principal coordinate analysis. To determine if specific bacterial or fungal taxa were more common or more rare, we evaluated relative abundance (which refers to the percent composition of a taxa relative to the total number of taxa) of the five most abundant genera and 10 most abundant OTUs (here on referred to as species). We focused on these relatively abundant bacteria because they are more likely to represent 'true' nasal commensals in these low microbial biomass samples. Furthermore, these taxa together represent 92% of all sequences obtained, thus reflecting the dominant microbiota present. In exploratory analyses, differential relative abundance was also investigated between subgroups of GPA according to ANCA type, sinonasal damage, disease activity and medications.

Detailed profiling of the nasal mycobiota or fungal communities included examination of total fungal abundance and was initially planned to evaluate the relative abundance of the 10 most abundant taxa; however, due to the predominance of the order *Malasseziales* and the small number of other fungal OTUs detected and their sparse distribution among samples, further investigation of relative abundance focused only on *Malasseziales*.

Statistical analyses

Categorical variables were compared between groups using χ^2 test; continuous variables were compared using the Wilcoxon rank-sum test. One-way analysis of variance was used to test for differences in alpha diversity (Shannon index) between groups. Beta diversity (UniFrac) was analysed using permutational analysis of variance (PERMANOVA).[32] Relative abundance values were log-transformed and compared using the Wilcoxon rank-sum test. Potential fungal–bacterial interactions were assessed by examining associations between abundance of total fungi as well as *Malasseziales* and the following: (1) UniFrac distance using PERMANOVA and (2) abundance of five most abundant bacterial genera and 10 most abundant species using Wilcoxon rank-sum test adjusting for study group (GPA vs control).

Correction for multiple comparisons was performed by the Benjamini-Hochberg FDR procedure for all analyses except for exploratory analyses performed within GPA subgroups of interest.[33] All statistical analyses were conducted with R V.3.4.1.

RESULTS

Participant characteristics

There were 101 participants enrolled in this study: 60 participants with GPA and 41 healthy controls. Characteristics at time of sampling are shown in table 1. Demographics were similar between the two groups. Participants with GPA were more likely to have received antibiotics within the prior 6 months (48% vs 29%). Among participants with GPA, 25% had active disease and a majority of participants had received immunosuppressive agents within the prior 6 months.

Bacterial composition and diversity in nasal cavities of participants with GPA and healthy controls

A total of 9 235 460 raw sequence reads were generated from all participant samples and, after quality filtering and removal of contaminants, a total of 9 213 337 high-quality reads were used for analysis (mean reads per sample 91 221±56 533). *Corynebacterium*, *Staphylococcus* and *Propionibacterium* featured as prominent bacterial taxa in the nasal cavities of the cohort, consistent

Table 1 Participant characteristics at time of nasal sampling

	GPA n=60	Control n=41	P values
Age	55 (37–66)	61 (47–68)	0.06
Female	35 (58%)	24 (59%)	0.98
White race	57 (95%)	36 (88%)	0.18
Ever smoker	19 (32%)	19 (48%)	0.11
Current allergies	14 (23%)	15 (37%)	0.15
GPA manifestations			
ANCA type (ever)		NA	–
Proteinase-3	36 (60%)		
Myeloperoxidase	15 (25%)		
Negative	9 (15%)		
Disease duration, years	4 (2–7)	NA	–
Newly diagnosed (within 30 days)	5 (8%)	NA	–
Current disease status		NA	–
Remission	45 (75%)		
Severe flare	2 (3%)		
Limited flare	10 (17%)		
Persistent disease	3 (5%)		
Currently active disease of sinonasal area	10 (10%)	NA	–
Ever had flare involving sinonasal area	26 (43%)	NA	–
Vasculitis Damage Index			
Any sinonasal item	28 (47%)	NA	–
Nasal blockade/chronic crusting	21 (35%)		
Nasal bridge collapse	11 (18%)		
Chronic sinusitis	10 (17%)		
Medication use			
Systemic glucocorticoid			
Current	25 (42%)	0	<0.01
Current or in past 6 months	31 (52%)	3 (7%)	<0.01
Other immunosuppressive drug			
Current	32 (53%)	0	<0.01
Current or in past 6 months	42 (70%)	0	<0.01
Current intranasal glucocorticoid	12 (20%)	5 (12%)	0.30
Antibiotic			
Current	15 (25%)	6 (15%)	0.21
Current or in past 6 months	29 (48%)	12 (29%)	0.06
Current trimethoprim–sulfamethoxazole			
Full dose*	0	0	–
Low dose†	10 (17%)	0	<0.01

Values expressed as median (IQR) or percentage.
*Full dose is trimethoprim–sulfamethoxazole 160–800 mg twice daily.
†Low dose refers to doses used for prophylaxis of *Pneumocystis jirovecii* pneumonia (trimethoprim–sulfamethoxazole 160–800 mg three times a week or trimethoprim–sulfamethoxazole 80–400 mg daily).
ANCA, antineutrophil cytoplasmic antibody; GPA, granulomatosis with polyangiitis; NA, not applicable.

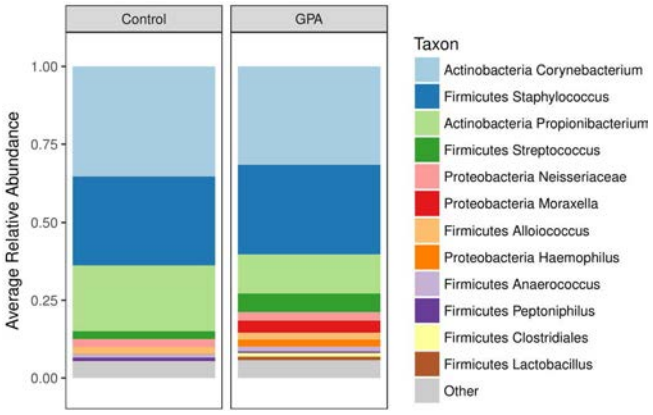

Figure 1 Twelve most abundant bacteria that are present in the samples are depicted in a stacked barplot. *Corynebacterium*, *Staphylococcus* and *Propionibacterium* featured as most abundant genera, similar to previously published studies of nasal microbial composition in healthy individuals. GPA, granulomatosis with polyangiitis.

with previous descriptions of the nasal microbial composition in healthy individuals[34] (figure 1 and online supplementary figure 1 for heatmap). Within these genera, the most abundant species identified were *Corynebacterium tuberculostearicum*, *Propionibacterium acnes*, *Staphylococcus aureus* and *S. epidermidis*.

To compare overall composition of bacterial communities, alpha and beta diversity were measured. The Shannon Diversity Index (alpha diversity) was not significantly different between participants with GPA and healthy controls (p=0.51). The analysis of beta diversity calculated on UniFrac distances revealed a borderline difference by unweighted UniFrac (ie, based on the identify of community members) (p=0.05), and a significant difference in weighted UniFrac (ie, accounting for both community membership and abundances), between participants with GPA and healthy controls, suggesting that microbial composition between the two groups is different (p=0.04) (figure 2A). We then compared the relative abundances of individual bacterial genera between groups and found that participants with GPA had a lower relative abundance of *Propionibacterium* (FDR-corrected p=0.03) compared with healthy controls. When we examined the most abundant species, both *P. acnes* and *S. epidermidis* had a significantly lower abundance in GPA compared with controls (for both species, FDR-corrected p=0.02). No difference was seen in the relative abundance of *S. aureus* (FDR-corrected p=0.49).

Use of non-glucocorticoid immunosuppressive agents is associated with 'healthy' nasal bacteria in GPA

When evaluating the association of covariates such as medications and disease characteristics with microbial communities in GPA and controls, we found that the nasal microbial communities in participants with GPA receiving non-glucocorticoid immunosuppressive therapies either currently or within the prior 6 months were similar to healthy controls, whereas participants with GPA not receiving non-glucocorticoid immunosuppressive therapies were significantly different from controls. Specifically, there was a significant difference in weighted UniFrac distance between participants with GPA off immunosuppressive therapies compared with controls (p=0.01) while no difference was observed between GPA on immunosuppressive therapies versus controls (p=0.16) (figure 2B). No significant difference in unweighted UniFrac distance was found according to use of immunosuppressive therapies.

When we evaluated the relative abundance of *Propionibacterium* and then stratified participants with GPA based on their use of non-glucocorticoid immunosuppressive therapy, we found that data from the participants with GPA who were off immunosuppressive therapy were driving the differences observed between GPA and controls; specifically, participants off immunosuppressive therapy had a significantly lower abundance of *Propionibacterium* compared with controls (p<0.01) while participants with GPA on immunosuppression were similar to controls (p=0.68) (figure 3). Analysis of the most abundant bacterial species revealed similar differences in relative abundance between groups for *P. acnes*, *P. granulosum* and *S. epidermidis* (figure 4). Relative abundances for each sample and OTU are available in online supplementary table 2.

When examining the association between disease activity in GPA and microbial communities, univariate and multivariate

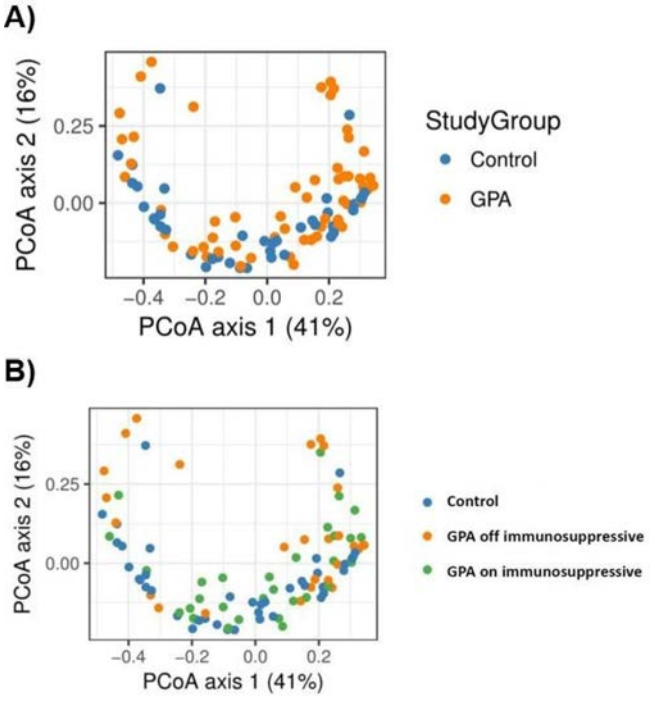

Figure 2 Difference in overall microbial composition between (A) controls and granulomatosis with polyangiitis (GPA), and (B) controls and GPA stratified by use of non-glucocorticoid immunosuppressive medications. The principal coordinates analysis (PCoA) plots depict differences in beta diversity based on weighted UniFrac distance, which accounts for phylogenetic differences as well as abundance. (A) There is a significant difference in microbial composition between controls (blue) and participants with GPA (orange) (p=0.04). (B) Differences in microbial composition are primarily driven by participants with GPA off non-glucocorticoid immunosuppressive therapy (orange) who had a significantly different weighted UniFrac from controls in blue (P=0.01) while those on immunosuppressive therapies (green) are similar to controls (p=0.16).

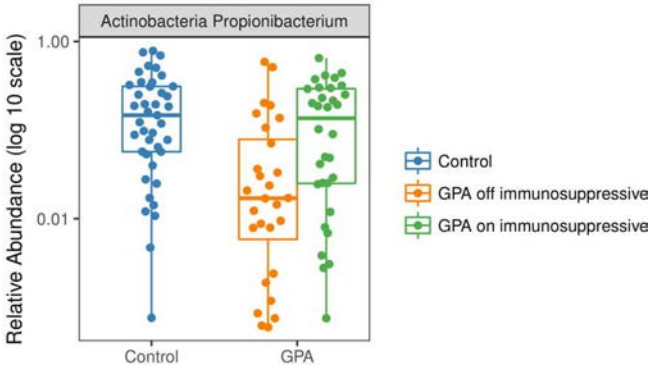

Figure 3 Differential abundance of *Propionibacterium* between controls and participants with granulomatosis with polyangiitis (GPA) stratified by use of non-glucocorticoid immunosuppressive therapy. Participants off immunosuppressive therapy (orange) had a significantly lower abundance of *Propionibacterium* compared with controls (blue) (false discovery rate (FDR)-corrected p<0.01). No difference was observed between participants with GPA on immunosuppression (green) and controls (FDR-corrected p=0.68) or participants with GPA off immunosuppression (FDR-corrected p=0.06).

analyses found no relationship between disease activity and UniFrac distances, either weighted or unweighted, or relative abundance of bacterial taxa. Similarly, use of antibiotics or prednisone was not associated with UniFrac distance or relative abundance. Additional exploratory analyses within clinically distinct subgroups of GPA are shown in online supplementary text 1.

Participants with active GPA harbour an altered nasal mycobiota

We found a non-significant trend towards lower total fungi abundance in participants with GPA compared with controls (p=0.06). However, we found that participants receiving prednisone had a lower total abundance of nasal fungi (p=0.02) compared with healthy controls, and those receiving other immunosuppressive therapies had a significantly higher abundance of fungi (p=0.03), independent of disease activity, antibiotics, ANCA type and sinonasal damage. When we investigated the nasal fungal community composition, unclassified *Malasseziales* was the most abundant fungal taxa present, followed by the genera *Penicillium* and *Aspergillus*. Evaluation of *Malasseziales* demonstrated a significantly lower abundance in GPA compared with controls (p=0.04). In particular, participants with GPA with active disease appeared to be driving these differences, as they had the lowest abundance compared with participants in remission (p=0.04) and healthy controls (p=0.01), while participants with GPA in remission were similar to controls (p=0.19) (figure 5). Significant correlations were also found between the five most abundant bacterial species and all fungi (online supplementary figures 2 and 3) as well as just *Malasseziales* (online supplementary text 2).

DISCUSSION

This study used deep sequencing methods to comprehensively define the entire community of nasal bacteria and fungi in GPA, and found significant differences in nasal microbial composition between participants with GPA and healthy controls. Furthermore, being off immunosuppressive therapy and disease activity were associated with bacterial and fungal dysbiosis, respectively.

For many decades, there has been speculation that infections may have a major influence on disease activity in GPA. With a growing understanding of the human microbiome, a shift occurred within the field of microbiology from focusing only on individual disease-causing organisms to studying the effects of endogenous microbes or commensals as communities. In this study, we examined the relationship between microbes and GPA from a different perspective than prior studies, by characterising the entire community of resident bacteria and fungi in the nasal cavity, an active site of immunity and a known reservoir for pathobionts such as *S. aureus*. We found that participants with GPA have dysbiosis in the nose and, in particular, a lower abundance of *P. acnes* and *S. epidermidis*, both of which have been suggested to be negative competitors of *S. aureus*.[1 4 35] Most interestingly, differences in microbial composition were primarily driven by participants with GPA who were off non-glucocorticoid immunosuppressive therapy independent of disease activity, suggesting that immunosuppressive therapy is associated with a healthy nasal microbiome in GPA (and conversely, absence of immunosuppressive therapy is associated with nasal dysbiosis). This was an unexpected finding since we had anticipated immunosuppressive therapy would cause more dysbiosis, not less, based on prior studies.[36] Similar observations have been made in rheumatoid arthritis.[37]

We postulate there are two potential explanations for this association between immunosuppressive use and improvements in nasal dysbiosis: (1) immunosuppressive therapy has

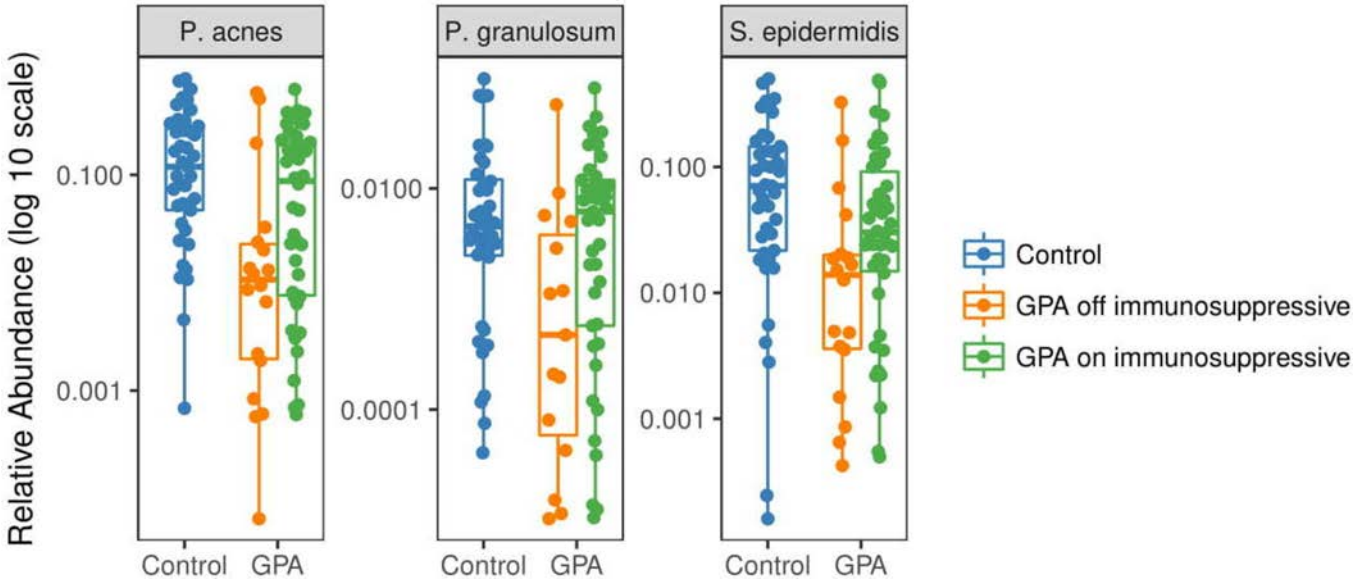

Figure 4 Differential abundance of *Propionibacterium acnes*, *P. granulosum* and *Staphylococcus epidermidis* between control and participants with granulomatosis with polyangiitis (GPA) stratified by use of non-glucocorticoid immunosuppressive therapy. Participants with GPA who are off immunosuppressives had a significantly lower abundance of *P. acnes*, *P. granulosum* and *S. epidermidis* (for all, FDR-corrected p<0.01) compared with controls and compared with participants on immunosuppression (FDR-corrected p=0.03, p=0.01 and p=0.03, respectively), while participants with GPA on immunosuppression were similar to controls (p>0.05).

direct benefits on the nasal microbiota, or (2) subclinical disease activity, not identified using standard methods of assessment, was present in participants who were off immunosuppressive therapy, which in turn is associated with an altered nasal microbiota. These results suggest either that the manipulation of the nasal microbiome may be a novel therapeutic target and/or that the nasal microbiome may be a more sensitive, non-invasive biomarker of disease activity.

Unlike prior studies, no difference was found in the abundance of *S. aureus* between GPA and controls, although differences were seen among GPA subgroups (however, these differences were no longer significant after correction for multiple comparisons).[38 39] Several differences in methodology may explain this discrepancy. The current study used sequencing data to identify bacteria, which may be more sensitive than culture data for identifying bacteria present; alternatively, sequencing data do not discriminate between viable versus non-viable bacteria. The location within the nasal cavity where swabbing was performed also differed: we sampled the middle meatus to study a mucosal surface whereas prior studies swabbed the anterior nares, which is lined with skin-like squamous epithelium. Despite their proximity, spatial variation in microbiota composition (in particular *S. aureus* colonisation) has been found within the nasal cavity, particularly between the middle meatus and anterior nares.[15]

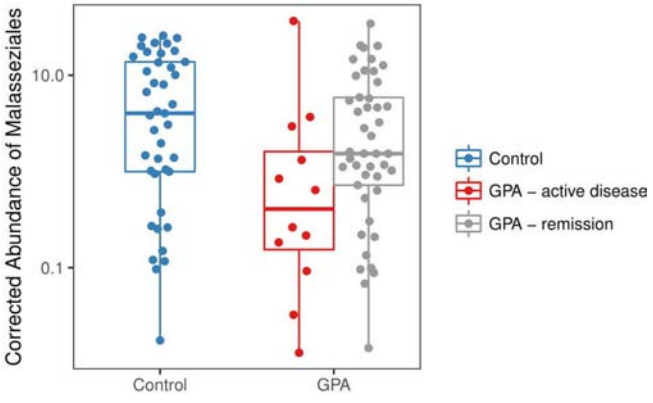

Figure 5 Differential abundance of *Malasseziales* between controls and participants with granulomatosis with polyangiitis (GPA) stratified by disease activity. PicoGreen-corrected abundance of *Malasseziales* was significantly lower in participants with GPA with active disease (red) compared with controls (blue) (p=0.01) and participants with GPA in remission (grey) (p=0.04). No difference was seen between participants with GPA in remission and controls (p=0.19).

To our knowledge, only two prior studies have examined the nasal mycobiome using culture-independent methods, and this is the first to examine the mycobiome in GPA.[40 41] *Malasseziales*, a fungal taxa that has previously been demonstrated to be abundant in the nose, mouth and skin, was the most abundant fungal taxa in the nasal cavity in this cohort.[40 42 43] Furthermore, *Malasseziales* was significantly lower in abundance in GPA compared with controls, and this difference was most pronounced in participants with active disease. The abundance of *Malasseziales* correlated with the abundance of several bacteria, suggesting interactions between bacteria and fungi occur in the nose. Prior studies also have suggested that interactions between fungi and bacteria may impact microbial composition and specifically the possibility has been raised that fungi may stabilise microbial community organisation.[44] Future studies further exploring the role of fungi in GPA are warranted.

Limitations to this study include the cross-sectional design, which restricts investigations of temporal dynamics and is unable to assess for interindividual heterogeneity. Given the variability in the microbiome between individuals, a future longitudinal study would have the advantage of controlling for potential confounders through repeated sampling within individuals. Furthermore, causal relationships cannot be determined and confounders such as inflammation itself can perturb the nasal microbiota. Validation of these results in other cohorts along with mechanistic studies are needed to interpret these findings. Although the sample size was adequate to evaluate differences between GPA and controls, the ability to explore differences

between GPA subgroups may have been underpowered. Lastly, measurement of disease activity relied on a validated disease activity measure, the BVAS/WG, which may not be sensitive enough to identify subclinical disease activity.

In conclusion, this study found an altered nasal microbial composition associated with GPA, both at the bacterial and fungal levels, and suggests that immunosuppressive therapies and inactive disease status are associated with healthy microbial communities. These findings justify further investigation of host–microbe interactions in GPA, a potentially exciting new dimension to our understanding of the disease, and may be the first step to elucidating critical components of the pathophysiology of disease and identification of novel therapeutics.

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Competing interests None declared.

Patient consent Obtained.

Ethics approval Institutional Review Board of the University of Pennsylvania.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement All sequence data related to this study are available from the United States National Center for Biotechnology Information (NCBI) Sequence Read Archive under accession number SRP149341.

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EXTENDED REPORT

Systematic approach demonstrates enrichment of multiple interactions between non-*HLA* risk variants and *HLA-DRB1* risk alleles in rheumatoid arthritis

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ABSTRACT

Objective In anti-citrullinated protein antibody positive rheumatoid arthritis (ACPA-positive RA), a particular subset of *HLA-DRB1* alleles, called shared epitope (SE) alleles, is a highly influential genetic risk factor. Here, we investigated whether non-*HLA* single nucleotide polymorphisms (SNP), conferring low disease risk on their own, interact with SE alleles more frequently than expected by chance and if such genetic interactions influence the *HLA-DRB1* SE effect concerning risk to ACPA-positive RA.

Methods We computed the attributable proportion (AP) due to additive interaction at genome-wide level for two independent ACPA-positive RA cohorts: the Swedish epidemiological investigation of rheumatoid arthritis (EIRA) and the North American rheumatoid arthritis consortium (NARAC). Then, we tested for differences in the AP p value distributions observed for two groups of SNPs, non-associated and associated with disease. We also evaluated whether the SNPs in interaction with *HLA-DRB1* were cis-eQTLs in the SE alleles context in peripheral blood mononuclear cells from patients with ACPA-positive RA (SE-eQTLs).

Results We found a strong enrichment of significant interactions (AP $p<0.05$) between the *HLA-DRB1* SE alleles and the group of SNPs associated with ACPA-positive RA in both cohorts (Kolmogorov-Smirnov test $D=0.35$ for EIRA and $D=0.25$ for NARAC, $p<2.2e-16$ for both). Interestingly, 564 out of 1492 SNPs in consistent interaction for both cohorts were significant SE-eQTLs. Finally, we observed that the effect size of *HLA-DRB1* SE alleles for disease decreases from 5.2 to 2.5 after removal of the risk alleles of the two top interacting SNPs (rs2476601 and rs10739581).

Conclusion Our data demonstrate that there are massive genetic interactions between the *HLA-DRB1* SE alleles and non-*HLA* genetic variants in ACPA-positive RA.

INTRODUCTION

In rheumatoid arthritis (RA (OMIM: 180300)), a particular subset of *HLA-DRB1* gene variants (major alleles at *01, *04 and *10 groups), commonly called shared epitope (SE) alleles, is the most important genetic contributor for the risk of developing anti-citrullinated protein antibody (ACPA)-positive RA.[1–3] It is noteworthy that the strength of the association between non-*HLA* genetic variants and ACPA-positive RA risk is, in general, very moderate in comparison to that of the *HLA-DRB1* SE alleles[4–7] (figure 1A). This prompted us to investigate whether the *HLA-DRB1* SE alleles could be a genetic hub[8] that captures multiple interactions. Indeed, previous studies have demonstrated interactions between the *HLA-DRB1* SE alleles and several single nucleotide polymorphisms (SNP), including variations in *PTPN22*, *HTR2A* and *MAP2K4* with regard to the risk of developing ACPA-positive RA,[9–12] where the combination of both risk factors shows significantly higher risk (measured as OR) than the sum of their separate effects. Departure from additivity is a way to demonstrate interaction between risk factors regarding the risk of disease. The additive scale, defined by attributable proportion (AP) due to interaction, has the advantage of a straightforward interpretation in the sufficient-component cause model framework.[9 13–16]

In our current study, we aimed to investigate whether there is an enrichment of genetic interactions between non-*HLA* SNPs, conferring low disease risk on their own, and the major *HLA-DRB1* related disease risk to develop ACPA-positive RA. We also explored to what extent the top interactions influence the association between *HLA-DRB1* and risk to ACPA-positive RA. First, we assessed departure from additivity regarding the interaction between the *HLA-DRB1* SE alleles and SNPs at the genome-wide level. The outcome of this analysis was used to investigate the potential enrichment of significant interactions among certain variants by comparing the distribution of the p value of interaction between two defined groups of SNPs: the pool of variants which exhibited a significant nominal association with ACPA-positive RA in comparison to SNPs that are not associated with disease risk. We thereafter performed a replication study with the same approach in an independent ACPA-positive RA cohort. Second, we performed an expression quantitative trait loci (eQTL) analysis for non-*HLA* SNPs in interaction with *HLA-DRB1*, stratified by the *HLA-DRB1* SE alleles in order to identify genes influenced by the interactions. Finally, we analysed the effect size from the *HLA-DRB1* SE alleles concerning the risk of developing ACPA-positive RA before and after step-by-step removal of the effect of the risk alleles of the strongest SNPs in interacting with *HLA-DRB1* SE. Our observations

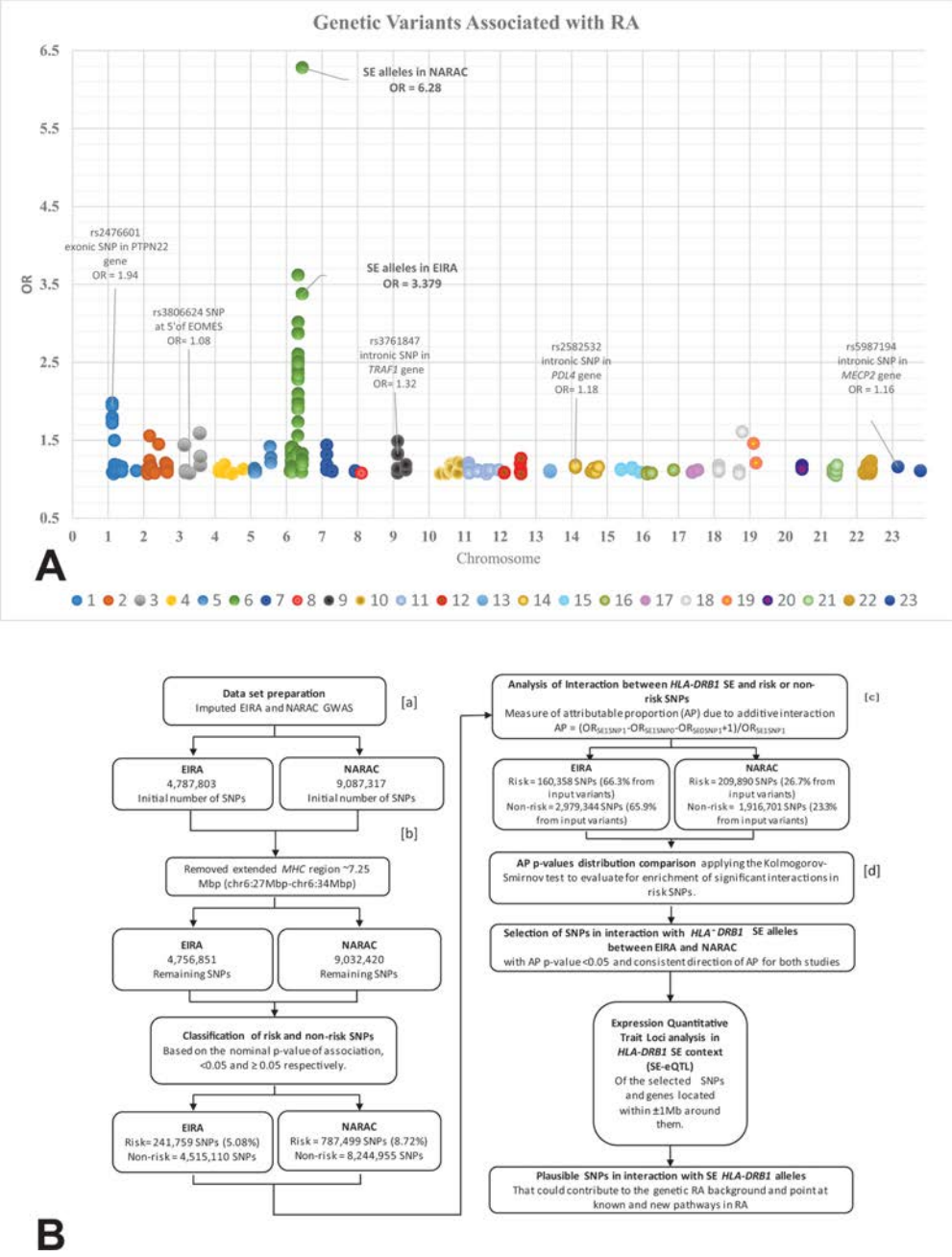

Figure 1 (A) Genetic variants associated with ACPA-positive RA. This plot represents the association signals (p<1.0e-05) from different GWAS in ACPA-positive RA, taken from the NHGRI-EBI GWAS catalogue (https://www.ebi.ac.uk/gwas/home).[46–48] X-axis: genomic positions, including chromosome X (marked as 23). Y-axis: the OR value observed for each SNP in different studies. Some examples are pointed. (B) Methodology workflow. (a) The workflow was also applied with non-imputed genotyping data (online supplementary table S2). (b) An alternative step excluding the PTPN22 locus was included at this point. (c) The AP value, its respective p value and CI (95% CI) were assessed using logistic regression implemented in GEISA (https://github.com/menzzana/geisa).[13 27 28] (d) The classification of risk and non-risk SNPs was permuted 10 000 times and each time the KS test was applied. The workflow was implemented until this step for each of the 1000 SE permuted variables, a lower number of permutations due to computational constrains. Both types of permutations showed that less than 5% of the KS test will exhibit a p value less than 2.2e-16, strongly indicating that differences in the AP p value distribution detected by the KS test from the original data are unlikely to be by chance. ACPA-positive RA, anti-citrullinated protein antibody positive rheumatoid arthritis; EBI, European Bioinformatics Institute; EIRA, epidemiological investigation of rheumatoid arthritis; GWAS, genome-wide association study; KS, Kolmogorov-Smirnov test; LD, linkage disequilibrium; MAF, minor allele frequency; MHC, major histocompatibility locus; NARAC, North American rheumatoid arthritis consortium; NHGRI, National Human Genome Research Institute; PCA, principal component analysis; SE, shared epitope; SE0SNP1, absence of the HLA-DRB1 SE alleles and presence of the risk allele from the SNP; SE1SNP0: presence of the HLA-DRB1 SE alleles and absence of the risk allele from the SNP; SE1SNP1, presence of the HLA-DRB1 SE alleles and the risk allele from the SNP; SNP, single nucleotide polymorphism. *PTPN22* is abbreviation for the gene.

indicated that the *HLA-DRB1* SE alleles act as a hub of cumulative additive interactions with multiple genetic variants in the development of ACPA-positive RA. We proposed that the analytic approach used here provides a novel way to study the impact of gene–gene interactions and the role of *HLA* in other autoimmune diseases.

Table 1 Description of studied populations

Study	Number of individuals	Female:male ratio	Frequency of *HLA-DRB1* SE alleles	rs4507692 MAF and nominal p value of association*	Number of SNPs in GWAS †	Number of SNPs in GWAS after imputation †
EIRA			0.45	MAF=0.45 P=0.57	282 527	4 756 851
Cases ‡	1151	2.4:1	0.59			
Controls	1079	2.6:1	0.30			
NARAC			0.43	MAF=0.43 P=0.67	398 551	9 032 420
Cases ‡	867	2.8:1	0.68			
Controls	1194	2.5:1	0.26			

*The rs4507692 SNP (chromosome 7) was used as a negative control instead of *HLA-DRB1* SE alleles, since the rs4507692 SNP is not associated with RA but has the same minor allele frequency (MAF) as the *HLA-DRB1* SE alleles.
†After removing the extended major histocompatibility (MHC) region.
‡Patients with anti-citrullinated protein antibody positive rheumatoid arthritis (ACPA-positive RA).
EIRA, epidemiological investigation of rheumatoid arthritis; GWAS, genome-wide association study; NARAC, North American rheumatoid arthritis consortium; RA, rheumatoid arthritis; SE, shared epitope; SNP, single nucleotide polymorphism.

MATERIALS AND METHODS

The methodology workflow is represented in figure 1B.

Studied populations

This project was based on genome-wide association study (GWAS) data from two independent case–control studies of RA, epidemiological investigation of rheumatoid arthritis (EIRA)[6 14 17–20] and North American rheumatoid arthritis consortium (NARAC).[6 18 21 22] A total of 4291 individuals were included in this study, where 2018 are ACPA-positive RA cases and 2273 are healthy controls (table 1). A detailed description is found in the online supplementary information.

Genotyping and data filtering

HLA typing was performed by sequence-specific primer PCR assay as described elsewhere.[23 24] A standard quality control for GWAS was performed before and after imputation for both cohorts (see the description in the online supplementary information). The extended *MHC* region (chr6:27339429-34586722, GRCh37/hg19) was removed from the analyses, to exclude influence of the high linkage disequilibrium (LD) and independent signals of association with ACPA-positive RA in the locus. Association tests for EIRA and NARAC were applied using logistic regression model in plink (V.1.07).[25] Based on the nominal p values of association, the SNPs were grouped into risk ($p<0.05$) or non-risk SNPs ($p\geq0.05$) (figure 1B and table 2).

Interaction analysis

The additive model was used to test for interactions between the *HLA-DRB1* SE alleles and each SNP from the EIRA and NARAC GWAS. The null hypothesis of the additive model assumes that there is additivity between the different sufficient causes for a phenotype, while the alternative hypothesis is assumed when departure from additivity is observed. The departure from additivity is estimated by the AP due to interaction using OR as measure of association,[26] with the following equation:

$$AP = (OR_{SE1\,SNP1} - OR_{SE1\,SNP0} - OR_{SE0\,SNP1} + 1) / OR_{SE1\,SNP1}$$

where 1 and 0 refer to presence or absence of the risk factor/allele, respectively, the SE0SNP0 was used as a reference group. A cut-off of five observations for each of the cell frequencies was applied. The gender and the first 10 principal components (online supplementary information) were included as covariates in the model. The AP, its respective p value and CI (95% CI) were assessed using logistic regression implemented in GEISA (V.0.1.12)[13 27 28] (table 2).

Comparison of the distribution of AP p values between the risk and non-risk groups of SNPs and quality controls

The distribution of AP p values observed in the interaction analysis from the ACPA-positive RA risk SNPs was compared with the distribution of AP p values observed from the non-risk SNPs using the Kolmogorov-Smirnov (KS) test, implemented in

Table 2 The Kolmogorov-Smirnov (KS) test for AP p value distributions of the interaction analysis with the *HLA-DRB1* SE alleles and risk or non-risk SNPs in EIRA and NARAC imputed data

Case–control group	SNP group	Number of initial input SNPs	Number of SNPs after cut-off *	% of SNPs analysed	Number of SNPs with AP p<0.05	% of analysed SNPs with AP p<0.05	D^+value from KS test (risk vs no-risk)†	Group of SNPs with enrichment of significant interactions
EIRA	Risk	241 759	160 358	66.33	39 518	24.64	0.354	Risk
	No risk	4 515 110	2 979 344	65.99	83 287	2.80		
NARAC	Risk	787 499	209 890	26.65	31 992	15.24	0.247	Risk
	No risk	8 244 955	1 916 701	23.25	64 012	3.44		

*Interaction was estimated using sex and the 10 first eigenvectors as covariables. A minimum of five individuals in each of the four combinations that formed the basis for the OR calculations was required.
†The alternative hypothesis for KS test was that the empirical cumulative distribution function (ECDF) of AP p values for risk SNPs lies above that of non-risk SNPs (figure 2). KS test p<2.2e-16 for both EIRA and NARAC. As mentioned in the Materials and methods section, these KS test p values are lower than the machine precision, meaning that when the precise p value was calculated the result was 0.
AP, attributable proportion; EIRA, epidemiological investigation of rheumatoid arthritis; NARAC, North American rheumatoid arthritis consortium; SE, shared epitope; SNP, single nucleotide polymorphism.

the *stats* package of R software (V.3.3.2).[29] The KS test statistic quantifies the maximum distance (D) between the two empirical cumulative distribution functions of the AP p values from the risk and non-risk SNP groups.

The SNP category of risk and non-risk was permuted 10 000 times to evaluate whether the observed AP p value distribution with the original SNP classification remains with random SNP classification. Similarly, the *HLA-DRB1* SE allele variable was iterated 1000 times with the same purpose. Both types of permutations showed that less than 5% of the KS tests will exhibit a p value under 2.2e-16 (online supplementary information). The rs4507692 SNP (chromosome 7) was used as a negative control instead of *HLA-DRB1* SE alleles, since the rs4507692 SNP is not associated with RA but has the same minor allele frequency as the *HLA-DRB1* SE alleles (table 1).

The SNPs from the *PTPN22* locus (chr1:113679091-114679090, GRCh37/hg19) were removed to apply the same workflow, from step 1 to 9 in figure 1B, to determine the influence of this locus in the enrichment analysis, due to the known gene–gene interaction between the rs2476601variant (or SNPs in LD with this variant) and the *HLA-DRB1* SE alleles.[9] Additionally, to address possible inflation in the results due to imputation and LD, the same steps as described in figure 1B were implemented with (1) raw (non-imputed) GWAS data, (2) with the exclusion of all variants from chromosome 6, and (3) LD pruned GWAS data.

SNPs in interaction with SE between EIRA and NARAC

We selected those variants with AP p<0.05 and same AP direction from both the EIRA and NARAC studies to evaluate their distribution across the genome. False discovery rate (FDR) correction was applied to these SNPs with a 5% threshold (online supplementary table S3)

Conditional eQTL analysis in the context of the *HLA-DRB1* SE alleles (SE-eQTL)

The selected SNPs were tested for eQTLs in the carrier and non-carriers of *HLA-DRB1* SE alleles (SE-eQTLs) for genes±1 Mbp around the SNPs. Data from peripheral blood mononuclear cells (PBMC) of 97 patients with ACPA-positive RA (69% female) from the COMBINE study[30] were included. The mixed-linear model function in the nlme 3.1 package from R/Bioconductor (V.3.3.2)[31] was used for the analysis (details in the online supplementary information).

RESULTS

Interaction between the ACPA-positive RA-associated SNPs and *HLA-DRB1* SE alleles is more common than with non-associated SNPs

EIRA study was the discovery cohort to test for enrichment of significant interactions between the *HLA-DRB1* SE alleles and the predefined risk SNPs from this study. The risk SNPs represent 5% of the variants analysed for interaction in EIRA. Out of these risk SNPs, 24.5% exhibited an AP p value less than 0.05 (table 2, figure 2A). On the other hand, among the non-risk variants (nominal p values of association ≥0.05) representing the remaining 95% of analysed SNPs, only 2.8% displayed a significant interaction (AP p<0.05) with the *HLA-DRB1* SE alleles (table 2, figure 2B). This striking difference in the frequency of significant interactions is reflected in the KS test (D=0.35, p<2.2e-16) (table 2, figure 2C). The enrichment was also observed when only the segments of the AP p values below 0.05 for risk and non-risk SNPs were compared (KS test D=0.25, p<2.2e-16, figure 2D–F), suggesting that the enrichment of significant interactions corresponds mainly to the low AP p values of the risk group of SNPs.

Importantly, this enrichment of low AP p values in the risk variants is totally absent when the rs4507692 SNP was tested instead of the *HLA-DRB1* SE variable as negative control. The proportion of interacting risk SNPs with the rs4507692 variant dropped to 2.8% (table 1 and online supplementary table S1 and figure S1a–f). Since the same group of risk variants was tested for interaction with the *HLA-DRB1* SE alleles and rs4507692 SNP, both AP p value distributions were comparable (KS test D value=0.35, p<2.2e-16) (online supplementary figure S2a). This comparison confirmed that the enrichment of significant interactions is only present when the risk SNPs and the *HLA-DRB1* SE alleles are tested.

The enrichment of significant interactions was not remarkably affected after removing the *PTPN22* locus (KS test D=0.353, p<2.2e-16), highlighting that the strong increment of significant interactions is due to multiple ACPA-positive RA risk variants.

Consistent enrichment of significant interactions was observed when the workflow was applied to non-imputed genotyping data for EIRA, before (KS test D=0.33, p<2.2e-16) and after (KS test D=0.33, p<2.2e-16) removing all the variants from chromosome 6 and LD pruning (online supplementary table S2). The enrichment of significant interactions in the risk group of SNPs was detected also when more stringent thresholds of nominal p value for association (0.005 and 0.0005) were used to classify associated SNPs (online supplementary figure S3).

Altogether, the results from EIRA study indicate that there is a strong enrichment of significant interactions between non-*HLA* risk variants and *HLA-DRB1* SE alleles in ACPA-positive RA.

An independent replication supports the observed enrichment of significant interactions between the ACPA-positive RA-associated SNPs and the *HLA-DRB1* SE alleles

To confirm the results, the independent NARAC study was used. Consistently, a significant proportion of interactions with AP p values below 0.05 was detected between the *HLA-DRB1* SE alleles and the risk SNPs (15.2%), compared with the ones found with the non-risk SNPs (3.3%) (KS test D=0.25, p<2.2e-16, table 2, figure 2G–I). This observation was corroborated when only the fraction of AP p values below 0.05 was compared between the risk and non-risk groups of variants in the NARAC study (KS test D=0.17, p<2.2e-16, figure 2J–L). As in EIRA, there was no enrichment of significant interactions in the risk group (2.6%) compared with the non-risk group (3%) of SNPs when the negative control (rs4507694) was implemented (online supplementary table S1 and figure S1g–l). Consequently, the proportion of relevant interactions remained higher between the risk SNPs and *HLA-DRB1* SE alleles, when compared with the proportion of such interactions with the risk SNPs and the rs4507692 variant (KS test D=0.26, p<2.2e-16; online supplementary figure S2b). Analogous results to EIRA were observed when the workflow was applied to non-imputed GWAS in the NARAC study (online supplementary table S2).

Chromosomes 1 and 9 were highlighted by the top interactions with the *HLA-DRB1* SE alleles

The comparison of results from EIRA and NARAC studies identified 1492 SNPs in interaction with the *HLA-DRB1* SE alleles, with AP p<0.05 and the same direction of AP for both studies (figure 3A,B, online supplementary table S3). The signals within chromosomes were ranked based on the minimum AP p value,

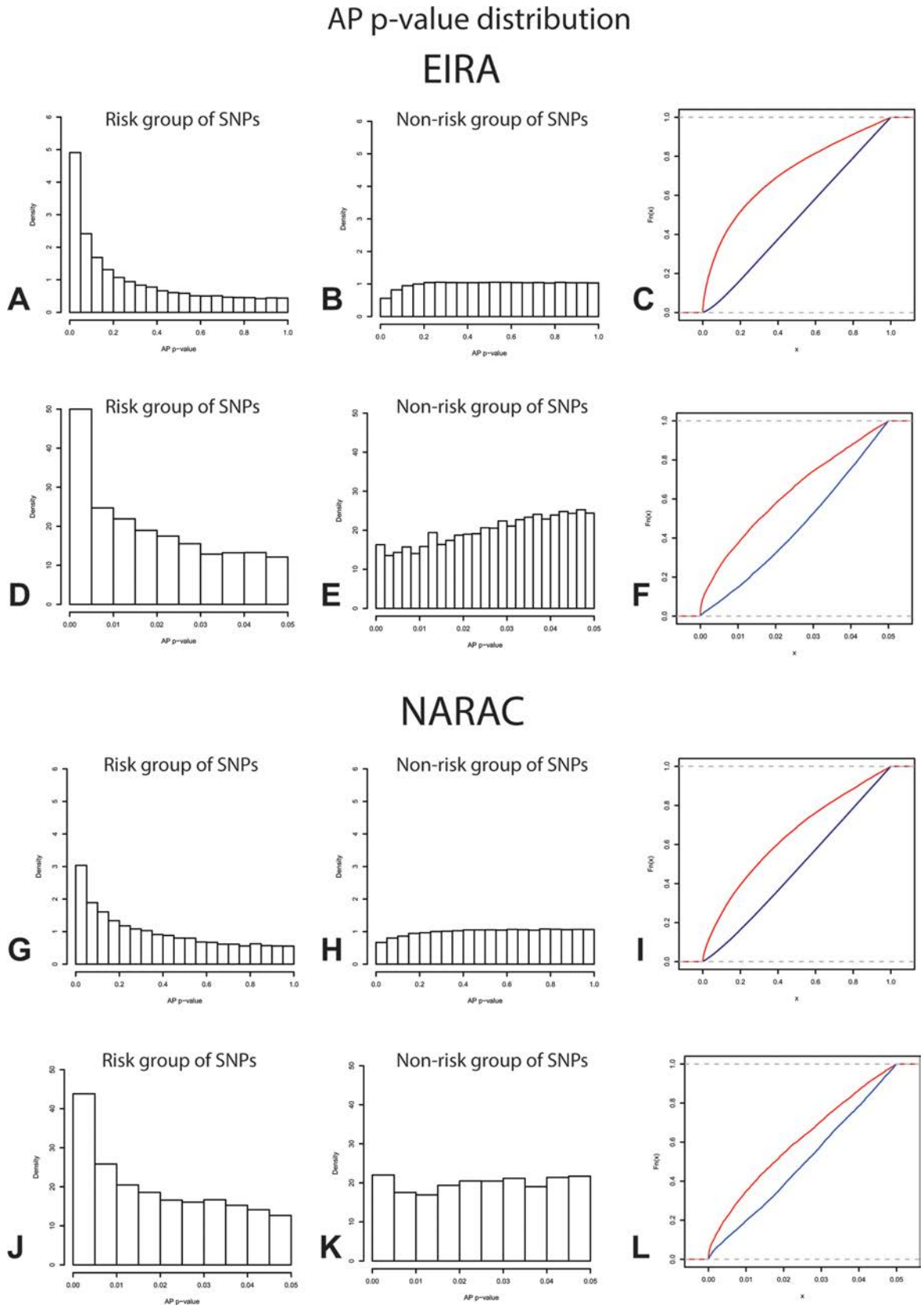

Figure 2 AP p value distributions for interaction between the *HLA-DRB1* shared epitope (SE) alleles and two groups of genetic variants. (A, G) Density plots of AP p values for the interaction between the *HLA-DRB1* SE alleles and the risk group of SNPs (nominal p value of association <0.05) or (B, H) non-risk group of SNPs (nominal p value of association ≥0.05) in the EIRA or NARAC studies, respectively. (C, I) The respective ECDF plots of the AP p values from risk (red line) or non-risk (blue line) SNPs in interaction with the *HLA-DRB1* SE alleles in the respective studies (table 2). The AP p value distribution and the KS tests on the fraction of AP p values less than 0.05 are represented in panels (D)–(F) for EIRA study and (J)–(L) for NARAC study. AP, attributable proportion due to interaction; ECDF, empirical cumulative distribution function; EIRA, epidemiological investigation of rheumatoid arthritis; KS, Kolmogorov-Smirnov test; NARAC, North American rheumatoid arthritis consortium; SNP, single nucleotide polymorphism.

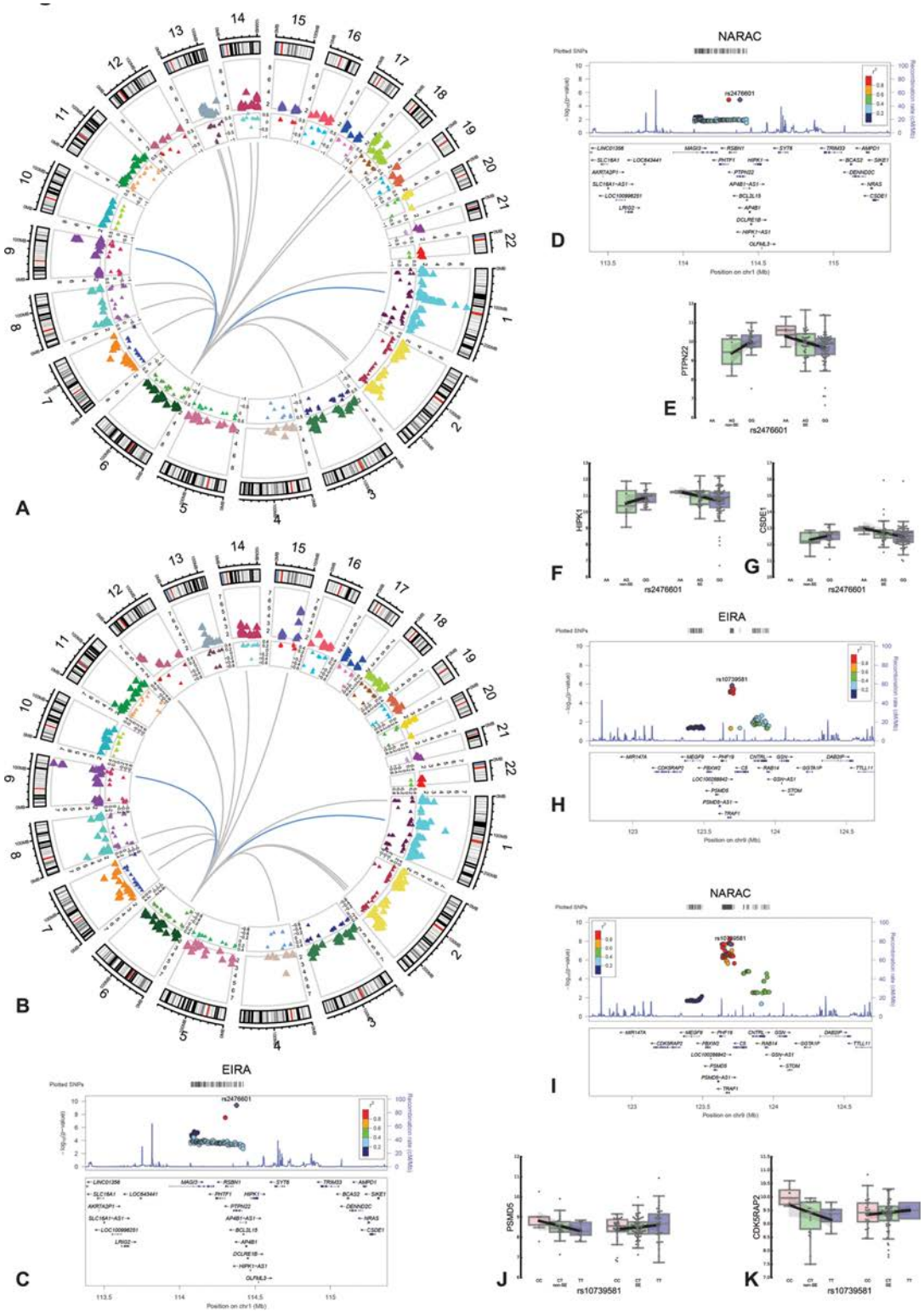

Figure 3 Selected SNPs from both studied cohorts with AP p<0.05 and the same direction of AP. The circos plots for (A) the EIRA study and (B) the NARAC study represent the 1492 selected SNPs in additive interaction with the *HLA-DRB1* SE alleles. The outermost track of the circos plots is the cytoband for 22 human chromosomes. The y-axis of the second track is the negative logarithm of the AP p values (additive interaction with the *HLA-DRB1* SE alleles). In the third track, the y-axis corresponds to the AP value. The internal connector lines highlight the interactions that exhibited an AP p<1e-03. (C) and (D) panels represent the 1p13 locus centred on the rs2476601 SNP. This variant is a conditional eQTL in the *HLA-DRB1* SE allele context for (E) *PTPN22*, (F) *HIPK1* and (G) *CSDE1* genes in PBMCs from the patients with ACPA-positive RA. (H) and (I) panels represent the 9q33 locus centred on the rs10739581 SNP. This variant is a conditional eQTL in the *HLA-DRB1* SE allele context for (J) *PSMD5* and (I) *CDK5RAP2* genes. Panels (C), (D), (H) and (I) were done using LocusZoom (V.0.4.8) (http://locuszoom.org/genform.php?type=yourdata).[49] AP, attributable proportion due to interaction; EIRA, epidemiological investigation of rheumatoid arthritis; FDR, false discovery rate; LD, linkage disequilibrium; NARAC, North American rheumatoid arthritis consortium; PBMC, peripheral blood mononuclear cell; SE, shared epitope; SE-eQTL, expression quantitative trait loci in shared epitope context; SNP, single nucleotide polymorphism. *PTPN22*, *HIPK1*, *CSDE1*, *PSMD5* and *CDK5RAP2* are abbreviations for the genes.

the maximum AP value and the percentage of these 1492 SNPs in interaction with the *HLA-DRB1* SE alleles (online supplementary table S4). Chromosomes 1 and 9 reach the highest position for both studied cohorts (minimum AP p=4.3e-10 in EIRA and p=1.6e-08 in NARAC; online supplementary table S4). Specifically, the loci 9q33 and 1p13 contain the top SNPs in interaction with the *HLA-DRB1* SE alleles (figure 3C,D,H,I and online supplementary table S3). Chromosomes 2, 7, 8 and 13 followed in the ranking when the results from both EIRA and NARAC were considered. The majority (84.6%) of these SNPs in interaction with the *HLA-DRB1* SE alleles exhibited positive AP values, and most of them were under 0.5 (figure 3A,B).

SNPs in interaction with the *HLA-DRB1* SE alleles show functional features

The genotypes of 564 variants out of 1492 (37.7%) were considered to be SE-eQTLs in patients with ACPA-positive RA (online supplementary tables S5 and S6). The four top SE-eQTL pairs are graphically represented in the online supplementary figure S4.

The non-synonymous variant rs2476601 in the *PTPN22* gene (1p13) exhibited the most significant interaction with the *HLA-DRB1* SE alleles (figure 3C,D). In turn, this polymorphism is an SE-eQTL for *PTPN22* (protein tyrosine phosphatase, non-receptor type 22), *HIPK1* (homeodomain interacting protein kinase 1) and *CSDE1* (cold shock domain containing E1) genes (figure 3E–G, online supplementary table S5).

The rs10739581 SNP (9q33) is the second top replicated variant in interaction with *HLA-DRB1* SE alleles (EIRA: AP=0.4, 95% CI 0.24 to 0.57, AP p=1.4e-6, FDR q=0.04; NARAC: AP=0.43, 95% CI 0.28 to 0.59, AP p=2.1e-8, FDR q=2.5e-4, online supplementary table S3). This variant is in high LD (r^2>0.9) with the rs3761847 SNP that has previously been associated with RA.[4 6 19 23 24] The rs10739581 SNP is an SE-eQTL for the *CDK5RAP2* (CDK5 regulatory subunit associated protein 2) and *PSMD5* (proteasome 26S subunit, non-ATPase 5) genes (figure 3J,K, online supplementary table S5).

These results suggest a plausible involvement of the 1492 selected SNPs in the pathogenesis of ACPA-positive RA through interaction with the *HLA-DRB1* SE alleles.

The top interacting SNPs explain a considerable part of the influence from *HLA-DRB1* SE on ACPA-positive RA

Finally, we observed that the step-by-step removal of the effect from the risk alleles of the rs2476601 and rs10739581, the two top replicated SNPs in interaction with the *HLA-DRB1* SE alleles, decreases the effect size of SE alleles for ACPA-positive RA in the studied cohorts (figure 4). This observation clearly suggests that there is a strong mutual influence, reflected in the additive interactions detected, between non-*HLA* genetic variants and the *HLA-DRB1* SE alleles in the development of ACPA-positive RA.

DISCUSSION

Our study demonstrates that there is an important enrichment of RA-associated SNPs interacting with *HLA-DRB1* SE alleles concerning the risk to develop ACPA-positive RA. Importantly, there is a gradual decrease in the effect size of the *HLA-DRB1* SE alleles in the risk of ACPA-positive RA after adjusting for top interacting SNPs. Based on these findings, we propose a concept called the *dominion hypothesis*, which suggests that the

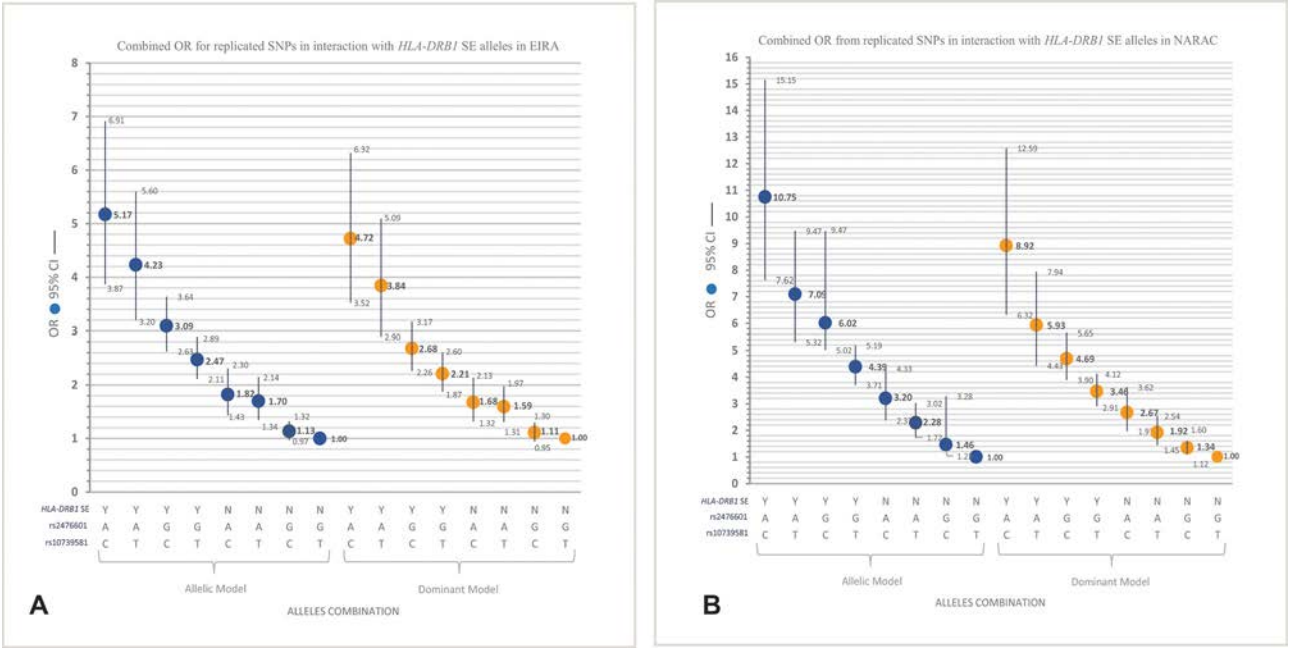

Figure 4 Three-factor OR calculation, the *HLA-DRB1* SE alleles and the top two SNPs in significant interaction. Panel (A) shows data from EIRA study while panel (B) shows data from NARAC study. The x-axis corresponds to the combinations of presence or absence of risk alleles from three factors in two models. The factors are the *HLA-DRB1* SE alleles (presence—Y, or absence—N), the rs2476601 SNP (G>A, 1p13 locus) and the rs10739581 SNP (T>C, 9q33 locus). The rs2476601 and rs10739581 SNPs are in significant interaction with the *HLA-DRB1* SE alleles after FDR correction in both EIRA (AP=0.45, 95% CI 0.31 to 0.60, p=4.3e-10, FDR q=5.2e-5 and AP=0.40, 95% CI 0.24 to 0.57, p=1.4e-6, FDR q=0.04, respectively) and NARAC (AP=0.41, 95% CI 0.23 to 0.6, p=1.1e-5, FDR q=0.04 and AP=0.43, 95% CI 0.28 to 0.6, p=2.1e-8, FDR q=2.5e-4, respectively). The y-axis represents the combined OR with 95% CI. AP, attributable proportion due to interaction; EIRA, epidemiological investigation of rheumatoid arthritis; FDR, false discovery rate; NARAC, North American rheumatoid arthritis consortium; SE, shared epitope; SNP, single nucleotide polymorphism.

HLA-DRB1 SE alleles function as a genetic hub that is involved in multiple interactions with non-*HLA* genetic variants that by themselves have a modest effect size in RA. These interacting non-*HLA* variants do cumulatively contribute to the high effect size of the *HLA-DRB1* SE alleles concerning risk to develop ACPA-positive RA. The *dominion hypothesis* has its foundations in the sufficient-component cause model,[16] which suggests that several diverse components are part of a sufficient cause for a disease in a given affected individual, where each sufficient cause can include one or more component causes. Together, these component causes can form a minimal set of conditions that drive disease.[32] Our hypothesis integrates the *HLA* alleles with other genetic variations across the human genome thereby potentially providing knowledge about the mechanisms behind this autoimmune disease.

The statistical approach in our study resulted in a list of 1492 SNPs (~270 independent loci, online supplementary table S7) as good candidates interacting with the *HLA-DRB1* SE alleles in conferring risk for developing of ACPA-positive RA. Out of these SNPs 37.7% are SE-eQTLs that define genes that could be involved in the development of ACPA-positive RA. These findings suggest that the now identified additive interactions may reflect biological processes involved in the pathogenesis of ACPA-positive RA. For instance, the rs2476601 SNP appears to be an SE-eQTL for *PTPN22*, *HIPK1* and *CSDE1* genes in PBMCs of patients with ACPA-positive RA (figure 3 and online supplementary table S5). Furthermore, Capture Hi-C studies showed that the rs2476601 is in physical contact with the *HIPK1* and *CSDE1* genes in CD4+ and CD8+ T cells (https://www.chicp.org, data accessed: February 2018),[33–35] which are relevant in RA.[36] Another illustration is the rs1506691, rs6804917 and rs12630663 SNPs (online supplementary table S6) that are in physical contact in T cells with the *EOMES* gene (https://www.chicp.org),[33 35] important in CD4+ T cell differentiation in the context of RA.[37] Moreover, the *HLA-DRB1* genotype appears to have a key role in shaping the T cell receptor repertoire.[38] Our findings reinforce previous observations that have suggested that the *HLA* locus plays a special role in transgenomic regulatory mechanisms.[39–41] The observed interactions might in some cases also reflect altered protein interactions in a given molecular pathway. For instance, it has been shown that the minor allele of rs2476601 SNP affects the LYP (protein encoded by *PTPN22* gene)-CSK protein complex on the TCR signalling context,[42] which in turn could have consequences for functions that involve TCR-peptide-MHC complexes. Additionally, one of the top interacting SNPs, the rs10739581, seems to be an SE-eQTL for the *PSMD5* gene that is involved in antigen presentation and proteasome regulation.[43] Interestingly, *H3F3A* and *TNC* genes that encode proteins that are known citrullinated autoantigens in RA[44 45] are in the proximity of SNPs in interaction with *HLA-DRB1* SE alleles (online supplementary table S6). Further interpretation and study of these interactions are required to understand their probable mechanisms in the RA pathogenesis.

When calculating the narrow heritability for ACPA-positive RA that could be explained by the current interaction analysis and the confirmed RA-associated loci,[6] we obtained values of 54% for EIRA and 64% for NARAC (online supplementary information and supplementary table S8). However, these values should be considered cautiously since the calculation does not completely integrate interaction effects.

In conclusion, we used a systematic approach to investigate interactions at the genome-wide level between non-*HLA* and *HLA-DRB1* alleles in ACPA-positive RA and we were able to identify a significant enrichment of interacting with *HLA-DRB1* SE alleles among disease-associated non-*HLA* SNPs. We suggest that the *dominion hypothesis* might be used to explore the next level of complexity in this disease as well as in other multifactorial immune-mediated diseases that involve *HLA*.

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Contributors LMDG: conceptualisation, data curation, formal analysis, investigation, methodology, software, project administration, validation, visualisation, writing—original draft preparation and review and editing. DR: conceptualisation, formal analysis, methodology, resources, writing—review and editing. KS: conceptualisation, investigation, methodology, software, writing—review and editing. LF: conceptualisation, formal analysis, writing—review and editing. KC: conceptualisation, validation, writing—review and editing. BB: conceptualisation, writing—review and editing. SU: data curation. YO: data curation, resources, writing—review and editing. LA: conceptualisation, investigation, resources, software, writing—review and editing. LK: conceptualisation, funding acquisition, resources, writing—review and editing. LP: conceptualisation, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, writing—original draft preparation and review and editing.

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EXTENDED REPORT

Methotrexate and BAFF interaction prevents immunization against TNF inhibitors

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ABSTRACT

Objectives TNF inhibitors (TNFi) can induce anti-drug antibodies (ADA) in patients with autoimmune diseases (AID) leading to clinical resistance. We explored a new way of using methotrexate (MTX) to decrease this risk of immunisation.

Methods We treated BAFF transgenic (BAFFtg) mice, a model of AID in which immunisation against biologic drugs is high, with different TNFi. We investigated the effect of a single course of MTX during the first exposure to TNFi. Wild-type (WT) and BAFFtg mice were compared for B-Cell surface markers involved in MTX-related purinergic metabolism, adenosine production and regulatory B-cells (Bregs). We translated the study to macaques and patients with rheumatoid arthritis from the ABIRISK cohort to determine if there was an interaction between serum BAFF levels and MTX that prevented immuniation.

Results In BAFFtg but not in WT mice or macaques, a single course of MTX prevented immunisation against TNFi and maintained drug concentration for over 52 weeks. BAFFtg mice B-cells expressed more CD73 and CD39 compared to WT mice. MTX induced adenosine release from B cells and increased Bregs and precursors. Use of CD73 blocking antibodies reversed MTX-induced tolerance. In patients from the ABIRISK cohort treated with TNFi for chronic inflammatory diseases, high BAFF serum level correlated with absence of ADA to TNFi only in patients cotreated with MTX but not in patients on TNFi monotherapy.

Conclusion MTX and BAFF interact in mice where CD73, adenosine and regulatory B cells were identified as key actors in this phenomenon. MTX and BAFF also interact in patients to prevent ADA formation.

INTRODUCTION

TNF inhibitors (TNFi) have revolutionised the care of rheumatic diseases due to their high specificity allowed by their biologic nature. However, these biologic drugs cause immunogenicity that can lead to a neutralising immune response[1] characterised by the production of anti-drug antibodies (ADA). ADA may also be generated against immune checkpoint inhibitors used in the treatment of cancer[2] but at a lower frequency than in patients with autoimmune diseases (AID). Thus, generation of ADA may be influenced by the immune status of the patient.

Methotrexate (MTX) is used as an antirheumatic drug and leads to a higher therapeutic effect of TNFi, if used concomitantly.[3] This effect might be explained by the diminished production of ADA when patients are treated with TNFi and MTX.[3 4] But MTX does not prevent ADA in all patients and the mechanism of action of MTX for preventing ADA formation still remains to be elucidated. Used as a disease-modifying antirheumatic drug, the main proposed mechanism of action of MTX is an increase in adenosine, a powerful anti-inflammatory agent, by decreasing its conversion into inosine and the conversion of AMP into IMP. This occurs mainly by inhibition of AICAR transformylase which leads to a release of AMP outside the cell. AMP is further converted into adenosine by CD73, an ecto-5′-nucleotidase[5 6] (online supplementary figure 1). In a mouse model of Pompe's disease, MTX was successfully used to prevent ADA formation against alpha-glucosidase, a highly immunogenic recombinant enzyme used as a treatment for this disease. Surprisingly, a single course of MTX at the time of first enzyme exposure was able to significantly reduce anti-enzyme antibody concentration. This MTX-induced tolerance to alpha-glucosidase was transferred to naïve mice using splenic B cells.[7]

The BAFF (B cell activating factor of the TNF family) or BLyS (B-lymphocyte stimulator) cytokine, discovered in 1999, is a key driver of B cell activation. BAFF is secreted mainly by monocytes, macrophages and neutrophils, and may explain pathogenic B cell activation in several systemic AID and lymphomas.[8] BAFF targets three receptors present in B cells: BAFF-R, TACI and BCMA. BAFF transgenic (BAFFtg) mice develop AID reminiscent of systemic lupus erythematosus (SLE) and primary Sjögren's syndrome (pSS) with an increased risk of developing lymphoma.[9] In humans, patients with pSS and SLE have elevated serum levels of BAFF,[10 11] sometimes correlated with the serum level autoantibodies.[10 12 13] Interestingly, belimumab, an anti-BAFF monoclonal antibody, was approved in 2011 for the treatment of SLE and open studies are encouraging in pSS.[14]

The study aimed to elucidate the role of MTX in the immune response against TNFi in a setting of high immunisation against TNFi using the model of BAFFtg mice and to translate the observed findings to prevent ADA formation in patients.

METHODS

Mice and treatment

BAFFtg C57BL/6J mice were kindly provided by F Vincent and F Mackay (Monash University Australia) and have been described previously.[9] Wild-type (WT) C57BL/6J mice purchased from Janvier Labs (Le Genest-Saint-Isle, France) were used as controls in this study. Genotyping was performed by PCR using primers spanning the SV40 polyA tail of the BAFF transgene construct. The experiments included male and female mice in equal numbers. The experiments were performed either on 2-month-old mice or older mice, depending on the length of the treatment with TNFi. Experiments were performed on matched numbers of males and females with a total of 140 mice. Treatment with MTX (5 mg/kg) was administered intraperitoneally within minutes of the first TNFi injection and repeated only twice at 24 and 48 hours, as reported in the model of Pompe's disease.[7] The dose of 5 mg/kg of MTX administered here three times is equivalent to a dose of 0.1 mg/kg in humans. This is based on pharmaco-kinetics analysis from the literature in rodents and humans.[15 16] Adalimumab was administered weekly at 20 mg/kg and etanercept biweekly at 8 mg/kg for 52 weeks. TN3 (a hamster anti-mouse TNFα antibody) was injected once a week at 20 mg/kg for 52 weeks also. To reverse MTX-induced tolerisation an anti-CD73 (clone TY23, BioXcell, West Lebanon, USA) monoclonal antibody was used. Animals were treated with anti-CD73 biweekly either for 1 or 9 weeks, with a first intraperitoneal injection of the anti-CD73 antibody 100 µg just before receiving the MTX plus adalimumab regimen.

Macaques and treatment

Adult captive-bred 3–5 year-old male cynomolgus macaques (*Macaca fascicularis*) were used.

Animals were treated with four 2 mg/kg intravenous adalimumab monthly injections which were similar to the schedule described in the Food and Drug Administration preclinical studies. Animals in the MTX group were injected prior to the adalimumab injection with 0.25 mg/kg MTX at day 0 and day 1.

Patients and treatment

Patients from the ABIRISK[17] (Anti-Biopharmaceutical Immunization: prediction and analysis of clinical relevance to minimize the RISK), designed to prospectively identify risk factors of ADA against TNFi, were included in this study. Authorisation from each local ethics committee was obtained as well as patient's informed consent. Patient's underlying disease was rheumatoid arthritis (RA) (fulfilling 2010 American College of Rheumatology/European League Against Rheumatism criteria) or inflammatory bowel disease (IBD). Patients were considered treated with MTX when this treatment was concomitantly administered with the newly introduced TNFi. In ABIRISK patients, ADAs were screened at 1, 3, 6 and 12 months after TNFi therapy for rheumatologic cohorts (RA) and at 6 weeks and 3, 6 and 12 months after TNFi therapy for IBD cohort. Patients with at least one time-point with detectable ADA were considered immunised (ADA+).

Statistics

All analyses of flow cytometry and animals were performed using non-parametric test (Mann-Whitney and Fisher tests). In the group comparisons of the human data a Welch's unequal variances t-test was used. This test is well suited for when groups have unequal variance and as in this case unequal sample sizes.[18] All authors had access to primary clinical trial data. Analyses were performed using GraphPad Prism V.7 software (La Jolla, USA).

Further methods are available in online supplementary materials.

RESULTS

A short course of MTX prevents ADA formation and maintains TNFi concentration for over a year in BAFFtg mice but not in cynomolgus monkeys or WT mice

BAFFtg mice treated with three different TNFi (adalimumab, etanercept and TN3) had an undetectable concentration of the drug due to ADA as soon as 8 weeks after initiation (figure 1). Intraperitoneal MTX administration at a dose of 5 mg/kg on days 0, 1 and 2 was able to maintain drug concentration of all tested TNFi, and to prevent the appearance of ADA in almost all animals. Strikingly, this tolerance was maintained for over a year with all three TNFi despite recurrent administration of TNFi without MTX. Thus, one course of three MTX injections was sufficient to prevent TNFi immunogenicity and induce long-term tolerance in BAFFtg mice. Following the same procedure as in BAFFtg mice, the rate of immunisation was low in WT mice (3/10 WT mice immunised) but MTX did not seem to have an effect (5/10 WT mice immunised with MTX) (online supplementary figure 2A,B).

To provide evidence for translation to patients we tried to reproduce these results in cynomolgus monkeys. In striking contrast, monthly adalimumab administration to cynomolgus monkeys with or without a 2-day MTX regimen showed no effect on immunogenicity. All animals showed a rapid appearance of ADA and drug concentrations were undetectable as soon as the second injection (online supplementary figure 2C,D). This suggests that high BAFF levels observed in BAFFtg mice compared to WT and macaques (online supplementary figure 3) are required for MTX to exert its effect

CD73 overexpression and increased adenosine production by B cells from BAFFtg mice

Since high BAFF levels seemed necessary for MTX-induced immune tolerance, we sought to compare the main molecular players involved in MTX mechanism of action between BAFF and WT mice. Between different metabolic pathways modulated by MTX, the adenosine synthesis pathway seemed particularly interesting in the context of tolerance induction studies. We thus analysed the expression of CD73, an ecto-5′nucleotidase involved in adenosine generation from AMP on a subset of peritoneal B1 cells (B220+ CD23 low). As shown in figure 2A, the expression of CD73 was markedly increased in the BAFFtg mice B1 subset compared with WT mice. Similar striking results were obtained in whole B220+ splenocytes in whom 21.7% of cells were CD73+ in BAFFtg mice versus 10.9% in WT mice (mean, p=0.0018) (figure 2B). Interestingly, the expression of the CD39 enzyme that acts upstream to CD73 in the adenosine synthesis cascade was also increased in B cells from BAFFtg mice compared to WT mice (figure 2C).

Functional characteristics of splenic B cells were next assessed: CD73 enzymatic activity on AMP exposure was quantified. Sorted splenic B cells were first exposed to low (20 µM) or high (200 µM) concentration of AMP for 2 hours, then adenosine and inosine productions were quantified. BAFFtg B cells were able to convert significantly more AMP into adenosine than WT mice (255.5 vs 120.7 ng/mL of adenosine, respectively, p<0.0001, at 20 µM AMP). This capacity was even increased at higher AMP concentrations (figure 2D). Cells without AMP exposure were used as controls and showed no

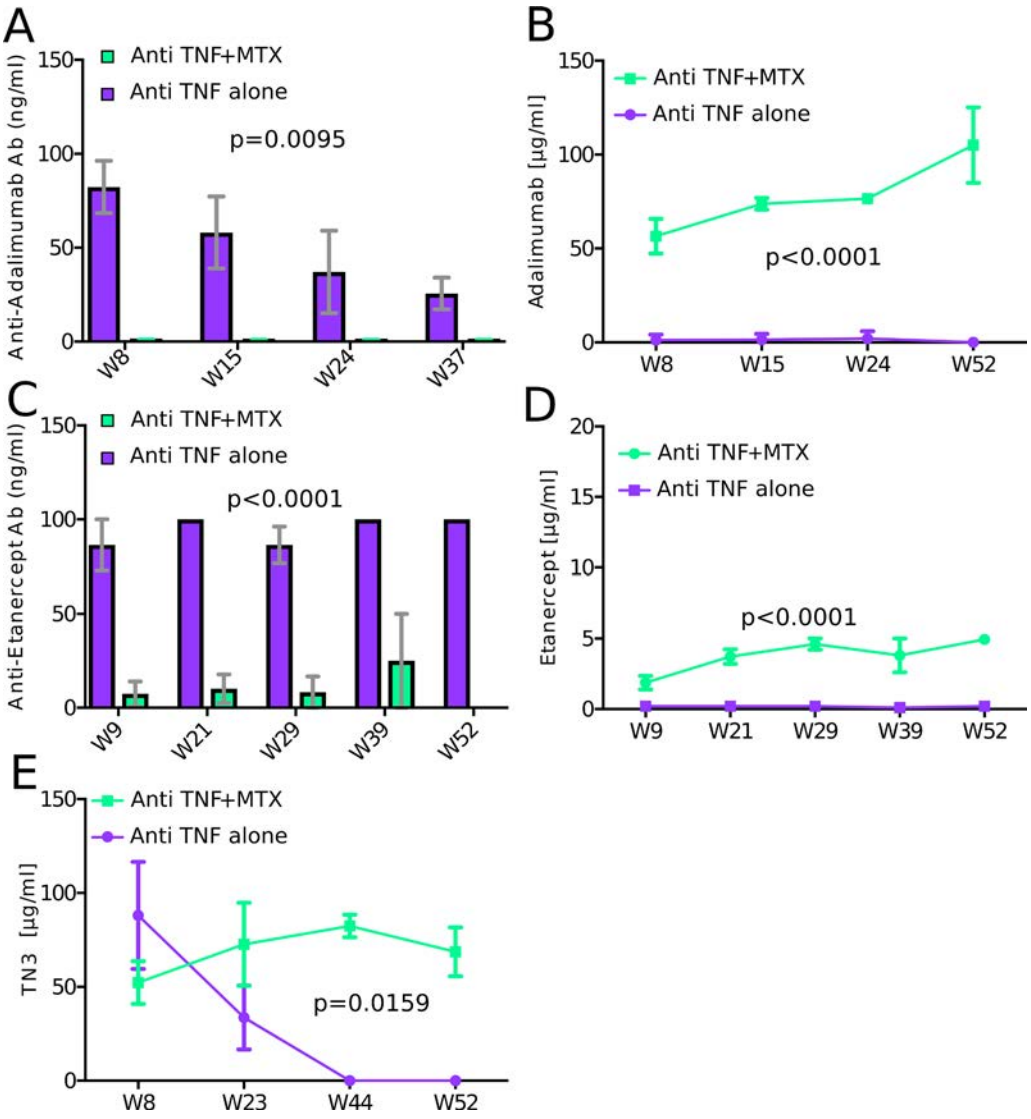

Figure 1 In BAFFtg mice, methotrexate (MTX) prevents immunisation against TNF inhibitors (TNFi) for over 52 weeks. BAFFtg were treated with TNFi with or without (H_2O) a course of MTX. Anti-drug antibodies and drug concentration were monitored regularly for a period of 52 weeks. Anti-adalimumab antibodies (A) and drug concentration (B) were monitored in BAFFtg animals with (n=15) or without (n=5) a short course of MTX. Anti-etanercept antibodies (C) and drug concentration (D) were monitored regularly in BAFFtg animals treated with (n=15) or without (n=5) a short course of MTX. (E) BAFFtg animals were treated with TN3 antibody (weekly injections) with (n=15) or without (n=5) a short course of MTX while drug concentrations were regularly monitored. Results are shown as mean and SEM of individual animals over time. The area under the curve was compared using Mann-Whitney test.

difference in adenosine production between BAFFtg and WT mice. Since adenosine can be rapidly degraded into inosine, the sum of adenosine+inosine production was also assessed and consistently showed the same difference between BAFFtg and WT mice (figure 2E). Taken together, these data suggest that in the highly autoimmune context of BAFFtg mice, MTX could promote tolerance induction through stimulation of extracellular ATP/AMP degrading system and adenosine synthesis pathway in B cells.

BAFFtg mice have an increased pool of regulatory B cells and this population expands further with MTX treatment

Bregs are important players in the negative regulation of immune responses and might be involved in tolerance induction mechanisms. We thus compared the regulatory B cell population between BAFFtg and WT mice. Surprisingly, B10 cells defined by their ability to produce interleukin (IL)-10 were significantly increased among splenocytes in BAFFtg mice, despite their autoimmune phenotype as compared to WT mice (3.4% vs 1.16% of CD19+ cells, respectively, p=0005, figure 3A). When BAFFtg mice were subjected to MTX tolerisation, B10 cells were further significantly increased compared with untreated BAFFtg mice (6.3% vs 3.4%, respectively, p=0046, figure 3A). Moreover, B10 cells from BAFFtg mice showed higher CD73 expression: 41.1% vs 26% in WT of CD19+ IL10+ cells (p=0009, figure 3B). BAFFtg and WT mice were next compared for their percentage of Breg precursors among splenocytes. BAFFtg mice had a similar percentage of CD21hiCD24hi CD23+ cells compared to WT mice. But, when BAFFtg mice were tolerised with MTX against adalimumab, this percentage significantly increased from 33.4% to 52.5% (p=0.02, figure 3C).

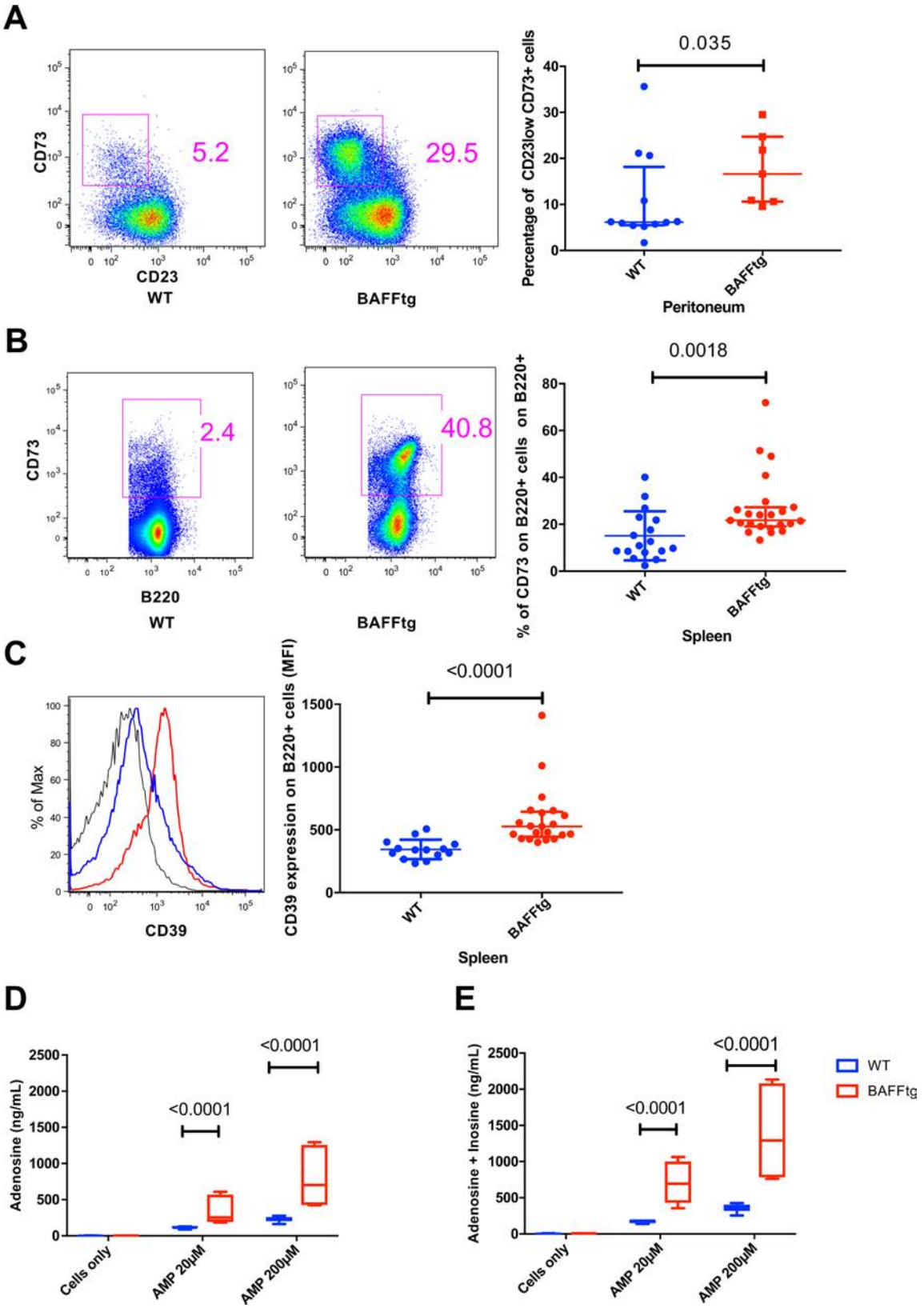

Figure 2 B cells from BAFFtg mice express more CD73 and CD39 and leading to more adenosine production. Wild-type (WT) and BAFFtg animals were compared. (A) Percentage of CD73+ positive cells on peritoneal B1 cells (B220+ CD23 low). (B) Percentage of CD73+ positive cells among B220+ splenocytes. (C) Mean fluorescence intensity of CD39 expression on B220+ splenocytes. Black line isotype control, blue line WT and red line BAFFtg animals. Adenosine (D) and adenosine+inosine (E) production was assessed on sorted B cell from the spleen on exposure to 20 and 200 µM AMP for 2 hours. Results are shown as mean and SD for panels (B) and (C), median and IQR for panel (A) and median 25th and 75th percentiles for the box, and min and max for whiskers for panels (D) and (E). Median and mean are of individual animals performed on at least three separate experiments. Purinergic metabolite quantification was performed in triplicate in three different animals for each condition. Results are compared with Mann-Whitney test.

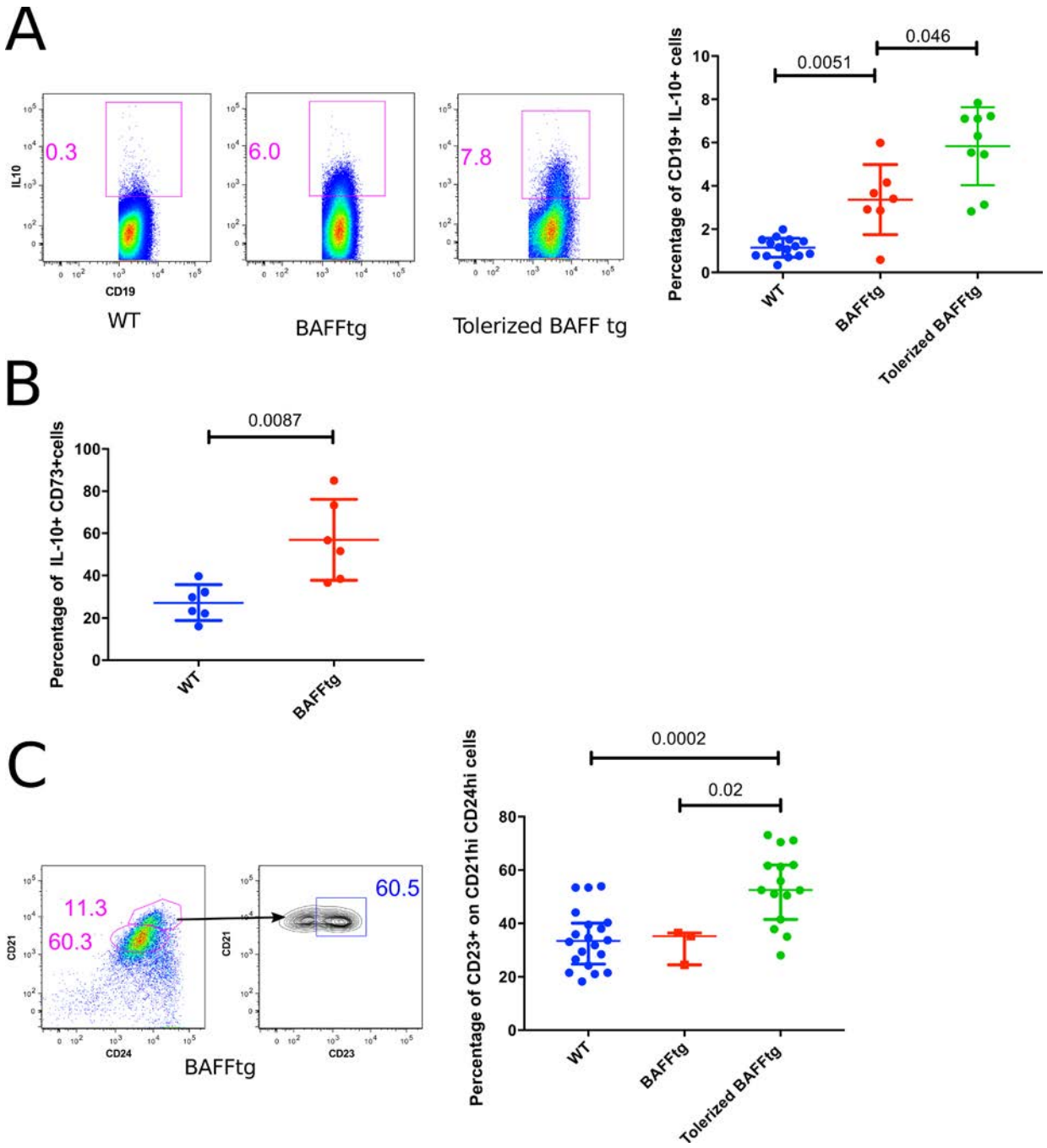

Figure 3 BAFFtg mice express more regulatory B cells and this is increased by methotrexate (MTX) tolerisation. Wild-type (WT), BAFFtg and BAFFtg tolerised by MTX animals were compared. (A) Sorted B cells were cultured for 48 hours with CD40L and intracellular staining for interleukin (IL)-10 was performed. (B) Percentage of CD73 positive cells among IL-10 cells. (C) Percentage of CD23 positive cells among CD24hi CD21hi B cells are defined as Breg precursors. On panels (A) and (B), means and SD are displayed and on panel (C) median and IQR. Medians and means of individual animals were performed on at least three separate experiments. Results are compared with Mann-Whitney test.

In vivo CD73 inhibition prevents MTX-induced tolerance

Animals tolerised with the MTX regimen at the beginning of adalimumab treatment were treated or not with anti-CD73 antibodies. Anti-CD73 antibodies were administered twice a week either during the first week only (beginning before MTX and adalimumab administration) or continuously for 9 weeks. The two methods of anti-CD73 antibodies administration yielded the same results and were therefore pooled. After 9 weeks of treatment with one cycle of MTX and adalimumab, the mice treated with anti-CD73 antibodies lost their ability to induce tolerance to adalimumab. Anti-CD73-treated mice had a significantly lower serum concentration of adalimumab (0 vs 6.8 ng/mL, p=0.02, figure 4A). The number of immunised animals was also significantly higher when the anti-CD73 regimen was added to MTX and adalimumab in BAFFtg animals (p=0.03, figure 4B).

Translation to patients: in MTX-treated patients, BAFF is higher in patients without ADA

BAFF quantification was performed at baseline before the first administration of TNFi in the serum of 292 patients included in the ABIRISK consortium (135 RA and 157 IBD, characteristics in table 1) and these patients were prospectively followed for 18 months with regular quantification of

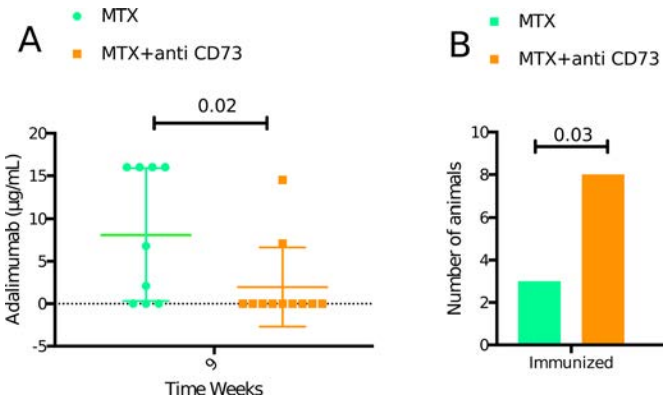

Figure 4 Anti-CD73 antibodies reverse methotrexate (MTX)-induced tolerance. BAFFtg animals tolerised to adalimumab with MTX were treated with or without anti-CD73 antibodies (TY 23) for 9 weeks. (A) Serum levels of adalimumab were measured at 9 weeks and compared between animals treated (n=10) or not (n=9) with TY 23 antibodies (Mann-Whitney test). (B) Absolute number of immunised animals are analysed and compared between animals treated (n=10) or not (n=9) with anti-CD73 antibodies (X^2 test).

ADA. In IBD, the percentage of patients with ADA was the same in patients cotreated or not with azathioprine/6-mercaptopurine (12% and 15%, respectively) and few patients were treated with MTX. In patients with RA, ADA occurred in 10% of the MTX-treated patients and in 25% of the non-MTX-treated patients ($p<0.05$, Fisher exact test). In MTX-treated patients, the mean serum level of BAFF was significantly lower (0.49 ng/mL) in ADA-positive patients than in patients without ADA (0.69 ng/mL, $p=0.02$, figure 5A). Conversely, in the non-MTX-treated patients, there was no significant difference in the serum level of BAFF between patients with or without ADA ($p=0.88$, figure 5B). Other baseline data (age, smoking, activity of the disease, cotreatment with steroids, C-reactive protein level) which could have influenced ADA occurrence were not different among RA MTX-treated patients between ADA+ and ADA− patients cotreated with MTX+TNFi (online supplementary table 1). Only three etanercept-treated patients had a transient low level of ADA only at month 1, with normal PK and thus without any clinical relevance since they are not neutralising antibodies. The removal of all patients treated with etanercept reinforced the association between low-serum BAFF level and ADA (0.68 ng/mL vs 0.46 ng/mL, $p=0.0006$, figure 5C). This removal did not affect the results in the non-MTX-treated patients ($p=0.61$, figure 5D). This confirms the interaction between MTX and BAFF to prevent ADA formation in TNFi-treated patients.

Table 1 ABIRISK patient characteristics evaluated for BAFF serum level

	Patients with RA (n=135)	Patients with IBD (n=157)
Age mean (SD)	52.4 (12.8)	37.4 (13.6)
Female (%)	75	77
ACPA positive (%)	71.3	NR
Rheumatoid factor positive (%)	66.9	NR
Baseline DAS28 mean (SD)	4.3 (1.3)	NR
Baseline Harvey-Bradshaw Index for Crohn's disease mean (SD)	NR	6.0 (3.9)
Baseline Mayo score for ulcerative colitis mean (SD)	NR	6.6 (3.4)
Baseline CRP (mg/L) mean (SD)	10.4 (12.8)	17.4 (26.6)
Percentage of MTX-treated patients (%)	79.3	10.2

ACPA, anti-citrullinated peptide antibodies; CRP, C-reactive protein; DAS, Disease Activity Score; IBD, inflammatory bowel disease; MTX, methotrexate; NR, not relevant; RA, rheumatoid arthritis.

DISCUSSION

In this study, we showed that a single short regimen of MTX abolished immunogenic response to TNFi in BAFFtg mice but not in WT mice or in macaques. This suggests that high BAFF levels observed in BAFFtg mice compared to WT mice and macaques (online supplementary figure 3B) are required for MTX to exert its effect. Our data suggest an interaction between BAFF and MTX for this active tolerisation induction. We demonstrated that BAFFtg mice had constitutively elevated levels of CD39 and CD73 on B cells. Using a transgenic mouse model was the most reliable way to increase BAFF homogeneously but other strategies could be explored such as treatment of WT mice with exogenous BAFF or with BAFF-containing plasmids, but these approaches will be probably much less reproducible. This increased CD73 expression in B cells is required for the tolerisation action of MTX. MTX is in turn responsible for IL-10 producing Breg induction and increased adenosine production by B cells. This interaction between BAFF and MTX suggested in the BAFFtg mouse model was confirmed in a prospective human study. Hence, high BAFF serum level was protective against ADA formation to TNFi only in patients cotreated with MTX but not in patients on TNFi monotherapy.

The main action of BAFF is to stimulate activated B cells. This is confirmed by mouse models and by the efficacy of belimumab, a therapeutic anti-BAFF antibody in human lupus. However, a recent work shows that when type 1 interferon and IL-6 increase activation of immune cells, there is a concomitant activation of regulatory B cells enriched among Breg precursor cells.[19] We describe a similar regulation in BAFFtg mice. Here, we showed that CD73 expression, a marker known to be expressed on regulatory T cells and on peritoneal B1 cells,[20 21] was enhanced in BAFFtg mice. CD73 was upregulated in B1 cells from the peritoneum, where the first contact between MTX and TNFi occurs. This was also confirmed in the spleen which has been shown to be responsible for MTX-induced tolerance transfer in mice.[7] Moreover, we found an increase of CD73 in B10 cells in BAFFtg mice especially when they were tolerised with MTX. Interestingly, it has been previously shown that BAFF stimulation of B cells increased IL-10 production in the supernatant[22] and that B cells from BAFFtg mice could secrete more IL-10.[23] Since B10 cells require in vitro activation to be studied, we sought to also explore Breg precursors as described previously.[19]Again, Breg precursors were increased in BAFFtg mice tolerised with MTX. Interestingly, a possible role of IL-10 in ADA prevention could also be suggested based on the association of anti-adalimumab ADA with IL-10 gene polymorphisms.[24]

MTX is the cornerstone of RA treatment but its mechanism of action remains to be elucidated. Through inhibition of AICAR transformylase, MTX increases the release of AMP outside the cell and in presence of CD73 the transformation into adenosine, a powerful immunosuppressive agent (online supplementary figure 1). CD73 is required for low-dose MTX-induced immunomodulatory effect observed in the air pouch model.[5 6] CD39, the directly upstream ectoenzyme of CD73, has also been shown to influence clinical response to MTX in patients with RA.[25] The interaction between MTX and BAFF can be explained, at least partly, by the increase of CD73 expression on B cells from BAFFtg

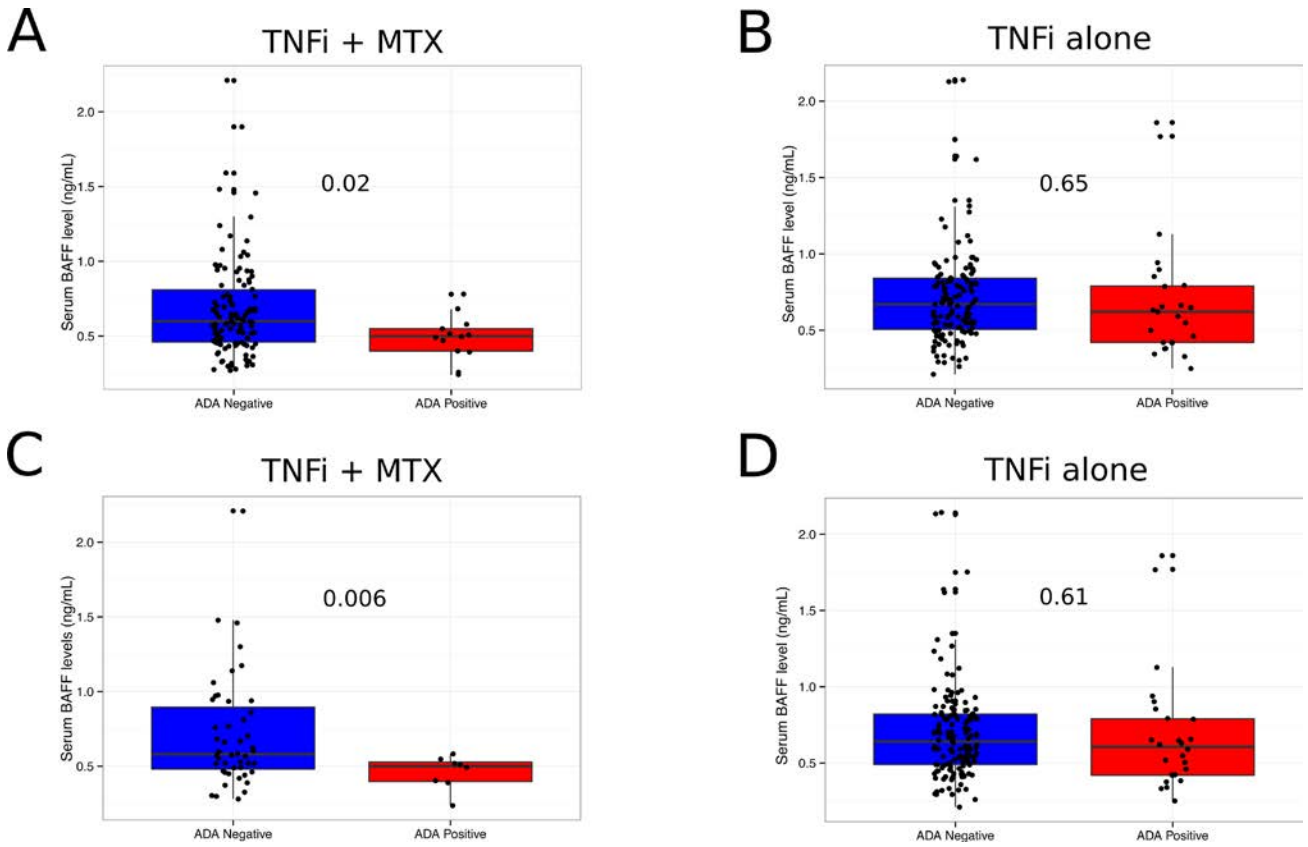

Figure 5 Antidrug antibody (ADA)-negative patients treated with methotrexate (MTX) have higher baseline BAFF levels compared with ADA-positive patients. TNF inhibitors (TNFi) were introduced in patients with rheumatoid arthritis (RA) or inflammatory bowel disease (IBD) concomitantly treated or not with MTX. Immunisation against TNFi was assessed prospectively using adapted Theradiag assay. According to the presence of antibodies, patients were classified as ADA positive or negative. (A) Baseline BAFF levels were compared between ADA+ andADA− patients treated with any TNFi and MTX (p=0.02). (B) Baseline BAFF levels were compared between ADA+ andADA− patients treated with any TNFi without MTX (p=0.65). (C) Baseline BAFF levels were compared between ADA+ andADA− patients treated with adalimumab and infliximab (etanercept excluded) and MTX (p=0006). (D) Baseline BAFF levels were compared between ADA+ andADA− patients treated with adalimumab and infliximab (etanercept excluded) without MTX.

mice. Indeed, CD73, present on BAFFtg mice B cells has a functional effect: exposition of these cells to AMP induced a higher level of adenosine synthesis. This was confirmed by treating mice with an anti-CD73 antibody which markedly decreased the ability of MTX to induce tolerisation against TNFi.

The MTX and BAFF interaction hypothesis is further confirmed by results obtained in patients from the ABIRISK cohort. For the first time in a clinical study, the serum level of BAFF was assessed to predict immunogenicity. The results obtained in mice were confirmed since only in patients cotreated with MTX, a high-serum BAFF level was correlated with less immunisation against TNFi. This effect was identical for different TNFi in two different inflammatory diseases. It was even higher if we excluded the patients treated with etanercept in whom only three presented with transient ADA, which probably has no clinical signification.

Besides RA treatment and prevention of ADA formation, MTX is also known to diminish immune response against vaccines. This effect is stronger than that induced by TNFi and similar to that induced by rituximab, an anti-CD20 B cell depleting agent.[26] But the time frame between MTX administration and the vaccine exposure, required to inhibit the immune response, remains unclear. The mechanism of action for impairing the vaccine response could be the same as the mechanism we propose for inhibiting the ADA formation: activation of specific Bregs. In this study, we showed that MTX had to be present at the very beginning of antigen exposure and that this specific window was sufficient to induce long-term tolerance. Interestingly, a recent randomised controlled trial showed that, in order to impair the response to vaccines, MTX had to be present just before and at the same time of vaccination.[27] We show in this report that a similar time frame is required to impair ADA formation against TNFi. Conversely to the action on RA activity which may take up to 3 months, this effect of MTX on ADA or vaccine requires the presence of MTX at the time of first exposure to the antigen.

In conclusion, just as IL-2 may activate effector or regulatory T cells depending on the dose or the environment, BAFF could be considered as a Janus cytokine able to balance between effector and regulatory B cells, depending on signals of the environment like MTX or other factors. In case of increased BAFF, using one single dose or one single cycle of MTX just before the first injection of a therapeutic monoclonal antibody may induce a specific tolerisation against this biologic drug for life. Another possible application would be to screen patients for BAFF levels and use MTX only in patients with elevated BAFF to prevent immunisation against TNFi and more generally to all immunogenic biologic drugs. Thus, it could be possible to prevent immunisation against any kind of therapeutic monoclonal antibodies in all domains of medicine by a very simple, cheap and safe procedure.

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Contributors SB, GN and XM designed the study, and supervised the experimental design and the data analysis. SB, GN, BL, PR, RK, RLG, APruvost, APaoletti, JP, KF, FM and AG made the experiments. PD and AHM performed statistical analysis. MA, AG, SHBA and MP gave substantial contributions from the ABIRISK cohort. SB and XM wrote the original draft of the manuscript. All authors reviewed and edited the manuscript, and provided final approval of the version published.

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Competing interests KF is an employee of GSK, PD is employed by SciCross and AHM is an employee of Sanofi.

Patient consent Not required.

Ethics approval The mouse study was approved by the regional Animal Care and Ethics Committee (Comité Régional d'Ethique sur l'Expérimentation Animale Île de France Sud, Fontenay-Aux-Roses, France; decision number 4281). Animal care and use was in accordance with the EU Directive 2010/63/EEU. The macaque study was approved by the regional Animal Care and Ethics Committee (Comité Régional d'Ethique sur l'Expérimentation Animale Île de France Sud, Fontenay-Aux-Roses, France; decision number A15_016). The CEA Institute was approved as compliant with ETS123 recommendations for animal breeding (European Union Directive 2010/63/EU, 22 September 2010) and with Standards for Human Care and Use of Laboratory Animals (Animal Welfare Assurance, OLAW No A5826-01). The study was also approved by the French department of education and research (MENESR; study number 2015070114504151v3) as defined in French law 'décret 2013-118 from 2013 Feb 1st'. The human study was approved by the local IRB named 'Paris Ile de France VII' under number 13-048 and by the French agency for drugs (ANSM) under number 2013-A01268-37. The ABIRISK study was registered as study NCT02116504 by ClinicalTrials.gov.

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EXTENDED REPORT

Restricted immune activation and internalisation of anti-idiotype complexes between drug and antidrug antibodies

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ABSTRACT

Objectives Therapeutic antibodies can provoke an antidrug antibody (ADA) response, which can form soluble immune complexes with the drug in potentially high amounts. Nevertheless, ADA-associated adverse events are usually rare, although with notable exceptions including infliximab. The immune activating effects and the eventual fate of these 'anti-idiotype' complexes are poorly studied, hampering assessment of ADA-associated risk of adverse events. We investigated the in vitro formation and biological activities of ADA-drug anti-idiotype immune complexes using patient-derived monoclonal anti-infliximab antibodies.

Methods Size distribution and conformation of ADA-drug complexes were characterised by size-exclusion chromatography and electron microscopy. Internalisation of and immune activation by complexes of defined size was visualised with flow imaging, whole blood cell assay and C4b/c ELISA.

Results Size and conformation of immune complexes depended on the concentrations and ratio of drug and ADA; large complexes (>6 IgGs) formed only with high ADA titres. Macrophages efficiently internalised tetrameric and bigger complexes in vitro, but not dimers. Corroborating these results, ex vivo analysis of patient sera demonstrated only dimeric complexes in circulation. No activation of immune cells by anti-idiotype complexes was observed, and only very large complexes activated complement. Unlike Fc-linked hexamers, anti-idiotype hexamers did not activate complement, demonstrating that besides size, conformation governs immune complex potential for triggering effector functions.

Conclusions Anti-idiotype ADA-drug complexes generally have restricted immune activation capacity. Large, irregularly shaped complexes only form at high concentrations of both drug and ADA, as may be achieved during intravenous infusion of infliximab, explaining the rarity of serious ADA-associated adverse events.

INTRODUCTION

Millions of patients are nowadays treated with therapeutic monoclonal antibodies that can be immunogenic. Antidrug antibody (ADA) formation is a concern during the development of any biopharmaceutical; the potential impact of immunogenicity is highlighted by the recent discontinuation of the PCSK9 inhibitor bococizumab, which was partly due to immunogenicity issues, despite promising clinical results.[1]

For efficacy and safety reasons, immunogenicity assessment of therapeutic protein products, including monoclonal antibodies, is presently scrutinised by regulatory agencies such as the Food and Drug Administration. High ADA titres towards therapeutic proteins are associated with lower efficacy and reduced clinical response, although low amounts of ADA have little apparent consequences.[2–6] Concerning safety, serious ADA-induced adverse events have been reported to various therapeutic proteins; erythropoietin (EPO) likely being the most notorious example, with cases of ADA formation cross-reacting to endogenous EPO leading to pure red cell aplasia.[7]

For therapeutic antibodies, ADA formation may also positively correlate with incidence of adverse events. Particularly for infliximab and natalizumab, the risk of acute hypersensitivity reactions is increased in ADA-positive patients.[8 9] However, for the majority of monoclonal antibodies, ADA positivity does not increase the occurrence of (severe) adverse events even in cases where ADA formation is clearly associated with diminished clinical response (eg, adalimumab[2 10]). A better understanding on the possible biological consequences of ADA would aid in assessment of immunogenicity-related risk and guidance of strategies for immunogenicity testing.

In ADA-positive patients, immune complexes between ADA and drug will form in vivo on treatment. The contribution of these complexes to drug clearance and immune-mediated adverse events is not clear, but is expected to depend—among others—on size and shape. Therapeutic antibodies like adalimumab, infliximab or natalizumab predominantly induce ADA responses towards the antigen binding site (or idiotype),[9 11 12] thereby limiting the number of ADA molecules that may bind each Fab arm to just one.[13] Consequently, therapeutic antibodies may be expected to bind to ADA—of which IgG is the major isotype[14–16]—in alternating fashion, forming circular or string-shaped immune complexes such as dimers, tetramers and hexamers (see lower panels of figure 1B).

Several studies investigated the size of these anti-idiotype complexes. Dimer-sized complexes were found in the majority of ADA-positive patients

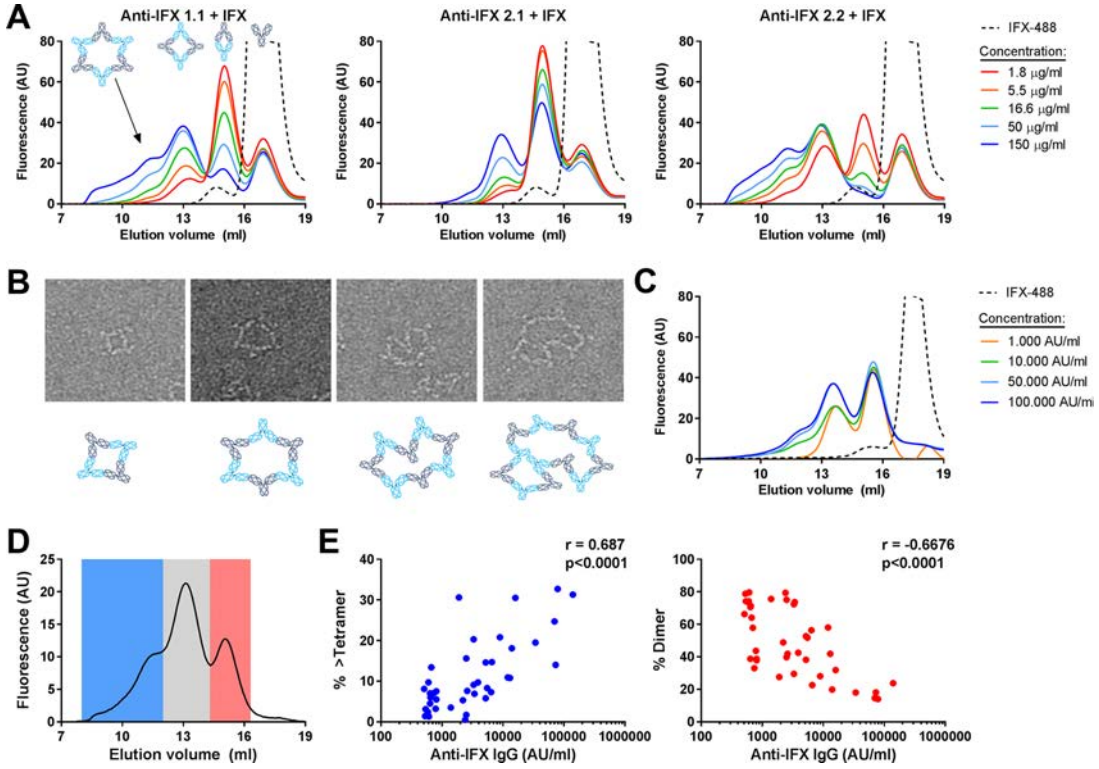

Figure 1 Immune complex size is highly dependent on concentration. (A) Infliximab (IFX)-488 and different monoclonal anti-IFX antibodies were mixed at increasing concentrations in a 1:1 ratio. Afterwards, samples were diluted so that each sample contained an equal amount of labelled drug, and analysed by high-performance size-exclusion chromatography (HP-SEC). (B) Transmission electron microscopic (TEM) analysis of complexes between monoclonal anti-IFX 2.2 and IFX at 150 µg/mL, and schematic representation of their conformation. See also online supplementary figure S5. (C) A titration of polyclonal anti-IFX antibodies from a single patient mixed with an equimolar amount of IFX-488, analysed by HP-SEC. n=3 separate experiments. (D, E) Serum of anti-IFX-positive patients was mixed with an equimolar amount of IFX-488 and analysed using HP-SEC. (D) Example of the elution pattern of one patient serum. Percentage of dimers (red box) and complexes bigger than tetramers (blue box) was determined by dividing the area under the curve (AUC) of these complexes by the total AUC. (E) Percentage of specified complexes was correlated to the anti-IFX IgG titre for each patient (n=41). Spearman's r for >tetramers: r=0.687, p<0.0001 and for dimers: r=−0.6676, p<0.0001.

treated with adalimumab 2 weeks after adalimumab administration.[12 17 18] In cynomolgus monkeys treated with infliximab, dimers as well as complexes larger than 670 kDa (>4 antibodies) could be demonstrated.[19] Infusion of (radiolabeled) infliximab into three ADA-positive patients led to dimer-sized complexes in two cases, without adverse events. However, complexes larger than 1000 kDa (>6 antibodies) were detected in a third patient that developed a severe infusion reaction, tentatively relating immune complex size to the adverse event.[20]

The differential effects of ADA on the occurrence of adverse events indicate that ADA-drug complexes are not invariably benign or harmful. However, for intravenously administered antibodies such as infliximab and natalizumab, high peak concentrations in serum of about 100–150 µg/mL are achieved, corresponding to roughly 1% of total serum IgG.[21 22] If ADAs are present in serum, administered drug will quickly be bound upon infusion, leading to rapid formation of soluble IgG complexes, potentially reaching high concentrations in case of high ADA titres.

In this study, patient-derived human monoclonal anti-infliximab antibodies are used to investigate the interplay between concentration and binding characteristics of ADA and drug, and the formation of different types of anti-idiotype immune complexes. Furthermore, the immune activating potential and clearance of ADA/drug complexes is investigated. The results of this study may advance the immunogenicity-related risk assessment of (new) therapeutic antibodies.

METHODS

See online supplementary materials and methods.

RESULTS

A panel of patient-derived recombinant monoclonal anti-infliximab antibodies was produced.[23] All monoclonal antibodies competed with tumour necrosis factor (TNF) for binding infliximab, as determined with a TNF competition assay (online supplementary table S1).[24] This indicates that, similar to polyclonal anti-infliximab antibodies,[24] they bind (close to) the drug's idiotype.

In vitro, size of monoclonal and polyclonal complexes depends on concentration and ratio

We sought to investigate which factors influence the size of immune complexes. High-performance size-exclusion chromatography (HP-SEC) analysis of monoclonal anti-infliximab clones combined with infliximab in a 1:1 molar ratio demonstrated different propensities of each anti-infliximab clone to form dimers, tetramers, hexamers and bigger complexes (online supplementary figure S1). Furthermore, for all ADA clones, the size of immune complexes depended highly on the concentrations in which drug and ADA were mixed. At a 1:1 ratio, higher concentrations resulted in larger complexes, whereas dimers eventually became the predominant type of complex at very low concentrations, regardless of the clone (figure 1A). This

concentration dependency was corroborated by a similar titration of polyclonal anti-infliximab antibodies from a patient's serum (figure 1C). Additionally, sera of 41 patients with anti-infliximab levels ranging from 510 to 140 000 AU/mL (median 1900 AU/mL) were mixed with an equimolar amount of infliximab and the size distribution of complexes was analysed (figure 1D). A strong correlation was found between the titre and the percentage of complexes larger than tetramers, whereas an inverse correlation was found for dimers (figure 1E).

During infusion, ADA and drug are not immediately present in equimolar concentrations. We therefore tested the influence of different antibody/drug ratios. As expected,[25] deviation of the 1:1 molar ratio caused a reduction of complex size (online supplementary figure S2A,B). A similar concentration and ratio dependency of immune complex size was also observed by combining monoclonal or polyclonal anti-adalimumab antibodies with adalimumab (online supplementary figure S3A,B), indicating that these observations are a general phenomenon for anti-idiotype complex formation.

Since HP-SEC analysis could not accurately resolve complex sizes larger than hexamers, monoclonal antibody complexes were additionally analysed with asymmetric flow field flow fractionation (AF4). Again, no distinct peaks of complexes larger than hexamers were observed (online supplementary figure S4), suggesting that they vary highly in their size and conformation. Transmission electron microscopic (TEM) analysis of these complexes supported this observation. Importantly, the conformation of complexes as shown by TEM indicated Fab-Fab interactions (figure 1B, online supplementary figure S5), in line with the anti-idiotypic nature of the interactions formed between ADA and drug.[9 11 12] Thus, ADAs towards infliximab and adalimumab form circular or string-shaped anti-idiotype immune complexes, the size of which is influenced by both the concentration and ratio of ADA and drug.

Ex vivo, large immune complexes are absent in serum

Upon infusion of drug, ADA-positive patients will form immune complexes in vivo. We sought to determine which complexes are still present ex vivo just before the next infusion (ie, trough level). Trough sera of patients with varying anti-infliximab titres (range 12–70 000 AU/mL, median 40 AU/mL) were fractionated and tested for presence of anti-infliximab. No large complexes were detected in any of the sera (figure 2A and online supplementary figure S6), although they could be observed in monoclonal antibody complexes subjected to the same fractionation procedures (figure 2B). However, small complexes resembling dimers were found in patients with low anti-infliximab titres.

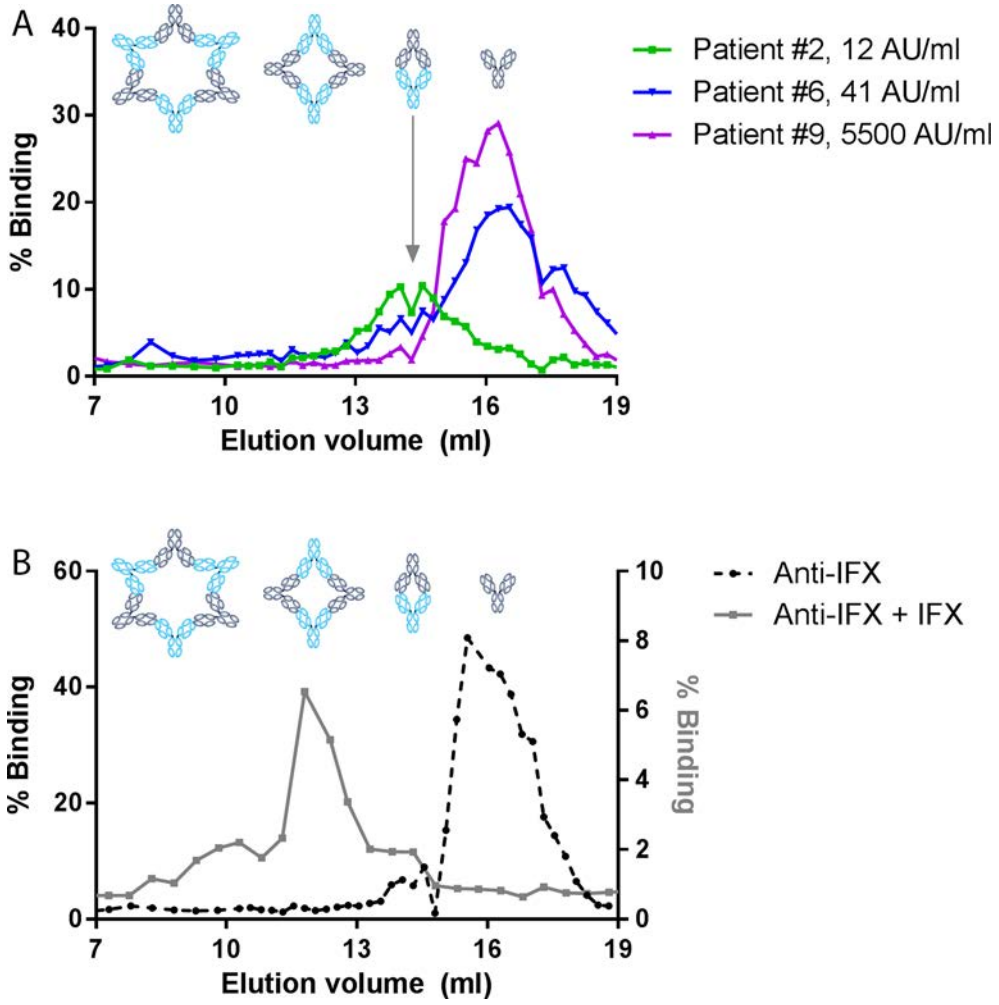

Figure 2 Only small complexes and monomeric anti-infliximab antibodies are detected in patients at trough. Anti-infliximab antibody presence was determined with a drug-tolerant assay in (A) fractionated anti-infliximab positive sera and (B) fractionated monomeric anti-infliximab 2.1 and complexes of anti-infliximab 2.4 and infliximab. Representative plots are shown for 3 out of 10 patients (A) and duplicate measurements of monoclonal complexes (B). Percentage binding is the sample measurement normalised to the total radioactively labelled input. IFX, infliximab.

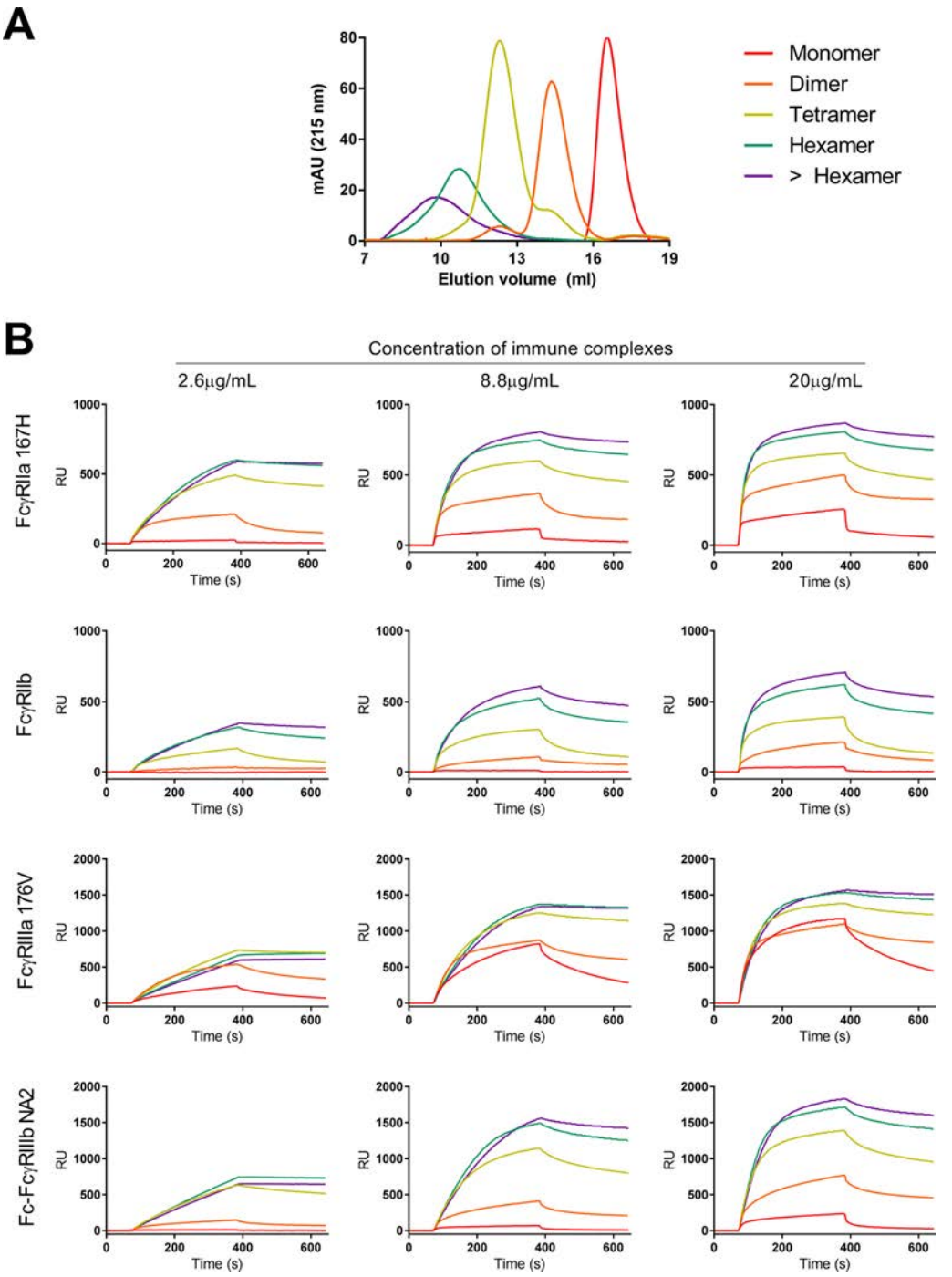

Figure 3 Binding to FcγRs increases with complex size. (A) Example of reanalysed fractions of immune complexes, done with high-performance size-exclusion chromatography (HP-SEC). (B) Representative sensograms of immune complexes binding to FcγRIIa, FcγRIIb, FcγRIIIa and FcγRIIIb on the chip. Receptor densities of shown sensograms are 10 nM for FcγRIIa and FcγRIIb, 3 nM for FcγRIIIa and 30 nM for FcγRIIIb, n=3 individual experiments. RU, response unit.

Large immune complexes are phagocytosed by macrophages

The discrepancy between the formation of large complexes in vitro and their absence ex vivo suggests that these complexes are preferentially and rapidly cleared in vivo. Since clearance is likely to occur via FcγRs, we first tested the binding of fractionated complexes (dimers, tetramers, hexamers or larger complexes; figure 3A) to different FcγRs immobilised on a biosensor chip using surface plasmon resonance. As expected, dimeric complexes showed enhanced binding compared with monomeric IgG for all receptors tested, and larger complexes bound substantially stronger, largely due to a further reduction of the dissociation rate (figure 3B).

Next, we evaluated FcγR-mediated phagocytosis by monocyte-derived macrophages in vitro. Of note, the exact immune complex size required for phagocytosis has not been identified so far. We generated three types of complexes (figure 4A): (A) virtually pure dimeric complexes using the anti-adalimumab clone 2.7, which has a strong preference for making dimers with adalimumab[13]; (B) pools of dimers and tetramers using anti-infliximab 2.1; and (C) pools containing significant amounts of

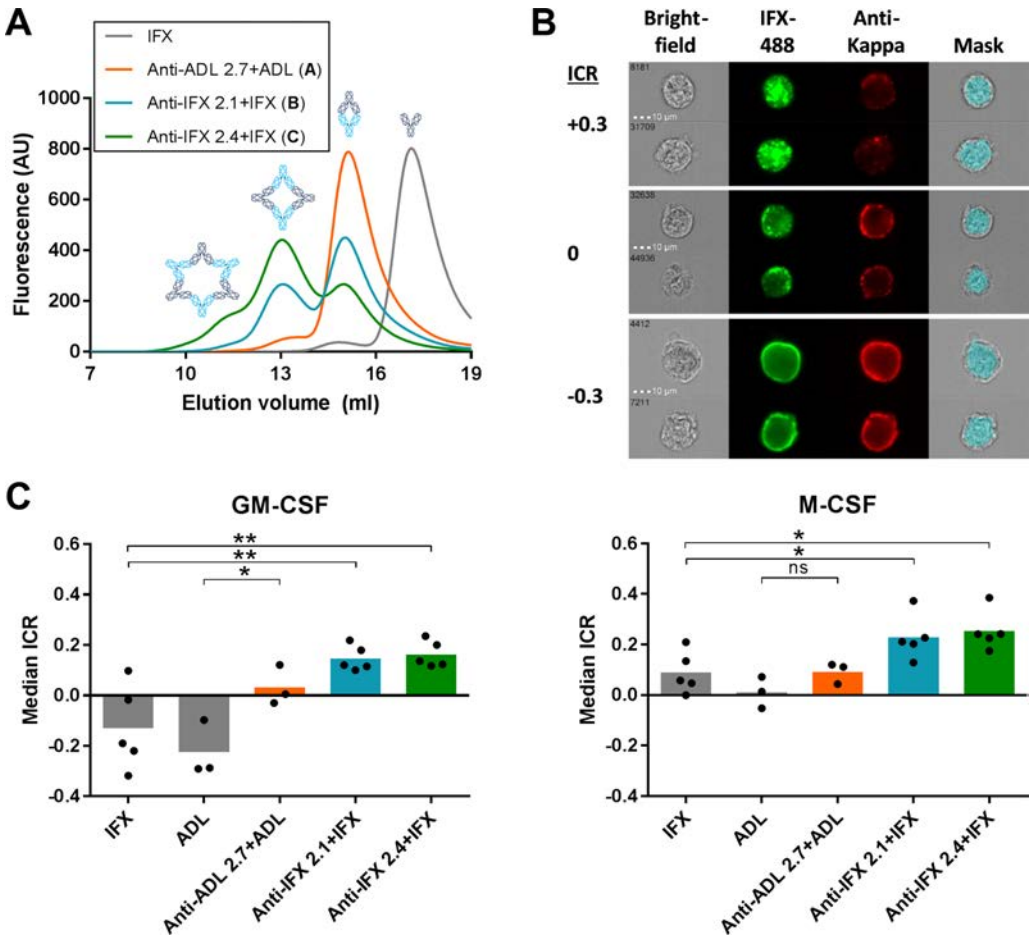

Figure 4 Complexes larger than dimers are efficiently internalised by macrophages. (A) Example of the fluorescent complexes used. (B) Representative examples of three different intensity concentration ratio (ICR) values. The ICR calculates the logit-transformed ratio between the fluorescent intensity inside the cell and the fluorescent intensity of the entire cell. From left to right: bright-field image, green fluorescence of infliximab (IFX)-488 (in complex with anti-IFX), red fluorescence of secondary staining with anti-kappa antibodies (not used in analysis), mask (blue) to determine the inside of a cell. (C) Median ICR of monomers, dimers or pools of larger immune complexes, determined for GM-CSF (left) or M-CSF (right) macrophages after 75 min. n≥3 individual donors measured in duplicate. Statistical differences were calculated by one-way analysis of variance (ANOVA), with Sidak's multiple comparison test; * and ** denote a statistical significance of p≤0.05 and p≤0.01, respectively. ADL, adalimumab; GM-CSF, granulocyte-macrophage colony stimulating factor; M-CSF, macrophage colony stimulating factor; ns, not significant.

large complexes (>tetramers) using anti-infliximab 2.4. Uptake of complexes by monocyte-derived macrophages polarised with granulocyte-macrophage colony stimulating factor (GM-CSF) (proinflammatory phenotype) or macrophage colony stimulating factor (M-CSF) (anti-inflammatory phenotype) was analysed using flow imaging (figure 4B,C). Monomeric infliximab and adalimumab were not phagocytosed by GM-CSF macrophages and only slightly by M-CSF macrophages. Interestingly, also dimers (pool A) were not efficiently internalised. However, efficient internalisation was observed for tetramers (pool B) and bigger complexes (pool C; see also online supplementary figure S7 for time-lapse confocal imaging). Blocking of all FcγRs strongly inhibited binding and/or phagocytosis of infliximab and complexes (online supplementary figure S8A). Immune complex phagocytosis was predominantly mediated by FcγRI in GM-CSF and by FcγRII in M-CSF macrophages, likely due to differential FcγR expression on these cells (online supplementary figure S8B). Together, these results show that tetramers and larger complexes are phagocytosed in an FcγR-mediated fashion, but internalisation is much less efficient for dimers, consistent with the serological analysis shown in figure 2A.

Immune complexes do not activate immune cells

During intravenous administration of drug, complex formation occurs in the blood and the primary effects of antibody complexes are thus likely to happen within the blood circulation. We therefore investigated a possible proinflammatory effect of immune complexes in a whole blood cell activation assay, using interleukin (IL)-6 production as read-out for activation. However, even very large complexes (anti-infliximab 2.4+infliximab made at 450 µg/mL, concentrations exceeding those achieved in patients) were not able to induce IL-6 production at 20 µg/mL (data not shown) nor at 50 µg/mL (figure 5). In contrast, biotinylated beads opsonised with anti-biotin antibodies strongly induced IL-6 production. These results demonstrate that anti-idiotype antibodies have little proinflammatory capacity, possibly due to their size and/or conformation.

Complement activation requires anti-idiotype complexes larger than hexamers

The lack of IL-6 production in whole blood cell cultures does not exclude the possibility that circulating immune complexes can activate the complement system. We therefore also tested pools

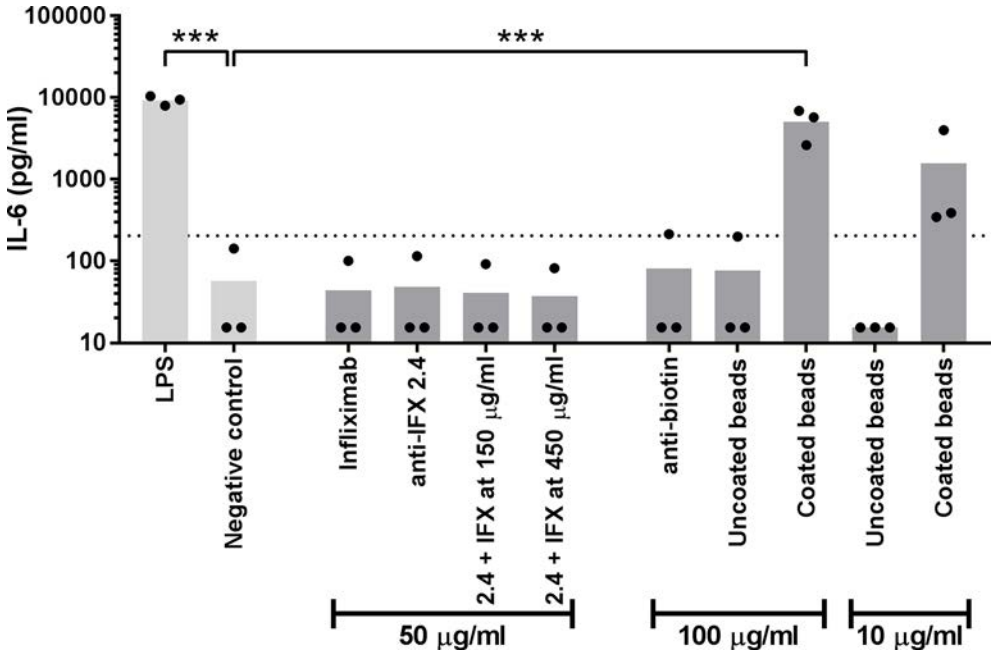

Figure 5 Large immune complexes do not have a proinflammatory effect on whole blood cell (WBC) cultures. Interleukin (IL)-6 production by WBC cultures was determined after addition of monomeric antibodies or large immune complexes (infliximab+anti-infliximab 2.4 mixed at 150 and 450 µg/mL of both antibodies). All samples were tested at 50 µg/mL of each antibody in 10-fold diluted blood, giving a supratherapeutic concentration. Anti-biotin-coated beads (100 or 10 µg/mL) and lipopolysaccharide (LPS) were used as positive control, WBC medium was used as negative control. Statistical differences were calculated by one-way analysis of variance (ANOVA), and further compared with the negative control by using Dunnett's multiple comparison test; *** denotes a statistical significance of $p \leq 0.001$. Unless indicated, no significant differences were found. Cut-off (dotted line) was set at the mean plus twice the SD of the negative control. IFX, infliximab.

of small and large anti-idiotype complexes for their capacity to activate complement. Pools of (predominantly) dimers and tetramers (anti-infliximab 2.1+infliximab) did not activate complement (figure 6A). However, pools containing—in addition to tetramers—hexamers and a variety of bigger complexes (anti-infliximab 2.4+infliximab) gave significant activation of the complement system. As expected, strong complement activation was also observed for the antibody-coated beads used in the whole blood cell assay (online supplementary figure S9).

To more specifically pinpoint the size of anti-idiotype complexes required for complement activation, we fractionated the pools of complexes to obtain samples with only dimers, tetramers, hexamers or complexes larger than hexamers (high molecular weight, HMW), and tested their individual activation capacity. No activation was seen for hexamers and smaller complexes, but a (non-significant) trend towards complement activation was found for the complexes with the highest molecular weight, suggesting that anti-idiotype complexes should be (substantially) larger than hexamers to activate complement (figure 6B). To exclude the possibility that abnormal Fc glycosylation of recombinant antibodies reduced the complement activating potential, Fc glycopeptides of all antibodies were analysed (online supplementary figure S10). However, the slight differences that were found compared with IVIg could not explain the lack of complement activation.[26]

Recently it was shown that RGY-mutant antibodies, which have a triple-mutated Fc, form fluid phase Fc-Fc interacting hexamers mimicking similar hexameric structures formed by IgG on opsonising a cellular target. These hexameric complexes potently activate the complement system via C1q.[27 28] Since our anti-idiotype hexamers do not activate complement, we investigated the potency of Fc-Fc interacting hexamers of IgG1-b12-RGY in our system, and indeed found strong complement activation (figure 6B). The contrasting results between anti-idiotype and Fc-Fc interacting hexamers strongly suggest that the conformation of immune complexes is crucial for complement activation.

DISCUSSION

Immunogenicity of therapeutic monoclonal antibodies is a recognised problem, and the relation between ADA formation and reduced efficacy has been demonstrated in numerous studies. Nevertheless, few studies addressed the pathophysiological effects of these immune responses, and therefore the impact of ADA on safety remains largely unknown. In this study, we investigated the factors influencing ADA-drug complex formation and determined the biological activities of these complexes. Data presented in this paper show for the first time that anti-idiotype complexes with distinct conformations are formed between drug and ADA, and that the majority of these complexes have a restricted capacity to cause immune activation. This would imply that for many therapeutic antibodies an ADA response will not ordinarily result in adverse events, given that such responses tend to predominantly consist of anti-idiotype antibodies.[9 11 12]

Antibody concentration and ADA/drug ratio were found to greatly influence complex size in patient sera. Although large complexes were formed in vitro, these were not detected ex vivo in trough serum samples of infliximab-treated patients. The rapid phagocytosis of tetramers and larger complexes by macrophages suggests that these complexes are cleared in vivo. On the other hand, our study shows that dimers can persist in circulation for prolonged times, in line with previous observations from our group for adalimumab.[12] Their impaired internalisation by macrophages suggests that dimers are too small to be efficiently cleared.

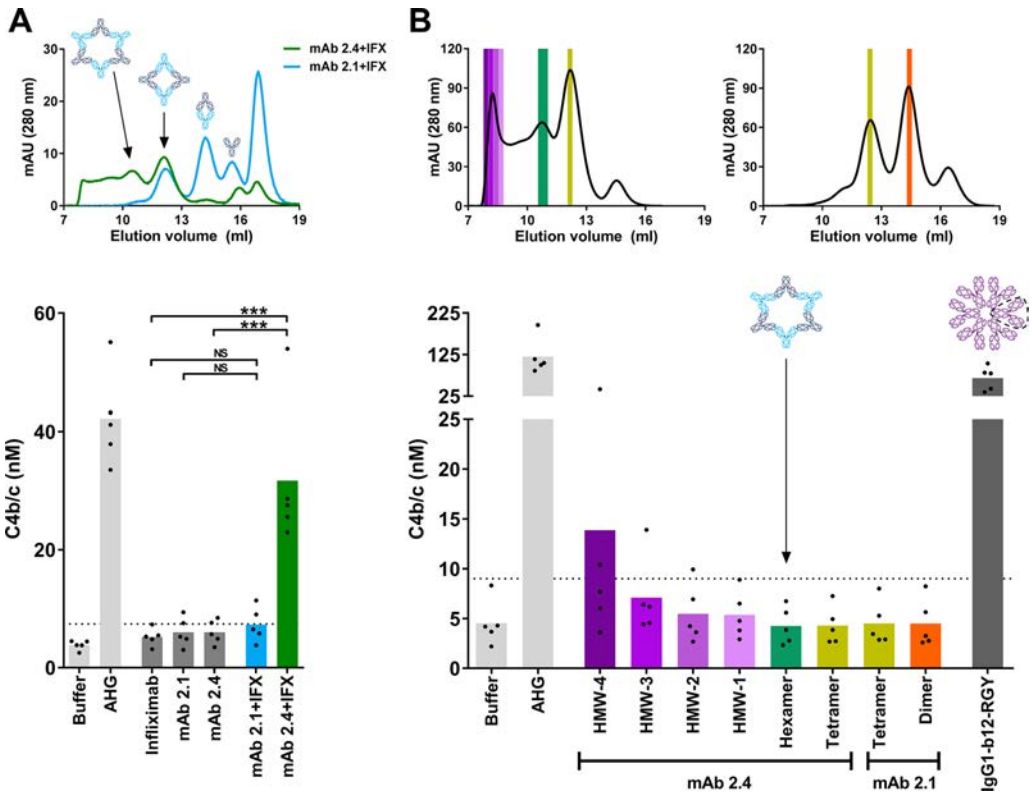

Figure 6 Anti-idiotype complexes larger than hexamers are required for activation of the complement system. (A) Representative elution patterns of complexes between infliximab with either anti-infliximab 2.1 or 2.4 made at 150 and 450 µg/mL of both antibodies, respectively. Bottom: complexes (final antibody concentration 100 µg/mL in serum) were tested in the C4b/c ELISA. n=5 serum donors, each dot represents the mean of two to five experiments per donor. Significant differences were calculated by one-way analysis of variance (ANOVA), with Sidak's multiple comparison test; *** denotes a statistical significance of p≤0.001. (B) Representative elution patterns of immune complexes consisting of infliximab with either anti-infliximab 2.4 (upper left) or 2.1 (upper right). Selected fractions are shown in coloured boxes. Bottom: all fractions and IgG1-b12-RGY were separately tested in the C4b/c ELISA (final antibody concentration 15 µg/mL in serum). n=5 serum donors, representative of ≥4 individual experiments. Buffer was used as negative control and aggregated human gammaglobulin (AHG) as positive control. Dotted line is twice the mean buffer value. Similar colours in top and bottom figures show corresponding samples. HMW, high molecular weight; IFX, infliximab; ns, not significant.

The valency required for FcγR-mediated inhibition of phagocytosis was also investigated by Ortiz *et al*,[29] showing that Fc trimers were not internalised by FcγR expressing THP-1 cells, in contrast to Fc pentamers. Our study now suggests that at least four Fc moieties are required for efficient internalisation.

Contrary to their findings, our results suggest a clear dependency on immune complex conformation, in addition to mere Fc-tail valency, as an important factor determining the immune-activating properties of an immune complex (see below).

We only observed complement activation for the very large—irregularly shaped—anti-idiotype complexes, whereas hexamers and smaller complexes did not activate complement. It has been known for decades that the classical pathway of the complement cascade is activated by multimerisation of antibodies on a surface or in fluid phase, but the detailed molecular mechanisms are only now being fully elucidated. In older studies, chemically cross-linked IgG forming dimers, trimers and tetramers were found to increasingly activate complement.[30 31] However, it is unclear to which extent these artificial complexes resemble complexes formed during an actual immune response. More recently, six Fc-Fc interacting antibodies were found to be the optimal amount for C1q docking and activation with the Fc tails pointing inward forming a hexameric structure also sometimes observed in crystal structures of human IgG.[27 28] This provides a platform for C1q to dock onto. Interestingly, the anti-idiotype complexes generated by ADA and drug have a completely opposite conformation compared with the Fc-Fc interacting hexamers: since ADA and drug interact through their Fab arms, the Fc tails will point outward. Therefore, ADA-drug-induced hexamers, but also dimers and tetramers, do not form a structurally optimal C1q platform like Fc-Fc interacting hexamers, probably explaining their lack of complement activity. Upon generation of larger complexes than hexamers the conformation is likely to become more disorganised (also suggested by our AF4 and TEM results), allowing some Fc tails to come into closer contact, thereby serving as a more optimal C1q docking platform. Large disorganised complexes may therefore activate complement to a certain extent, but this is expected to be restricted to situations of high ADA titres.

For infliximab[8 21 32] and natalizumab,[9 33] clinical studies show that ADA positivity and high ADA titres[34] are associated with infusion reactions. These encompass all symptoms that occur during or shortly after infusion, and may be mild (eg, dizziness, nausea), moderate (eg, chest tightening, urticaria) or severe (eg, significant hyper/hypotension, stridor).[35] While these symptoms resemble those of an IgE-mediated type I hypersensitivity, the vast majority of patients experiencing an infliximab-induced infusion reaction are negative for IgE anti-infliximab,[23 36 37] and no elevated tryptase levels are found in infusion reaction positive+ patients.[35 38] By contrast, our study provides a mechanistic link between high IgG ADA titres and the formation of

large, irregularly shaped immune complexes that—under privileged circumstances—can activate complement. Our results thus suggest that, instead of an IgE-mediated response, infusion reactions may represent type III hypersensitivity.

Anti-infliximab/adalimumab antibodies are largely (>90%)[24] anti-idiotype antibodies. We here demonstrate that ADA-drug complexes formed with anti-idiotypic antibodies have restricted immune activating capacities. It however remains unknown to what extent the small fraction of non-anti-idiotypic ADA influences this restricted activation.

During infusion of drug in ADA-positive patients, drug is effectively titrated to ADA in vivo. The concentration of ADA at the site of infusion and the infusion rate will influence the type of complexes that are formed. At first, ADA will theoretically be in excess, probably resulting predominantly in smaller, trimeric complexes (ie, one drug and two ADA molecules). However, when a ratio approaching equimolarity is reached, larger complexes will be formed. The concentration of both antibodies at equimolarity will determine the size of the complexes and their (clinical) effects. The current management to ameliorate infusion reactions by reducing the infusion speed may impact the effective (local) peak drug concentrations, thereby reducing the risk of a severe infusion reaction. In contrast to intravenous administered therapeutics, subcutaneous administration leads to a local high concentration of drug, thereby taking the ADA-drug ratio far from equimolarity. Although little is known about their actual in vivo formation, the formed complexes are expected to be small, and are likely even more slowly distributed than monomeric drug molecules.[39] These properties may prevent systemic reactions in a dual manner.

As shown in this study, immune complexes larger than dimers are efficiently taken up by macrophages in vitro. ADA formation thus may reduce the drug concentration in vivo,[19] but this inherently also reduces the ADA concentration. This complicates the assessment of immunogenicity, since one cannot measure ADA that is already cleared. So, immune complex clearance can lead to underestimation of the immunogenicity of antibody therapeutics. In any case, these results warrant caution against interpretation of quantitative aspects of ADA formation, and show that even multitiered testing approaches may not reveal the full extent of an ADA response.

Taken together, immune complex formation between ADA and drug results in large immune complexes when the concentration of both antibodies is high, but the anti-idiotype conformation limits the formation of complexes capable of complement or immune cell activation. Very large immune complexes may be formed and adverse events may occur in those cases where ADA levels are very high and the administered amounts of drug as well (upward of ca 50–100 µg/mL), typically only during intravenous administration.

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Contributors KAvS, SK, POdH, NILD, FSvdB, AS, AEHB, SR, EMS, CAMK, MW and EB performed the experiments, analysed the data and helped revise the manuscript. GV, AEHB, WJ, SR, RIK, GW and TR designed the experiments. KAvS, SK, GV, WJ, SR, GW and TR contributed ideas. GV, AEHB, WJ, SR, RIK, FSvdB and GW helped write the manuscript. KAvS, GW and TR designed the study. KAvS and TR wrote the paper.

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EXTENDED REPORT

Apolipoprotein B binds to enolase-1 and aggravates inflammation in rheumatoid arthritis

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ABSTRACT

Objective Immune cells from patients with rheumatoid arthritis (RA) express more enolase-1 (ENO1) on their surface than those from healthy subjects, and they elicit an enhanced inflammatory response. This study is aimed to identify the ligands of ENO1 that could promote inflammatory loops in vitro and enhance the arthritis severity in vivo.

Methods ENO1-binding proteins in RA synovial fluid were identified by mass spectrometry, and affinity to ENO1 was evaluated by means of a ligand blotting and binding assay, surface plasmon resonance and confocal microscopy. Proinflammatory response by the interaction between ENO1 and apolipoprotein B (apoB) was tested in vitro and in vivo using peripheral blood mononuclear cells and a K/BxN serum transfer arthritis model and low-density lipoproteins receptor (LDLR) knockout mice.

Results ApoB in the synovid fluid of patients with RA was identified as a specific ligand to ENO1 with a higher affinity than plasminogen, a known ENO1 ligand. ApoB binding to ENO1 on monocytes elicited the production of tumour necrosis factor-α, interleukins (IL)-1β and IL-6 through both p38 mitogen-activated protein kinase and NF-κB pathways. In the K/BxN serum transfer arthritis model, administration of apoB increased the production of proinflammatory cytokines and exaggerated arthritis severity. The severity of K/BxN serum transfer arthritis in LDLR knockout mice was comparable with wild-type mice.

Conclusions A key component of atherogenic lipids, apoB, aggravated arthritis by potentiating the inflammatory response *via* its interaction with ENO1 expressed on the surface of immune cells. This suggests a novel mechanism by which lipid metabolism regulates chronic inflammation in RA.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterised by persistent synovitis with joint destruction, systemic inflammation and autoantibody production.[1] Infiltrating macrophages and lymphocytes in inflamed synovium produce proinflammatory cytokines including tumour necrosis factor (TNF)-α, interleukin (IL)-1, IL-6 and IL-18, which contribute to articular and extra-articular inflammation.[2 3] The activity of these immune cells is largely regulated by a fine balance between stimulatory and inhibitory receptors on the cell surface. Previously, enolase 1 (ENO1) has been identified as a novel stimulatory receptor on monocytes.[4]

ENO1 is a multifunctional glycolytic enzyme that is expressed in the cytoplasm of all cells.[5] Surface expression of ENO1 can be markedly upregulated by its translocation to the cell surface during immune cell activation.[6–8] Previously, we reported that peripheral blood mononuclear cells (PBMCs) from patients with RA expressed more ENO1 on their surface compared with PBMCs from healthy controls (HCs) or patients with osteoarthritis (OA) and those cells elicited an enhanced inflammatory response after stimulation with anti-ENO1 antibody.[4] However, physiological and pathological ligands of surface ENO1 have not yet been fully elucidated, and the mechanisms by which ENO1 may drive the inflammatory process in arthritis are largely unknown.

Therefore, we sought to identify and characterise ligands of ENO1, using plasminogen, a known ENO1 ligand as a control, to help explain the role of ENO1's increased cell surface expression in inflammatory cells from patients with RA. We identified the low-density lipoprotein (LDL) component apolipoprotein B (apoB) as a novel and specific ligand of ENO1 and demonstrated that it aggravated arthritis by potentiating the inflammatory response *via* its interaction with ENO1 expressed on the surface of immune cells.

METHODS

Patient samples

RA was diagnosed according to 2010 American College of Rheumatology/European League Against Rheumatism classification criteria.[9] Peripheral blood was collected from HC (n=9) and patients with RA (n=38). Synovial fluid was obtained from patients with RA (n=5) and patients with OA (n=5).

PBMCs were isolated from heparinised blood by density gradient centrifugation using Ficoll-Paque Plus gradient (GE Healthcare Biosciences, Uppsala, Sweden). CD14-positive monocytes were isolated from PBMCs of patients with RA using a monocyte isolation kit II (MACS, Miltenyi Biotech, California, USA).

Affinity purification of ENO1-binding proteins

A 2 mg/mL of EZ-Link sulfo-NHS-SS-Biotin solution (Thermo Scientific, Rockford, Illinois, USA) was added to 1 mg/mL human recombinant ENO1 (Prospec, Ness-Ziona, Israel) and incubated for 10 min at 4°C. Free biotin was removed from biotinylated ENO1 using centrifugal filter units (Millipore, Masschusetts, USA). Pooled synovial fluid of patients with RA or patients with OA was incubated with biotinylated ENO1. Avidin agarose beads

(Thermo Scientific) were added to each pooled synovial fluid mixture and rotated for 2 hours at 4°C. After rinsing with phosphate buffered saline (PBS), ENO1-binding proteins were eluted by heating with 50 mM dithiothreitol (Sigma-Aldrich, Missouri, USA) in LDS sample buffer (Invitrogen, California, USA) at 70°C for 10 min.

Mass spectrometry for identification of ENO1 ligands

The eluate of ENO1 binding proteins was separated on a 4%–12% gradient Bis-Tris NuPage gel (Invitrogen), and in-gel tryptic digestion was conducted following the established protocol.[10] A detailed description of 'Mass spectrometry for identification of ENO1 ligands' and 'Relative quantitation of ENO1-binding proteins' is provided in online supplementary methods.

Ligand blotting

Equal amounts of ENO1, apoB100 (Calbiochem, California, USA) and plasminogen (Molecular Innovations, Michigan, USA) were loaded on Bolt 4%–12% Bis-Tris Plus gels (Life Technologies, New York, USA). Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skim milk in PBS with 0.05% Tween-20 (PBST), membranes were incubated with ENO1 or without ENO1, followed by staining with mouse anti-ENO1 antibody (1:1000, Abnova, Taipei, Taiwan) and then horseradish peroxidase (HRP)-labelled antimouse IgG antibodies (Jackson Immuno Research Laboratories, Pennsylvania, USA). Bands were visualised by enhanced chemiluminescence using Luminata Forte Western HRP Substrate (Millipore).

Ligand binding assay

A 96-well MaxiSorp microtiter plate (NUNC, Roskilde, Denmark) was coated with apoB or plasminogen (0.2–10 nM) in 50 mM sodium carbonate coating buffer (pH 9.6) at 4°C overnight. After washing with PBST and blocking with PBS containing 1% bovine serum albumin (BSA), 10 nM of ENO1 solution was added. Samples are then incubated with mouse anti-ENO1 antibody (1:1000 dilution) and HRP-conjugated antimouse IgG antibody (1:5000 dilution). Detection was performed using tetramethylbenzidine (KPL, Maryland, USA). Absorbance was measured at a wavelength of 450 nm using a microplate reader (VersaMax, Molecular Devices, California, USA).

Confocal microscopy

U937 cells were fixed with 4% paraformaldehyde for 15 min at room temperature (RT), blocked with 1% BSA in phosphate buffered saline (PBS) for 30 min at RT and incubated with apoB for 1 hour at RT. After cells were incubated with mouse anti-ENO1 antibody and goat anti-apoB antibody (Life Technologies) for 1 hour at RT, they were incubated with Alexa Fluor 488-conjugated donkey anti-mouse IgG (Life Technologies) and phycoerythrin-conjugated donkey antigoat IgG (BD Biosciences California, USA) for 1 hour at RT. Images were captured using a Leica TCS SP8 STED and analysed with Leica LAS AF Lite.

Surface plasmon resonance (SPR)

Immobilisation of ENO1 on a sensor chip CM5 (carboxymethylated dextran surface, BIAcore) was carried out using an amine coupling kit (BIAcore) in a continuous flow of HBS-N buffer (10 mM HEPES, PH=7.4) containing NaCl (150 mM). After immobilisation, various concentrations of the plasminogen or apoB (2.5–20 µg/mL) were injected for 120 s at a flow rate of 30 µL/min, and dissociation from the sensor surface was monitored for 480 s at the same flow rate. HEPES buffer (pH 8.0) was used as running buffer. Binding events were measured at 25°C. Data were analysed using BIAcore 3000 Evaluation software with a 1:1 binding model to calculate the association rate constant K_a ($M^{-1}S^{-1}$), dissociation rate constant K_d (S^{-1}) and equilibrium dissociation constant K_D for the interaction between ENO1 and apoB or plasminogen (GE Healthcare, Biosciences, Uppsala, Sweden).

ENO1 stimulation

PBMCs (5×10^6 cells/mL) from patients with RA or HC were stimulated with apoB protein in RPMI 1640 supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin and incubated for 24 hours at 37°C in a 5% CO_2 incubator.

siRNA knockdown of ENO1

All siRNAs were purchased from Santa Cruz Biotechnology (California, USA). The target sequence of the siRNA comprised a pool of three different siRNA duplexes for human ENO1: 5′-GAGAUGGAUG GAACAGAAAtt-3, 5′-GUACCGCUUCCU-UAGAAC Utt-3, and 5′-CGCUUCCUUAGAACUUCUAtt-3′. The sequence of the control siRNA was 5′-CCAGGGUUCCUAAU CGGAUUU GCUA-3′. The Neon transfection system (Thermo Fisher Scientific, USA) was used for transfection of siRNA into monocytes of RA in accordance with the manufacturer's instructions. As compared with the non-treated cells, 87.7% and 76.8% of monocytes were viable after transfection with control-siRNA and ENO1-siRNA, respectively.

Cytokine ELISA

IL-1β, IL-6 and TNF-α in supernatants were measured using cytokine ELISA kits (BD Biosciences). Mouse serum levels of IL-1β, IL-6 and TNF-α were measured using multiplex ELISA kits (Bio-Rad, California, USA).

Examination of signalling pathways

Specific inhibitors of NK-κB (BAY 11–7082), PI3K (LY294002), ERK (PD98059), p38 MAPK (SB203580) and JNK (SP600125) were purchased from Sigma-Aldrich. PBMCs from RA were pretreated with inhibitors or DMSO (negative control) for 1 hour. After washing with media, PBMCs were stimulated with apoB and incubated for an additional 24 hours. The levels of IL-1β and IL-6 were measured by ELISA, as described above.

Western blot analysis of phospho-p38 mitogen-activated protein kinase (MAPK)

After stimulation with apoB, proteins from cell extracts were separated on Bolt 4%–12% Bis-Tris Plus gels and transferred onto PVDF membranes. After blocking with 5% BSA in PBS, membranes were probed with anti-phospho-p38 MAPK antibody or p38 MAPK antibody at a 1:1000 dilution. The membranes were then incubated with HRP-labelled secondary antibody. Target proteins were visualised with enhanced chemiluminescence.

K/BxN serum transfer arthritis mouse experiments

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of our institution (IACUC No. 15–0058-C2A0). BALB/c mice were purchased from Orient Bio Inc. C57BL/6 wild-type mice were purchased from The Jackson Laboratory (Maine, USA).

To induce arthritis, 6-week-old male BALB/c mice were injected with 50 µL of K/BxN mice serum (provided by Professor Jeehee Youn at Han Yang University, Seoul, South Korea, with permission from Dr Diane Mathis) by intraperitoneal injection on day 0 as described previously.[11] ApoB was intravenously injected additionally on day 2 and day 7 after K/BxN serum administration.

C57BL/6 wild-type mice and low-density lipoproteins receptor (LDLR) knockout mice (provided by Professor Goo Taeg Oh at Ewha Womans University, Seoul, South Korea) were administered with K/BxN mice serum by intraperitoneal injection on days 0 and 4 to induce arthritis, and apoB was intravenously injected on day 2 and day 6.

Ankle thickness, arthritis scores and histological evaluation

The thickness of both ankles was measured every other day using a calliper. The arthritis score was determined for each paw using the following three-point scale (0 points: no swelling; 1 point: one digit swelling or mild swelling of the foot and ankle with the foot maintaining its original V shape; 2 points: long edges of the foot were parallel to each other, with disappearance of the normal V shape; 3 points: inversion of the V shape by expansion of the ankle and hindfoot to greater than the width of the forefoot). Scores of four limbs were added together to give an arthritis score with maximum 12 points. For histology evaluation, whole knee joint sections were stained with H&E. Six researchers who were blinded with regard to the study findings and sample identification evaluated knee joint sections by examining at 100× magnification.

Statistical analysis

All statistical analyses were performed using GraphPad Prism (V.5.01, La Jolla, California, USA). Results are presented as the means±SEM. Differences between two groups were assessed by the Mann-Whitney U test and Wilcoxon signed-rank test as appropriate. All reported p values were two sided. Statistical significance was considered at $p<0.05$.

RESULTS

Identification of ENO1 ligands in RA synovial fluid

To identify potential ligands in RA synovial fluid for ENO1, we first isolated proteins from synovial fluid of patients with RA or OA by affinity chromatography using ENO1 as a receptor and then performed mass spectrometry. Of the ENO1 binding proteins, 109 proteins were significantly enriched in RA synovial fluid compared with OA synovial fluid (signal to noise, signal to noise (STN) >7, p value <0.0005) (table 1). Plasminogen, a known ENO1 ligand, was enriched in RA synovial fluid (STN=7.29, p=0.0004), as expected. Among them, apoB was highly enriched (STN=34.71, p<0.0001), suggesting that it was a potential ligand for ENO1.

ApoB is a specific ligand of ENO1

To determine the interaction between ENO1 and apoB, we performed a ligand blotting assay in which immobilised apoB and plasminogen as a positive control were incubated with recombinant ENO1, followed by detection with an anti-ENO1 antibody. ENO1 bound to both apoB and plasminogen (figure 1A). In a ligand binding assay, ENO1 exhibited a higher affinity for apoB than for plasminogen (figure 1B). SPR analysis determined the association rate constant K_a of ENO1 with apoB as $1.499\times10^6\,M^{-1}S^{-1}$, which was fourfold greater than that of plasminogen ($K_a=3.559\times10^5\,M^{-1}S^{-1}$). The equilibrium dissociation constant K_D (binding affinity) of ENO1 with plasminogen was 1.505×10^{-7} M, which was threefold stronger than that of apoB ($K_D=4.694\times10^{-7}$ M) (figure 1C). Under fluorescence confocal microscopy, apoB and ENO1 colocalised on the cell surface of U937 cells, a human monocytic cell line with constitutive expression of ENO1 on the cell surface (figure 1D). Taken together, these results demonstrate that apoB is a specific ligand of ENO1 with stronger affinity than plasminogen.

ApoB mediates the production of proinflammatory cytokines

We examined the effects of apoB binding to ENO1 on inflammatory response. PBMCs isolated from patients with RA or HCs were treated with apoB for 24 hours. In response to apoB treatment, PBMCs from RA produced more IL-1β, IL-6 and TNF-α than PMBCs from HCs (figure 2A). When surface ENO1 expression was downregulated by transfection with ENO1-specific siRNA (online supplementary figure 1), production of proinflammatory cytokines by RA PBMCs in response to apoB stimulation decreased (figure 2B). Furthermore, apoB-induced IL-1β and IL-6 production was suppressed in the presence of ENOblock, a non-substrate analogue that can directly inhibit ENO1 activity[12] (figure 2C). Therefore, treatment of PBMCs with apoB induces proinflammatory response, and this response depends on expression of functional ENO1.

Downstream apoB signalling involves the p38 MAPK and NF-kB pathways

To elucidate the downstream signalling pathways of the aopB–ENO1 binding interaction, changes in the phosphorylation profiles of MAPKs and serine/threonine kinases in PBMCs from patients with RA incubated with apoB for 20 min were examined. The phosphorylation levels of ERK1, GSK3, MKK6, MSK2, p38 and RSK were increased in RA PBMCs after apoB treatment (figure 3A). Next, PBMCs from patients with RA were pretreated with signalling molecule inhibitors, such as LJ294002 (PI3K), PD98059 (ERK1/2), SB203580 (p38 MAPK), SP600125 (JNK) or BAY11-7082 (NF-κB), followed by stimulation with apoB for 24 hours. The production of IL-1β and IL-6 was suppressed by pretreatment with the p38 MAPK inhibitor and NF-κB inhibitor but not by the PI3K, ERK or JNK inhibitor (figure 3B,C). Accordingly, after stimulation with apoB, p38 MAPK phosphorylation increased in a time-dependent manner (figure 3D). These results suggest that surface ENO1 activation involves intracellular p38 MAPK and NF-κB pathways.

ApoB aggravates arthritis in a K/BxN serum transfer arthritis mouse model

After induction of arthritis by intraperitoneal injection of K/BxN serum, both the arthritis score and surface ENO1 expression on PBMCs increased progressively and peaked between day 5 and day 7. Thereafter, they decreased over time (figure 4A–D). Surface ENO1 expression correlated significantly with arthritis severity (figure 4E).

To determine whether the interaction between surface ENO1 and apoB might contribute to arthritis pathogenesis, BALB/c mice were treated with K/BxN serum and/or apoB protein. The ankle thickness measurements and the arthritis scores were substantially higher in mice treated with both K/BxN serum and apoB than in those treated with K/BxN serum alone (figure 5B,C). Serum levels of IL-1β, IL-6 and TNF-α also tended to be higher in the mice treated with both K/BxN serum and apoB than in those treated with K/BxN serum alone (figure 5D–F). Joints

Table 1 Proteins interacting with ENO1 in the pooled synovial fluid from patients with rheumatoid arthritis (n=5) and osteoarthritis (n=5)

			OA		RA		PLGEM	
Uniprot ID	Gene name	Identified proteins	1st	2nd	1st	2nd	STN	P values
P04114	APOB	Apolipoprotein B-100	418	368	1469	1285	34.71	0.0000
P00738	HP	Haptoglobin	42	29	603	587	32.69	0.0000
P02679-2	FGG	Isoform gamma-A of fibrinogen gamma chain	96	95	741	684	30.85	0.0000
V9HVY1	HEL-S-78p	Epididymis secretory sperm binding protein Li 78p	90	83	697	648	30.09	0.0000
P01009	SERPINA1	Alpha-1-antitrypsin	225	187	718	741	23.51	0.0000
A8K008	–	cDNA FLJ78387	175	163	536	525	17.99	0.0000
V9HWA9	HEL-S-62p	Epididymis secretory sperm binding protein Li 62p	733	666	1259	1141	16.62	0.0000
Q6PJF2	IGK@	IGK@ protein	85	74	357	326	16.10	0.0000
P08603	CFH	Complement factor H	25	22	210	178	14.10	0.0000
B7Z549	–	cDNA FLJ56821, highly similar to inter-alpha-trypsin inhibitor heavy chain H1	18	13	179	156	13.70	0.0000
B4E1Z4	CFB	Complement factor B	22	16	190	156	13.45	0.0000
P01023	A2M	Alpha-2-macroglobulin	473	436	831	735	12.83	0.0000
P02671	FGA	Fibrinogen alpha chain	75	72	266	262	12.58	0.0000
P01031	C5	Complement C5	8	7	129	117	12.21	0.0000
B3KS79	–	cDNA FLJ35730 fis, clone TESTI2003131, highly similar to alpha-1-antichymotrypsin	17	16	154	138	12.02	0.0000
Q6N093	DKFZp686I04196	Putative uncharacterised protein DKFZp686I04196 (Fragment)	40	30	194	177	11.98	0.0000
P01871	IGHM	Ig mu chain C region	60	51	212	204	11.09	0.0000
Q14624	ITIH4	Inter-alpha-trypsin inhibitor heavy chain H4	23	21	142	142	10.84	0.0000
Q8N355	IGL@	IGL@ protein	34	38	173	157	10.53	0.0000
P00450	CP	Ceruloplasmin	6	7	104	93	10.49	0.0000
Q96K68	–	cDNA FLJ14473 fis, clone MAMMA1001080, highly similar to Homo sapiens SNC73 protein (SNC73) mRNA	46	39	181	170	10.45	0.0000
P00734	F2	Prothrombin	9	11	102	91	9.48	0.0000
P13671	C6	Complement component C6	0	0	62	52	8.76	0.0004
P02790	HPX	Hemopexin	22	15	101	94	8.01	0.0004
P04217	A1BG	Alpha-1B-glycoprotein	0	2	55	51	8.00	0.0004
P13645	KRT10	Keratin, type I cytoskeletal 10	587	566	805	763	7.77	0.0004
P00747	PLG	Plasminogen	50	45	151	122	7.29	0.0004
P10643	C7	Complement component C7	0	0	47	41	7.29	0.0004

Liquid chromatography – mass spectrometry (LC-MS)/MS analysis integrated with an EASY-nLC system and LTQ-Velos mass spectrometry in duplicate. STN and p values were generated by PLGEM.
PLGEM, power law global error model; STN, signal to noise; OA, osteoarthritis; RA, rheumatoid arthritis.

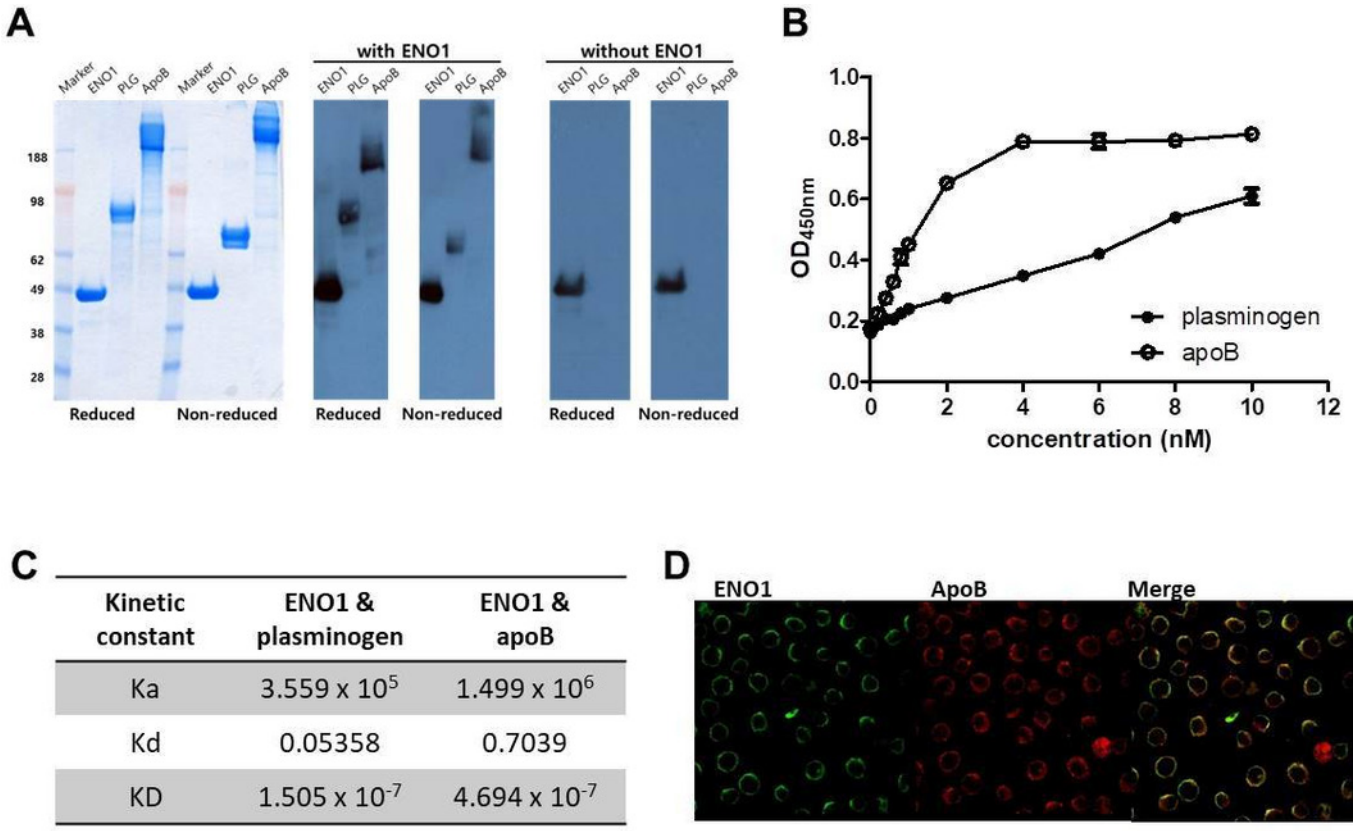
tended to have higher degree of inflammation and destruction in mice treated with both K/BxN serum and apoB than in those treated with K/BxN serum alone (figure 5G–J). Administration of apoB alone did not induce arthritis.

LDL receptor is not required for the inflammatory response induced by the ENO1-apoB interaction in vivo

As LDL containing apoB binds to LDLR, we next sought to investigate whether activation of immune cells by apoB requires LDLR. LDLR knockout and wild-type C57BL/6 mice were injected with K/BxN serum and apoB. Strikingly, the LDLR knockout mice developed a degree of arthritis comparable to wild-type mice (figure 5L,M). Serum levels of IL-1β, IL-6 and TNF-α were slightly higher or similar in the LDLR knockout mice as compared with the wild-type mice (figure 5N–P). Taken together, aggravation of inflammatory arthritis by apoB is independent of the interaction of apoB with the LDL receptor.

DISCUSSION

This study is the first to identify apoB as a novel, specific ligand of ENO1 and to show that apoB induces a proinflammatory response in PBMCs from patients with RA that is dependent on ENO1. ApoB signalling through ENO1 involved the activation of intracellular p38 MAPK and NF-κB pathways but did not require LDL-receptor. Administration of apoB further aggravated arthritis in a murine arthritis model, and this correlated with levels of surface ENO1 expression. Taken together, apoB might augment the detrimental inflammatory response in arthritis via an interaction with ENO1.



Kinetic constant	ENO1 & plasminogen	ENO1 & apoB
Ka	3.559×10^{5}	1.499×10^{6}
Kd	0.05358	0.7039
KD	1.505×10^{-7}	4.694×10^{-7}

Figure 1 ApoB is a specific ligand of ENO1. (A) Ligand blotting assay. ENO1, plasminogen (PLG) and apoB were subjected to SDS-PAGE (left) and transferred to PVDF membranes. These membranes were then incubated with ENO1 protein (middle) or without ENO1 protein (right) followed by incubation with anti-ENO1 antibody. ENO-1 bound to plasminogen (lane 2 in each blot) and apoB (lane 3 in each blot). (B) A 96-well MaxiSorp plate coated with increasing concentrations (0.2–10 nM) of plasminogen (●) or apoB (○) was incubated with ENO1. Binding of ENO1 to apoB or plasminogen was detected with anti-ENO1 antibody. Compared with plasminogen, apoB had a higher affinity for ENO1. (C) Surface plasmon resonance. Increasing concentrations (2.5–20 µg/mL) of plasminogen or apoB were injected over sensorchip surfaces coupled to ENO1. The binding affinity of apoB or plasminogen for ENO1 was calculated as described in *Materials and methods*. (D) Colocalisation of apoB and ENO on the cell surface. U937 cells were incubated with apoB and then stained with antibody against ENO1 (green) or apoB (red). ApoB colocalised with ENO1 on the cell surface (yellow in merged images). Magnification × 1000. ApoB, apolipoprotein B; ENO1, enolase 1.

Systematic screening of synovial fluid proteins using mass spectrometry and SPR identified apoB as a novel ligand with the highest affinity for ENO1 among the ENO1 binding proteins in the RA synovial fluid. This is striking for several reasons. First, apoB is abundant in the serum as part of lipoproteins such as LDL and VLDL, and there must be robust regulation of this interaction to avoid overactivation of immune cells. Second, apoB is enriched in synovial fluid of patients with RA, which can activate the immune cells in situ of joint.[13 14] Finally, LDL in patients with RA exhibits altered physical properties such as higher instability and proinflammatory potential that might contribute to both accelerated atherosclerosis and inflammation.[15]

We speculate that there are several regulatory mechanisms that could control ENO1 activation by apoB. As apoB is abundant in plasma, the upregulation of ENO1 on the cell surface might be a critical step in enabling the interaction between ENO1 and apoB. Structural modification of apoB or apoB-carrying lipoproteins might be an additional regulatory mechanism. For example, LDL from patients with RA is more unstable; it is susceptible to oxidation and already oxidised in patients with RA.[15] Indeed, oxLDL, which is enriched in RA, can induce a greater inflammatory response than naïve LDL,[15 16] which is supported by our observation of a stronger interaction of oxLDL than native LDL with ENO1 (online supplementary figure 2). Therefore, in patients with RA, apoB might be easily released from LDL and exposed on the surface of lipoproteins for a closer interaction with ENO1. An additional regulatory mechanism in the interaction of apoB and ENO1 might be achieved through citrullination, which is a critical post-translational modification in RA.[17–22] In our further studies, we found that citrullination of the apoB binding epitope of ENO1[23 24] increases its affinity for apoB (online supplementary figure 3). Since citrullination of ENO1 has the opposite effect on its affinity for plasminogen, decreasing its binding capacity,[20] this modification might shift the binding preference of ENO1 from plasminogen in favour to apoB, thus contributing to the enhanced inflammatory response in RA patients.

Our data showed that apoB-mediated aggravation of the inflammatory response was not mediated by LDL receptor, since arthritis severity was similar between LDLR knockout mice and the wild-type mice. As such, it appears that apoB is an important independent immune-modulator that links lipid metabolism with local and systemic inflammatory responses.

RA is associated with an increased risk of cardiovascular mortality due to accelerated atherosclerosis.[25 26] The inflammatory environment associated with RA, rather than traditional cardiovascular risk factors, has been postulated to be implicated in this accelerated atherosclerosis in patients with RA.[27] In atherosclerosis, monocytes infiltrate, accumulate and differentiate into macrophage-type foam cells in atherosclerotic

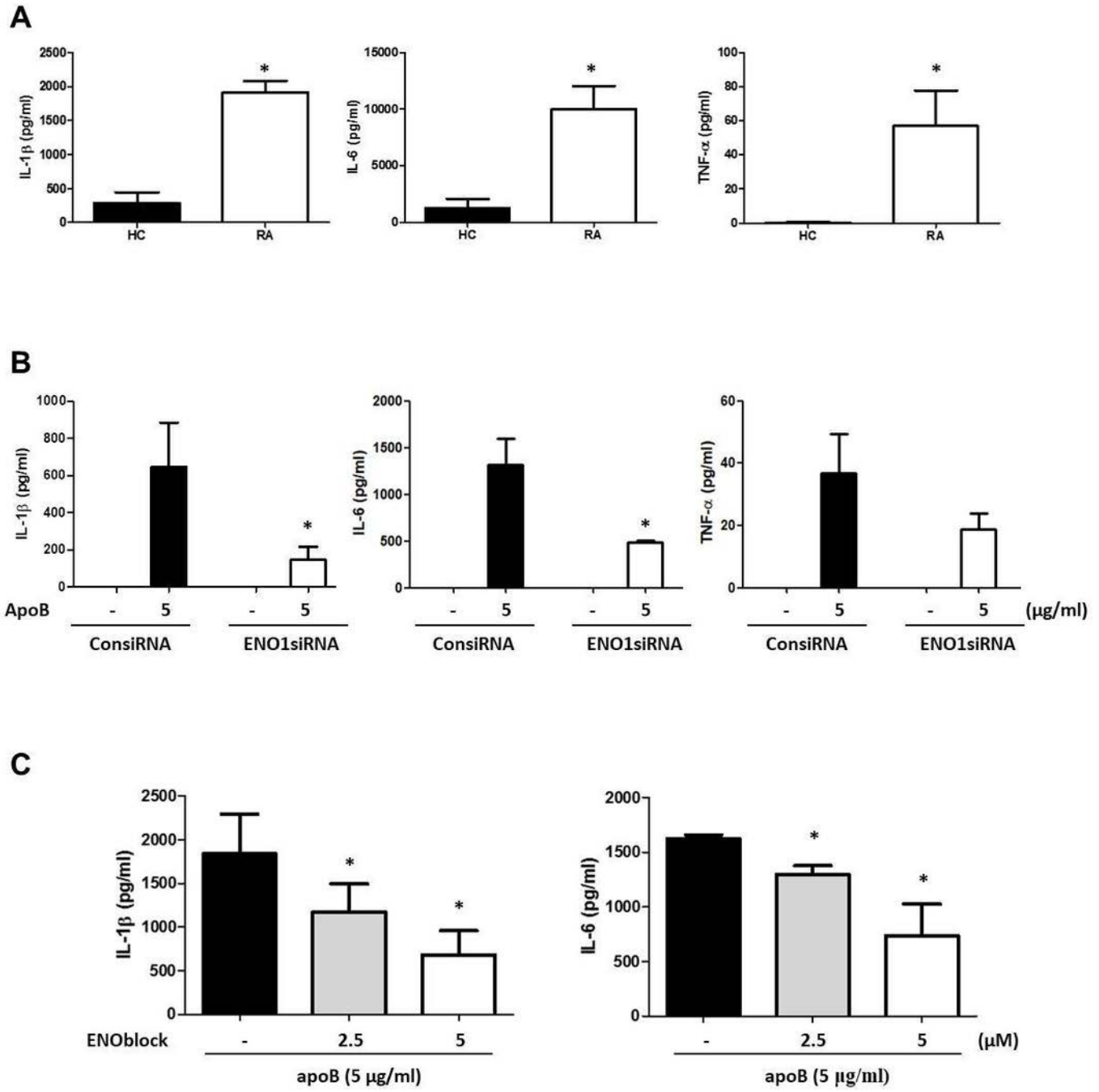


Figure 2 ApoB induces proinflammatory cytokines in PBMCs from patients with rheumatoid arthritis and is dependent on ENO1. (A) PBMCs from heathy controls (HCs; n=3) and from patients with RA (n=6) were stimulated with apoB (5 μg/mL). PBMCs from RA produced more IL-1β, IL-6 and TNF-α than PBMCs from HCs. * versus HC. (B) CD14+ cells from patients with RA (n=3) were transfected with ENO1-siRNA or control-siRNA (consiRNA) followed by treatment with apoB for 24 hours. Lower ENO1 expression was associated with decreased production of IL-1β, IL-6 and TNF-α. * versus ConsiRNA. (C) ENOblock inhibited IL-1β and IL-6 production by PBMCs from RA patients (n=3) after stimulation with apoB. Each * versus no treatment of ENOblock. All experiments were conducted in duplicates. Data are presented as the mean±SEM. *P <0.05. ApoB, apolipoprotein B; ENO1, enolase 1; IL, interleukin; PBMCs, peripheral blood mononuclear cells; RA, rheumatoid arthritis; TNF-α, tumour necrosis factor-α.

plaques.[28] LDL, which accumulates within the arterial wall in atherosclerosis, can induce monocytes to produce an inflammatory response both in vivo and in vitro[29–34] and that this process involves an autoimmune response to the protein moiety of LDL (ie, apoB).[35–37] Our findings suggest that apoB might represent a new pathophysiological link between RA and atherosclerosis via an interaction with ENO1 on immune cells, which promotes active inflammation in joints and potentially in the vascular wall.

There are outstanding questions that require further investigations. The ENO1–apoB interaction transmits inflammatory signals via activation of p38 MAPK and NF-κB to induce the production of inflammatory cytokines.[38] However, the signalling cascade linking surface ENO1 and p38 MAPK or NK-κB signalling remains a subject for further investigation. It is possible that surface ENO1 activation by apoB might influence cellular energy metabolism and, consequently, cell function.[39 40] Finally,

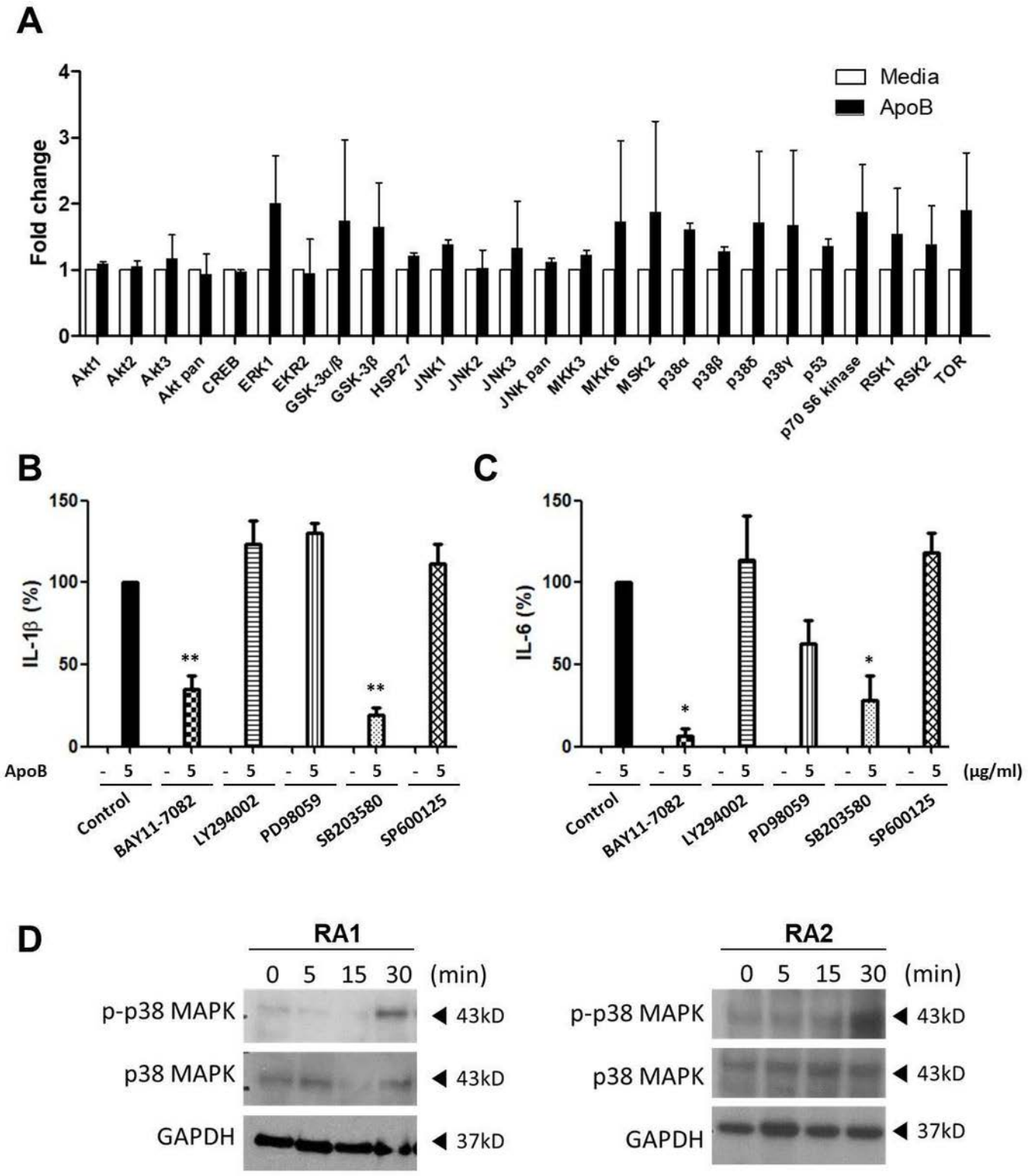


Figure 3 Induction of proinflammatory cytokines by apoB signalling via the p38 MAPK and NF-κB pathways. (A) RA PBMCs were either untreated (media) or treated with apoB (5 μg/mL) for 20 min. After incubation, the phosphorylation status of MAPK was detected in protein extracts from RA PBMCs using a human phospho-MAPK array. PBMCs from RA (n=6) were pretreated for 1 hour with BAY11-7082 (NF-κB inhibitor), LY294002 (PI3K inhibitor), PD98059 (ERK inhibitor), SB203580 (p38 MAPK inhibitor) and SP600125 (JNK inhibitor), followed by stimulation with apoB (5 μg/mL) for 24 hours. Production of IL-1β (B) and IL-6 (C) was inhibited in the presence of BAY11-7082 and SB203580. Data are presented as the mean±SEM. Experiments were conducted in duplicates. Each *, ** versus control (stimulation with 5 μg/mL of apoB without inhibitor). (D) PBMCs from two different patients with RA (RA1 and RA2) were stimulated with apoB for 5, 15 and 30 min. Changes in phosphorylated p38 MAPK levels were investigated using immunoblotting. The results are representative of six independent experiments. *p<0.05; **p<0.01. ApoB, apolipoprotein B; GADPH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; MAPK, mitogen-activated protein kinase; PBMCs, peripheral blood mononuclear cells; RA, rheumatoid arthritis.

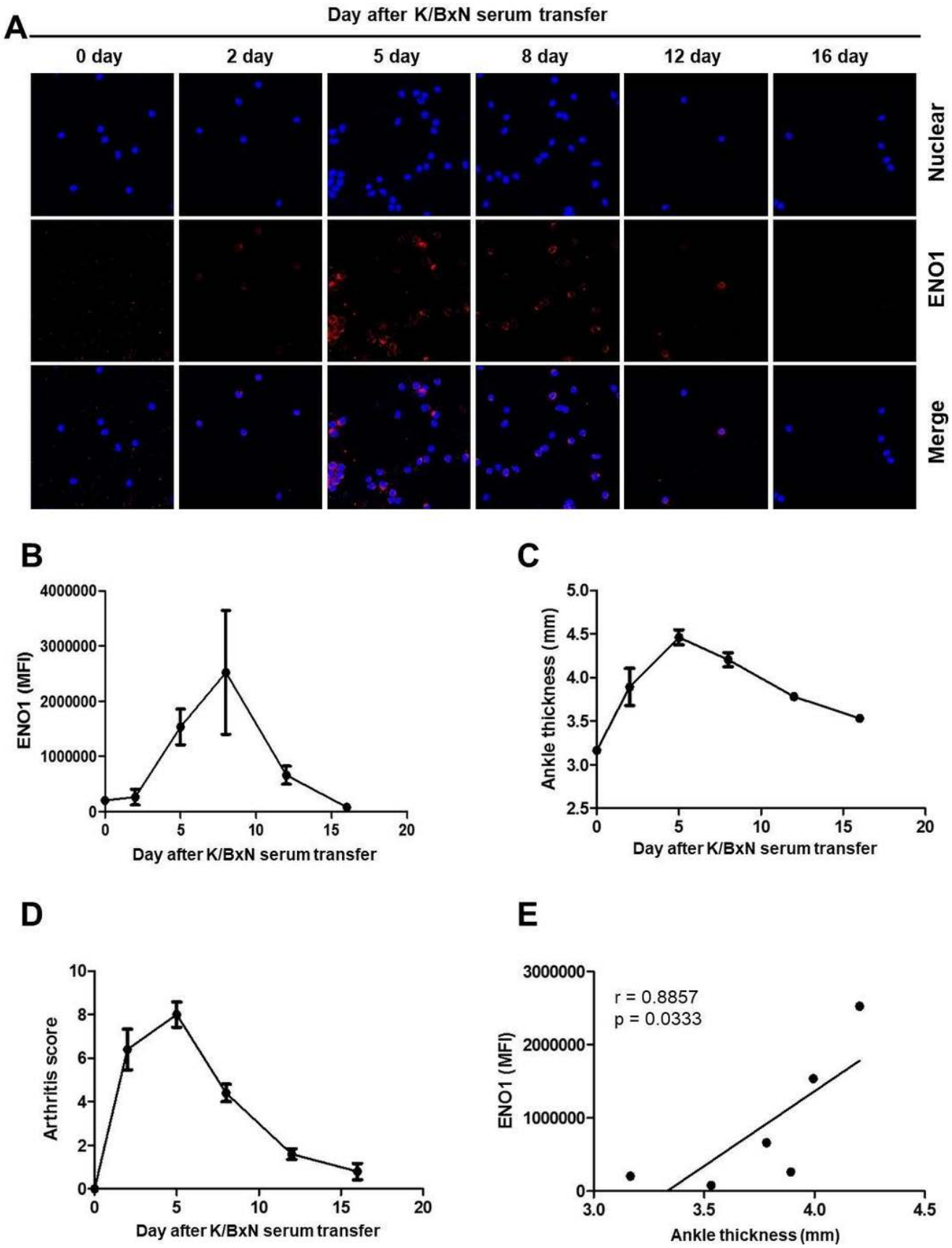


Figure 4 Increased surface ENO1 on PBMCs in K/BxN serum transfer arthritis model. BALB/c mice were injected with K/BxN mice serum on day 0 to induce arthritis. (A) Confocal microscopy revealed increased surface ENO1 expression on PBMCs after induction of arthritis by transfer of K/BxN serum. (B) The changes in ENO1 fluorescence intensity per cell increased after injection and peaked at day 8. (C) Ankle thickness and (D) arthritis scores were measured on days 0, 2, 5, 8, 12 and 16 after K/BxN serum transfer. (E) Expression levels of surface ENO1 were significantly correlated with ankle thickness. Each treatment group included five mice. ENO1, enolase 1; MFI, mean fluorescence intensity.

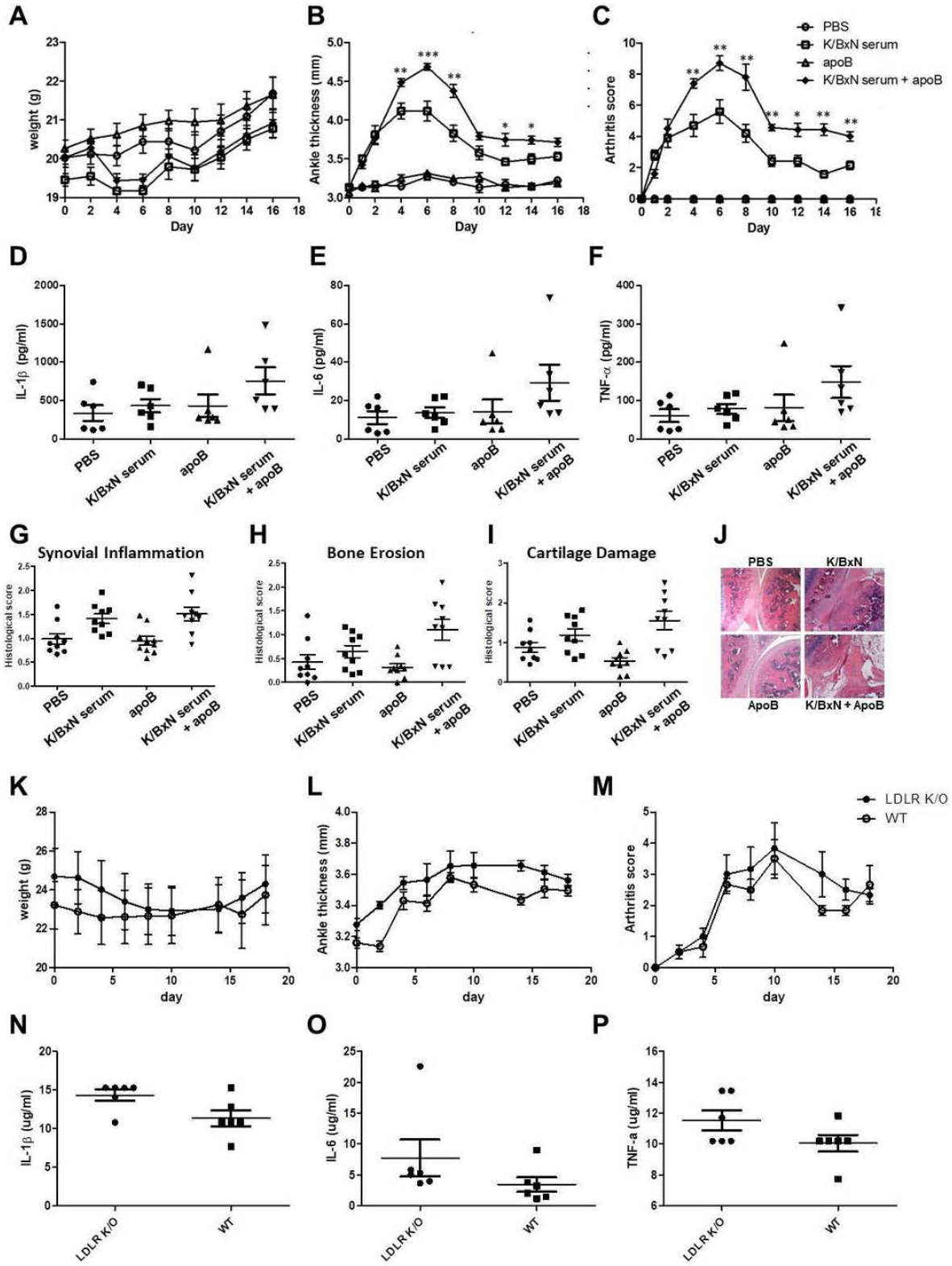


Figure 5 ApoB aggravates K/BxN serum transfer arthritis in mice. Mice were intraperitoneally injected with 50 µL of K/BxN serum on day 0 and intravenously injected with apoB (25 µg) on days 2 and 7. Weight (A), ankle thickness (B) and arthritis score (C) were measured every other day. (D–F) Serum cytokine levels (ie, IL-1β, IL-6 and TNF-α) were measured on day 7 after K/BxN serum transfer. Each *, ** or *** versus K/BxN serum transfer mice. (G–I) Histological structural change of knee joints isolated on day 16 after K/BxN serum transfer were analysed. (J) Representative images of mouse knee arthritis. LDLR knockout mice (LDLR K/O; ●) and wild-type mice (WT; ○) were intraperitoneally injected with 50 µL of K/BxN mice serum on days 0 and 4 and intravenously injected with apoB (25 µg) on days 2 and 6. Weight (K), ankle thickness (L) and arthritis score (M) were measured every other day. (N–P) Serum cytokine levels were measured on day 8 after K/BxN serum injection. Data are presented as the mean±SEM. *P<0.05; **p<0.01; ***p<0.001. For figure parts A–C, 10 mice per group; for figure parts D–F, 7 mice per group; for figure parts G–I, 9 mice per group; and for figure parts K–P, 6 mice per group were used. ApoB, apolipoprotein B; IL, interleukin; LDLR, low-density lipoproteins receptor; TNF-α, tumour necrosis factor-α; WT, wild type.

the ENO1–apoB interaction may not be specific for RA but is a general feature for any inflammatory condition. Further studies are needed to clarify these points.

In conclusion, we present a novel mechanism by which lipid metabolism regulates chronic inflammation and provide a link between the key component of artherogenic lipids, apoB and the cell surface receptor ENO1, and thus between vascular disease and chronic joint inflammation. Targeting apoB and its interaction with ENO1, therefore, offers a novel therapeutic strategy for inflammatory diseases such as RA.

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Contributors JYL and YWS conceived and designed the study. JYL performed experiments in vitro experiments. MJK performed the mass spectrometry. JYC, JSP and JYL performed the in vivo experiments. EYL, EBL, ECY, MJK, TP and JKP participated in study design and manuscript preparation. YWS directed and supervised all aspects of the study.

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Competing interests None declared.

Patient consent Obtained.

Ethics approval The study was approved by the institutional review board of the Seoul National University Hospital (IRB 1702-057-831) and was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice guidelines. Written informed consent was obtained from all patients before enrolment in the study.

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EXTENDED REPORT

Non-classical monocytes as mediators of tissue destruction in arthritis

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ABSTRACT

Objectives Bone destruction in rheumatoid arthritis is mediated by osteoclasts (OC), which are derived from precursor cells of the myeloid lineage. The role of the two monocyte subsets, classical monocytes (expressing CD115, Ly6C and CCR2) and non-classical monocytes (which are CD115 positive, but low in Ly6C and CCR2), in serving as precursors for OC in arthritis is still elusive.

Methods We investigated CCR2$^{-/-}$ mice, which lack circulating classical monocytes, crossed into hTNFtg mice for the extent of joint damage. We analysed monocyte subsets in hTNFtg and K/BxN serum transfer arthritis by flow cytometry. We sorted monocyte subsets and analysed their potential to differentiate into OC and their transcriptional response in response to RANKL by RNA sequencing. With these data, we performed a gene ontology enrichment analysis and gene set enrichment analysis.

Results We show that in hTNFtg arthritis local bone erosion and OC generation are even enhanced in the absence of CCR2. We further show the numbers of non-classical monocytes in blood are elevated and are significantly correlated with histological signs of joint destruction. Sorted non-classical monocytes display an increased capacity to differentiate into OCs. This is associated with an increased expression of signal transduction components of RANK, most importantly TRAF6, leading to an increased responsiveness to RANKL.

Conclusion Therefore, non-classical monocytes are pivotal cells in arthritis tissue damage and a possible target for therapeutically intervention for the prevention of inflammatory joint damage.

INTRODUCTION

In patients with inflammatory arthritis, joint destruction is the most serious consequence of the disease, as it leads to irreversible functional impairment.[1 2] Destruction of the articular bone has been demonstrated to be almost exclusively mediated by osteoclasts (OC), multinucleated cells of myeloid origin generated from monocytic/macrophage like precursors, which are still poorly defined.[3–7]

The importance of OC in erosive arthritis has been shown in various experimental murine models; in the absence of receptor activator NF-κB ligand (RANKL) or c-fos, arthritic mice are fully protected against bone damage despite the presence of severe synovial inflammation.[4 6] The life span of individual OC has been estimated to be only a few weeks; therefore, they have to be replaced by a perpetual supply of osteoclast precursors (pOCs).[8] Peripheral blood monocytes are potential precursors for OC, as both in humans and in mice monocytes can be driven to differentiate into OCs on stimulation with M-CSF and RANKL and increased frequencies of potential pOCs have been identified in patients with inflammatory arthritides.[3 8–11] Based on the expression of lymphocyte antigen 6 complex (Ly6C) and chemokine receptor 2 (CCR2) monocytes can be further subdivided into inflammatory or classical monocytes expressing CD11b, CD115, Ly6C and CCR2 and resident or non-classical monocytes, which do not express Ly6C and CCR2[12–14]. The chemokine receptor CCR2 has been shown to be important in the biology of inflammatory monocytes, as mice lacking this receptor show greatly reduced numbers of circulating inflammatory monocytes.[15 16] CCR2-deficient mice have been used in several experimental models of autoinflammatory/autoimmune diseases to show that classical monocytes are the culprits of pathology that mediate tissue damage, since CCR2-deficient mice were protected against these diseases.[17–19] However, in arthritis, the role of monocyte subsets is controversial. It is known that haematopoietic cells including monocytes are recruited to the site of inflammation and differentiate into macrophages and OCs locally in the synovial membrane.[20] However, it is still unclear if there are committed pOCs or if all monocytes/macrophages have the potential to become OCs. In this regard, the role of the two monocyte subsets in mediating joint destruction and in particular their role as pOCs during arthritis is conflicting, as some reports showed an important role of CCR2^{+} cells and thus classical monocytes as OC precursors in arthritis as well as osteoporosis, whereas others claimed non-classical monocytes to be critically important for joint inflammation.[21–23] Of particular note, CCR2-deficient mice develop more severe (and more destructive) arthritis in several experimental models.[21 22 24–26]

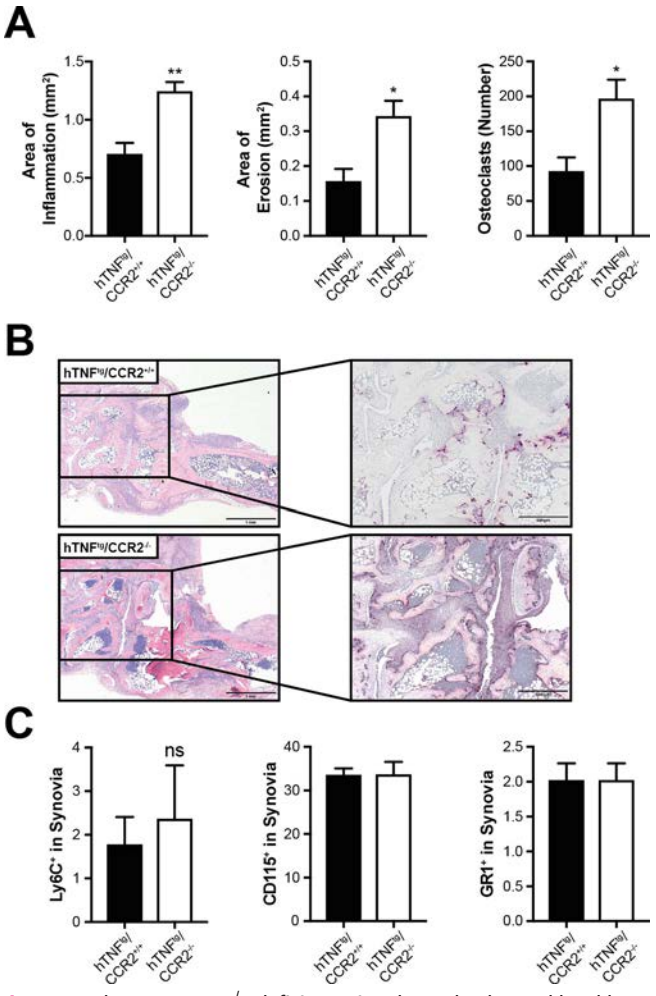

Figure 1 hTNFtg/CCR2$^{-/-}$-deficient mice showed enhanced local bone destruction and osteoclast formation. CCR2$^{-/-}$ mice were crossed into hTNFtg mice and histological analysis was performed. (A) Quantitative analysis of histological parameters of arthritis (hTNFtg, n=5; hTNFtg/CCR2$^{-/-}$, n=9)). Results are shown as mean±SEM (B) Representative histological sections of the hind paws of hTNFtg/CCR2$^{+/+}$ and hTNFtg/CCR2$^{-/-}$ mice stained with H&E and TRAP. Osteoclasts are displayed as purple-stained cells. *P<0.05. (C), Analysis of the percentage of Ly6C^{+} cells (left panel), CD115^{+} cells (middle panel) and Gr1^{+} cells (right panel) shown as per cent of total cells in the synovial membrane of hTNFtg/CCR2$^{+/+}$ and hTNFtg/CCR2$^{-/-}$ mice. TRAP, tartrate-resistant acid phosphatase.

MATERIALS AND METHODS

Animals and arthritis models

The heterozygous human TNF transgenic (hTNFtg) Tg197 mouse strain has been described previously.[27] CCR2 $^{-/-}$ mice on the C57bl/6 genetic background were purchased from Jackson Laboratories and crossed into hTNFtg animals. Arthritis was evaluated weekly in a blinded manner as described previously.[6]

Serum-transfer arthritis was induced by intraperitoneal application of 150 µl of K/BxN serum on day 0 and day 2 as described.[28]

All animal studies were approved by the animal ethics committee of the Medical University Vienna and comply with institutional guidelines.

Histology and immunohistochemistry

Histological sections of hind paws from wild type (WT) hTNFtg and hTNFtg/CCR2$^{-/-}$ mice, serum transfer arthritis as well as the Tissue Quest analysis were prepared and analysed as previously described.[6 20 28 29]

Flow cytometry and cell sorting

Blood was collected every other week starting at 4 weeks of age until the end of the experiment (week 10 for hTNFtg, day 12 for K/BxN serum transfer arthritis). Cells were simultaneously stained for CD11b (PerCP-Cy5.5-conj., clone M1/70), CD115 (PE-conj., clone AFS98) Ly6-G (FITC-conj., clone RB6-8C5, all eBioscience) and Ly6-C (PE-Cy7-conj., clone AL-21) and CD11c (APC-Cy7-conj., clone HL3, all BD Pharmingen). Classical monocytes were characterised as CD11b^{+}CD115^{+}Ly6C^{+} and non-classical monocytes as CD11b^{+}CD115^{+}Ly6C^{-} cells. For flow-cytometric analyses, we used a BD FACSCanto II (Becton Dickinson Immunocytometry Systems, San Jose, California, USA).

For cell sorting, pooled peripheral blood cells from either hTNFtg or WT mice were stained with CD11b, CD115 and Ly6-C antibodies and then isolated using a BD FACSAria II sorter.

In vitro OC generation

For in vitro OC formation, we sorted classical and non-classical monocytes from WT or arthritic hTNFtg mice. Cells were cultured in MEM-alpha supplemented with 10% fetal bovine serum (FBS) in the presence of 100 ng/mL M-CSF for 3 days, then MEM-alpha with 10% FBS and 30 ng/mL M-CSF and 50 ng/mL RANKL. After 4 days, we performed tartrate-resistant acid phosphatase (TRAP) staining and determined the amount of OCs (TRAP positive and three or more nuclei) and also counted the numbers of nuclei.

RNA-sequencing

Total RNA was prepared from approximately 1 million WT classical and non-classical monocytes by using mirVana miRNA Isolation Kit (AM1560, ABI). Two hundred nanograms of total RNA was subsequently used to prepare RNA-seq library by using TruSeq SR RNA sample prep kit (FC-122–1001, Illumina), following the manufacturer's protocol. The libraries were sequenced for 50 cycles (single read) with a HiSeq 2000 (Illumina). Raw sequencing data were processed with CASAVA 1.8.2 to generate FastQ files. Sequence reads were mapped onto the mouse genome build mm9 using TopHat 2.0. Gene expression values (RPKM, reads per kilobase exon per million mapped reads) were calculated with Cufflinks 2.0. All downstream statistical analyses, including PCA, were performed with Partek Genomics Suite 6.6, R 3.0.1 and GeneSpring GX 12.1 (Agilent Technologies).

Western blotting

Sorted classical and non-classical monocytes were cultured in the presence of 100 ng/mL M-CSF for 3 days or an additional 24 hours with RANKL (DC-STAMP) and then lysed in radioimmunoprecipitation (RIPA) buffer supplemented with phosphatase and protease inhibitors (Roche). Western blotting (WB) was performed as described.[29] Primary antibodies used: Actin (Cytoskeleton), TRAF6 (Santa Cruz), DC-STAMP (Merck).

GO term enrichment analysis

Functional gene annotations were obtained from the Gene Ontology (GO) database.[30 31] Gene set enrichment analyses were performed using Fisher's exact test with all approved protein coding genes from the HUGO Gene Nomenclature

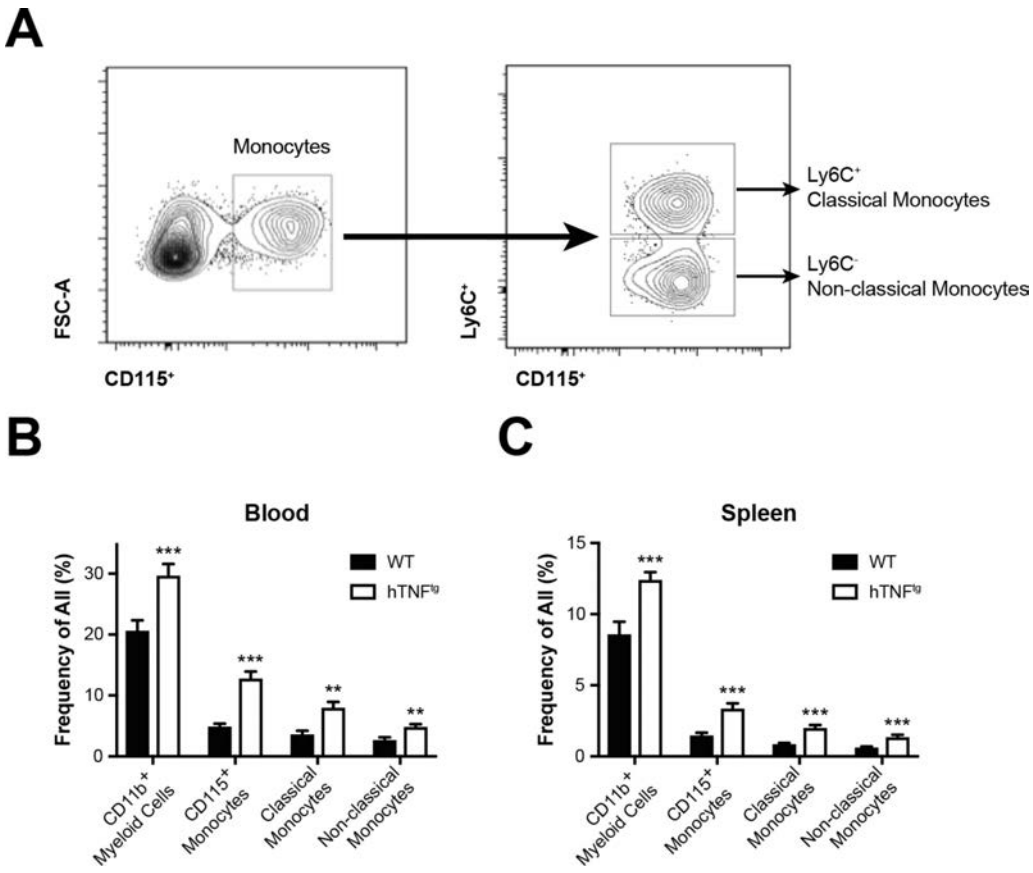

Figure 2 Monocytes in blood and spleen in hTNFtg arthritis. (A) Gating of flow cytometric analysis of myeloid populations in blood obtained from WT and hTNFtg mice. (B,C) Bar graph summarising frequencies of myeloid populations in blood (B) and spleen (C) of WT mice and hTNFtg mice (n=at least 10 per group). Results are shown as mean±SEM. **P<0.01; ***P<0.001. WT, wild type.

Committee (HGNC) as background set.[32] The resulting p values were corrected for multiple hypotheses testing according to the Bonferroni procedure using a cut-off of $p<0.05$. In order to remove redundant GO terms, we summarised all terms with a semantic similarity of 0.7 or higher and represented them by their most informative common ancestor with the GO tree.

Statistical analysis

Data are presented as the means±SEM. We compared group mean values, as appropriate, by Student's unpaired two-tailed t-test or one-way analysis of variance with Bonferroni's multiple comparison test (Graph Pad Prism). We considered $p<0.05$ significant.

RESULTS

Increased joint damage in CCR2-deficient hTNFtg mice

CCR2 deficiency has been shown to aggravate arthritis in interleukin1 receptor antagonist (IL1-RA)$^{-/-}$ mice as well as in collagen-induced arthritis (CIA).[25 26] In our first experiment we tested, whether reduction of circulating classical monocytes altered the extent of arthritis and joint damage also in hTNFtg mice. To this end, we crossed CCR2-deficient mice into hTNFtg mice, generating hTNFtg/CCR2$^{-/-}$ mice. We detected increased synovial inflammation (figure 1), consistent with the results obtained in other models of arthritis. hTNFtg/CCR2$^{-/-}$ mice also showed a significant increase in the extent of erosive bone damage and local generation of OC compared with hTNFtg mice (figure 1A, B). Analysing the composition of the inflammatory infiltrate, we detected only few Ly6C$^+$ cells in the inflamed synovium (figure 1C). There were numerically increased numbers of CD115$^+$ cells but fewer Gr1$^+$ neutrophil granulocytes in hTNFtg/CCR2$^{-/-}$ mice compared with hTNFtg mice (figure 1C). Of note, we detected a significant retention of Ly6C$^+$ cells in the bone marrow of hTNFtg/CCR2$^{-/-}$ mice, in accordance with the published phenotype of CCR2-deficient mice (online supplementary figure S1). Therefore, although Ly6C$^+$ cells are retained in the bone marrow also in the hTNFtg model in CCR2-deficient mice, tissue damage is not reduced, but even enhanced.

Non-classical monocytes are elevated in hTNFtg arthritis

We next analysed the relative numbers of myeloid cells in blood and spleens of WT and hTNFtg mice by flow cytometry. We detected significantly increased proportions of CD11b$^+$ myeloid cells and CD115$^+$ monocytes in blood and spleens of hTNFtg compared with WT mice (figure 2B, C). Both CD11b$^+$CD115$^+$Ly6C$^+$ classical and CD11b$^+$CD115$^+$Ly6C$^-$ non-classical monocytes were significantly elevated in blood and spleen, suggesting altered composition of myeloid cells in hTNFtg mice (figure 2B, C). In hTNFtg mice, arthritis can be detected around week 5 of age (figure 3A). We therefore analysed blood of mice starting at 4 weeks of age, when no clinical symptoms of arthritis are detectable. We found that total numbers of CD115$^+$ monocytes were significantly increased compared with WT animals already at 4 weeks of age and remained elevated at every time point measured (week, 4, 6, 8 and 10) (figure 3B). Analysing classical monocytes, we found a significant increase of these cells only from week 6 onwards. Non-classical monocytes, however,

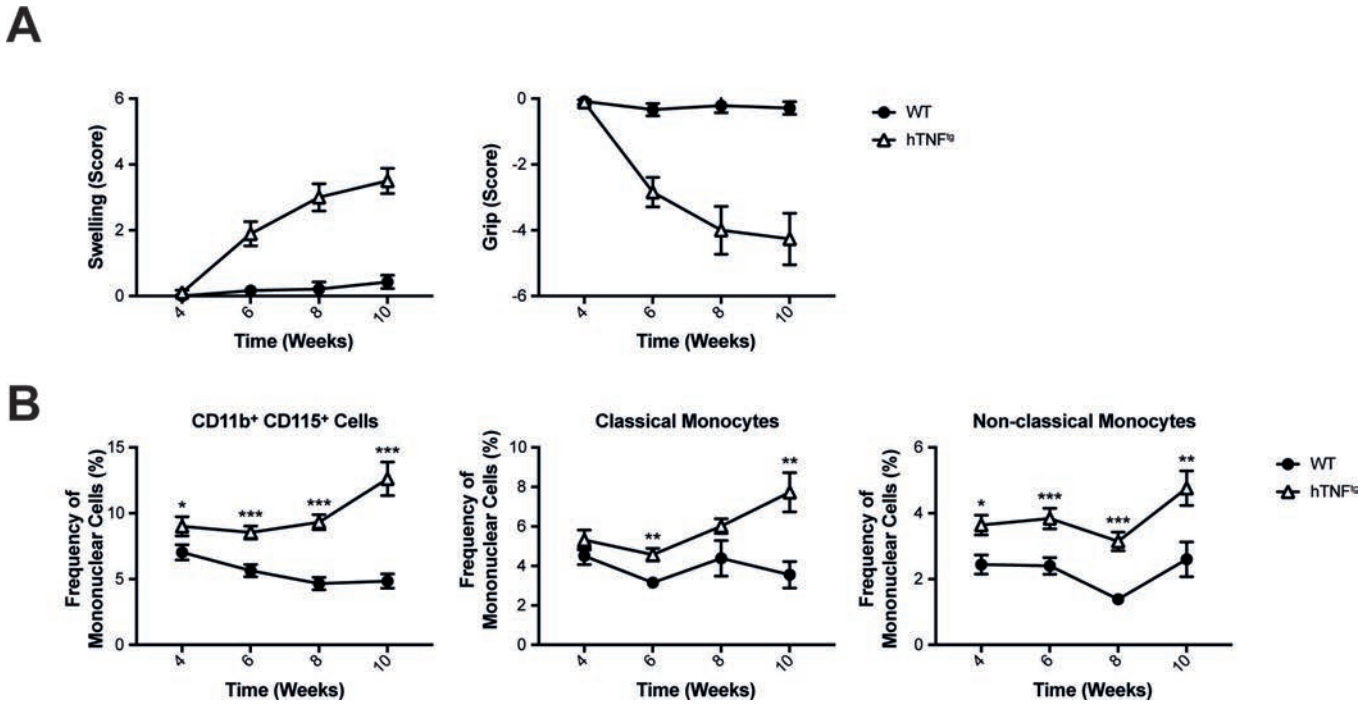

Figure 3 Development of arthritis is accompanied by accumulation of circulating mononuclear cells. (A) Clinical assessment of paw swelling and grip strength in WT mice (n=10) and hTNFtg mice (n=9). (B) Characterisation of monocytes under steady-state conditions and during hTNF driven arthritis. Blood from WT mice and hTNFtg were analysed using flow cytometric analysis over time (n=at least 12 per group and time point). Results are shown as mean±SEM. *P<0.05; **P<0.01; ***P<0.001. WT, wild type.

were increased in hTNFtg mice throughout the observation period, beginning from week 4 (figure 3B). These data reveal, that in arthritis prone hTNFtg mice, non-classical monocytes are increased already at the preclinical stage.

Moreover, when we plotted levels of classical or non-classical monocytes in blood against the number of OC found in the hind paws of arthritic mice, we detected a significant correlation between the number of non-classical monocytes in blood and the number of OCs in hind paws (r=0.64, p=0.02). Further, the levels of these cells correlated with the area of inflammatory bone destruction (r=0.66, p=0.01; figure 4A) and the area of synovial inflammation (r=0.65, p=0.02; figure 4A). In contrast, none of these variables correlated significantly with the number of classical monocytes in peripheral blood (figure 4B).

Non-classical monocytes accumulate at the site of tissue damage

We next asked, how Ly6C$^+$ cells were distributed in the inflamed synovial membrane and compared this to the general distribution of CD115$^+$ cells. While we did detect Ly6C$^+$ cells in the arthritic synovial membrane of hTNFtg mice, there were almost no Ly6C$^+$ cells in the vicinity of erosions (figure 4C). Moreover, OCs stained negative for Ly6C. However, erosions were packed with CD115$^+$ cells, with OCs also expressing CD115. Altogether these data reveal, that OCs in vivo are negative for Ly6C and that also the monocytes/macrophages present in the vicinity of OCs are negative for Ly6C.

To validate the data obtained in hTNFtg animals, we used another arthritis model, namely K/BxN serum transfer arthritis.[33] While we did not detect increased relative numbers of CD11b$^+$ cells or CD115$^+$ cells in blood or spleens after induction of K/BxN serum transfer arthritis (online supplementary figure S2), relative numbers of non-classical monocytes in blood positively correlated with markers of joint destruction (area of erosion and number of OCs) (online supplementary figure S3A). Again, the relative numbers of classical monocytes in blood did not correlate with area of inflammation, area of erosion or number of OC in the hind paw (online supplementary figure S3B). These findings further demonstrate a close correlation of non-classical monocytes with various parameters of bone destruction. We also stained for CD115 and Ly6C in the inflamed hind paws of mice after induction of K/BxN serum transfer arthritis. In line with the hTNFtg model, we detected CD115$^+$ monocytes in the vicinity of bone erosions also in K/BxN serum transfer arthritis (online supplementary figure S3C) and OC stained negative for Ly6C.

High osteoclastogenic potential of non-classical monocytes ex vivo

In order to test the osteoclastogenic potential of the two monocyte subsets, we isolated classical monocytes and non-classical monocytes from blood by FACS and cultured these cells with M-CSF and RANKL. Non-classical monocytes were significantly more potent in differentiating into mature multinucleated OCs than classical monocytes, irrespective of whether the monocytes derived from WT or arthritic hTNFtg mice (figure 5A). In order to investigate potential mechanisms related to the increased osteoclastogenic potential of non-classical monocytes compared with classical monocytes, we tested the global transcriptional response of both monocyte subpopulations to RANKL stimulation. Therefore, we performed RNA sequencing of non-classical monocytes and classical monocytes after 24 hours of RANKL stimulation. Transcriptome analysis revealed a large number of differentially regulated genes (figure 5B,C). In a gene set enrichment analysis, we detected statistically significant enrichment of all RANKL-induced genes in categories such as metabolic pathways, genes involved in growth, chemotaxis and proliferation as well as OC associated molecular markers in stimulated non-classical monocytes compared with classical monocytes.[34]

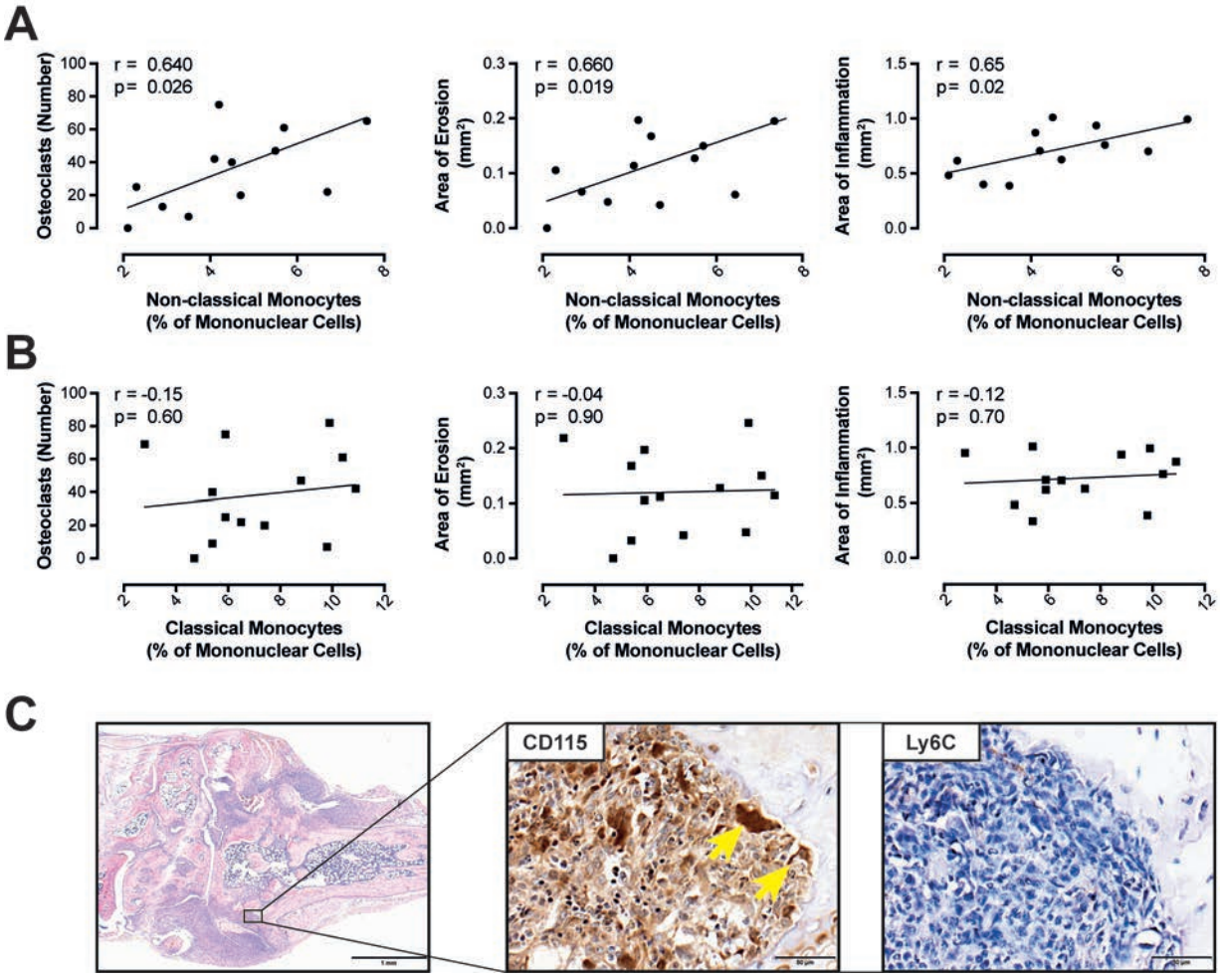

Figure 4 Characterisation of monocytes during hTNF driven arthritis. The number of circulating non-classical monocytes (A) and classical monocytes (B) was correlated with histological markers of joint destruction such as the number of osteoclasts, the area of erosion and inflammation. (C) H&E staining of a hind paw of a hTNFtg mouse (scale bars=1 mm) and immunohistochemical staining for monocyte markers CD115 and Ly6C in an erosion (scale bars=50 µm). Arrows indicate CD115 positive osteoclasts.

Vice versa, RANKL-repressed genes were significantly enriched in classical monocytes (online supplementary figure S4).

Gene ontology enrichment analysis showed increased expression of genes associated with the inflammatory response and response to microbial products in classical monocytes compared with non-classical monocytes. In contrast, genes associated with regulation of OC differentiation, iron metabolism and regulation of pH were enriched in non-classical monocytes (figure 5D).

Of note, we found a significantly increased expression of TRAF6 and Gab2, both proximal adaptors of RANK-mediated signal transduction with well-established roles in osteoclastogenesis,[35 36] in non-classical monocytes compared with classical monocytes (figure 6A). Increased expression of TRAF6 in non-classical monocytes versus classical monocytes was confirmed by qPCR and at the protein levels by Western blot (figure 6B and online supplementary figure S5). We did not detect differences in c-fms or RANK, the respective receptors for M-CSF and RANKL (figure 6C). However, among the differentially expressed genes, we found osteoclast-associated receptor (OSCAR) as well as Osteoclast Stimulatory Transmembrane Protein (OC-Stamp) and dendritic cell-specific transmembrane protein (DC-Stamp), the latter being important molecules regulating the fusion of OCs (figure 6C).[37–39] We confirmed increased expression of DC-STAMP on non-classical monocytes by Western Blot (figure 6D). We therefore determined the number of nuclei/OC that we found in OCs that had differentiated from non-classical monocytes or classical monocytes. As shown in figure 6E and F, OC derived from non-classical monocytes exhibited significantly more nuclei than OCs derived from classical monocytes.

DISCUSSION

Elucidating the mechanisms leading to irreversible joint damage and consequently functional impairment in patients suffering from inflammatory arthritis is of major importance for our understanding of the pathways to severe and disabling disease and for the development of new and even more effective remedies for treating these diseases than available today. In our study, we provide evidence that tissue damage in arthritis is predominantly mediated by non-classical monocytes lacking Ly6C.

The importance of different monocyte subsets in specific diseases, such as autoimmune/autoinflammatory and infectious diseases, has just begun to be appreciated. In many autoimmune models, the role of monocyte subsets in causing disease seems to be quite clear. In colitis, $CCR2^+$ monocytes have been established as culprits for tissue damage.[19 40] Similarly, $CCR2^+$ cells are the predominant infiltrating myeloid cells type in experimental autoimmune encephalomyelitis (EAE) and seem

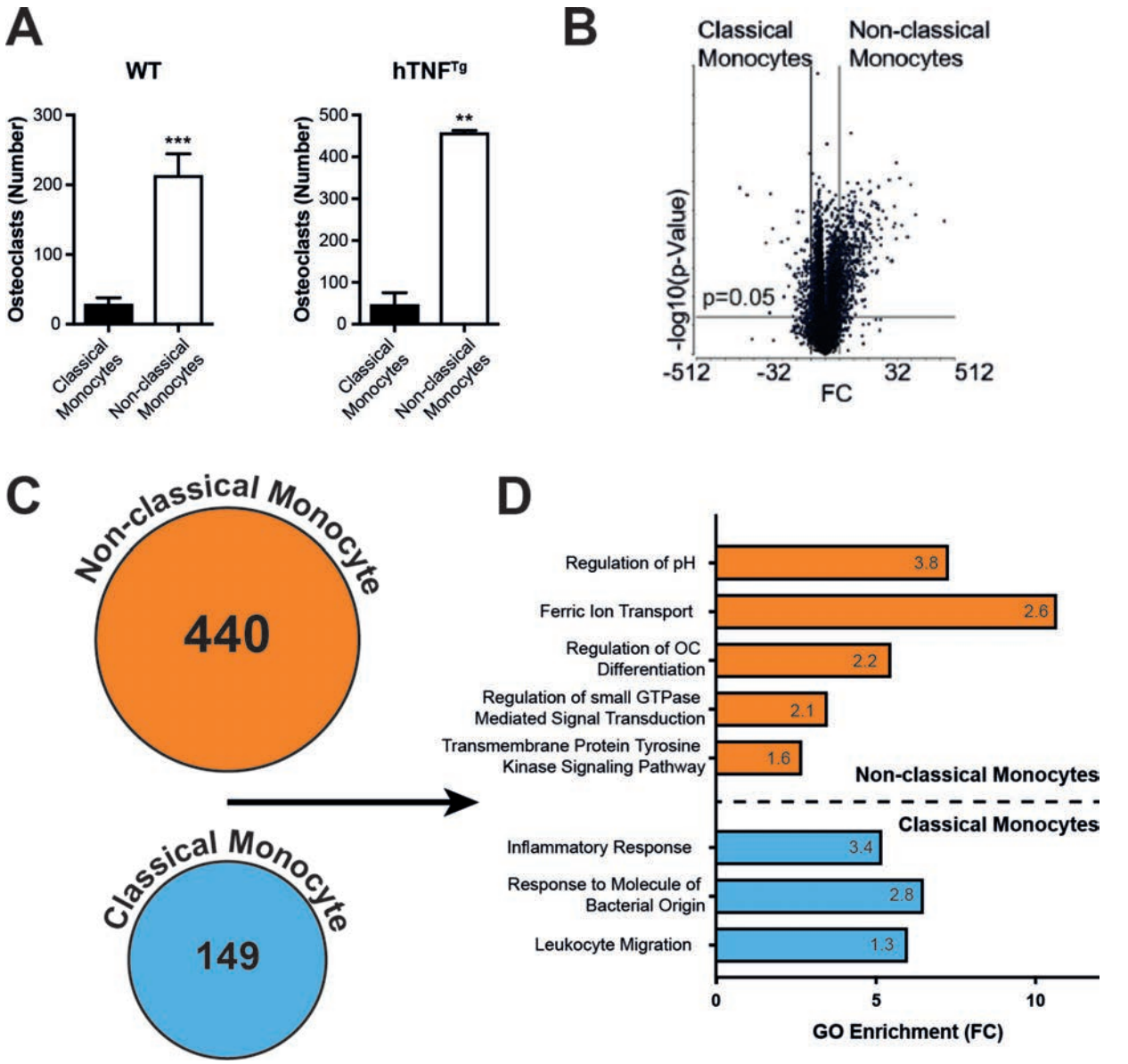

Figure 5 Identification of cell populations with osteoclastogenic potential from peripheral blood cells. (A) In vitro OC assay of sorted Ly6C$^+$ classical and Ly6C$^-$ non-classical monocytes of WT and hTNFtg mice stimulated with MCSF (4d) and RANKL (3d). Graph shows the number of TRAP positive multinucleated cells (data shown are mean values of five independent experiments±SEM). (B) Volcano plot representation of differential expression analysis of genes in RNA sequencing of WT Ly6C$^+$ classical vs Ly6C$^-$ non-classical monocytes after stimulation with MCSF (4d) and RANKL (1d) (n=3 each). The x-axis shows log2 FC in expression, the negative log10 of the p value is plotted on the y-axis. Each gene is represented by one point on the graph. (C) Number of differentially regulated genes (FC≥2, p≤0.05) in RNA sequencing in Ly6C$^+$ classical vs Ly6C$^-$ non-classical monocytes. (D) GO enrichment analysis of the transcriptome of Ly6C$^+$ classical vs Ly6C$^-$ non-classical monocytes: x-axis shows FC of significantly enriched GO terms in differentially regulated genes in RNA-sequencing of Ly6C$^+$ classical vs Ly6C$^-$ non-classical monocytes. The number in the columns represents the –log10 p value of the indicated GO term. **P<0.01; ***P<0.001. FC, fold change; GO, Gene Ontology; OC, osteoclasts; TRAP, tartrate-resistant acid phosphatase; WT, wild type.

to be directly responsible for axonal damage, and CCR2-deficient mice are resistant to EAE.[18 41–43] Also in atherosclerosis, CCR2 has been found to be essential in lesion generation.[17 44]

However, the pathogenesis of inflammatory arthritis seems to be different. It has been already reported that in both CIA as well as arthritis in IL1-RA deficient mice, CCR2 deficiency is associated with an increase in clinical signs and joint damage.[25 26] In addition, recent evidence also suggests an important role of non-classical Ly6C$^-$ monocytes in the pathogenesis of K/BxN serum transfer arthritis, as antibody-mediated depletion of CCR2$^+$ cells or in CCR2 deficiency did not alter arthritis development, whereas depletion of monocytes in general or specifically of non-classical monocytes ameliorated disease.[21] However, there have also been reports suggesting that Ly6C$^+$ classical monocytes to mediate tissue destruction in arthritis.[22] Therefore, the exact role of the two monocyte subsets is still controversial. Here, we have used two common models of inflammatory arthritis. The data obtained show positive correlations of non-classical monocytes with histological parameters of bone destruction in both hTNFtg and K/BxN serum transfer arthritis. Of note, we detected only small numbers of Ly6C$^+$ cells in hTNFtg synovitis, which is in agreement with the flow cytometric analyses of Misharin and colleagues in serum transfer arthritis.[21] Most importantly,

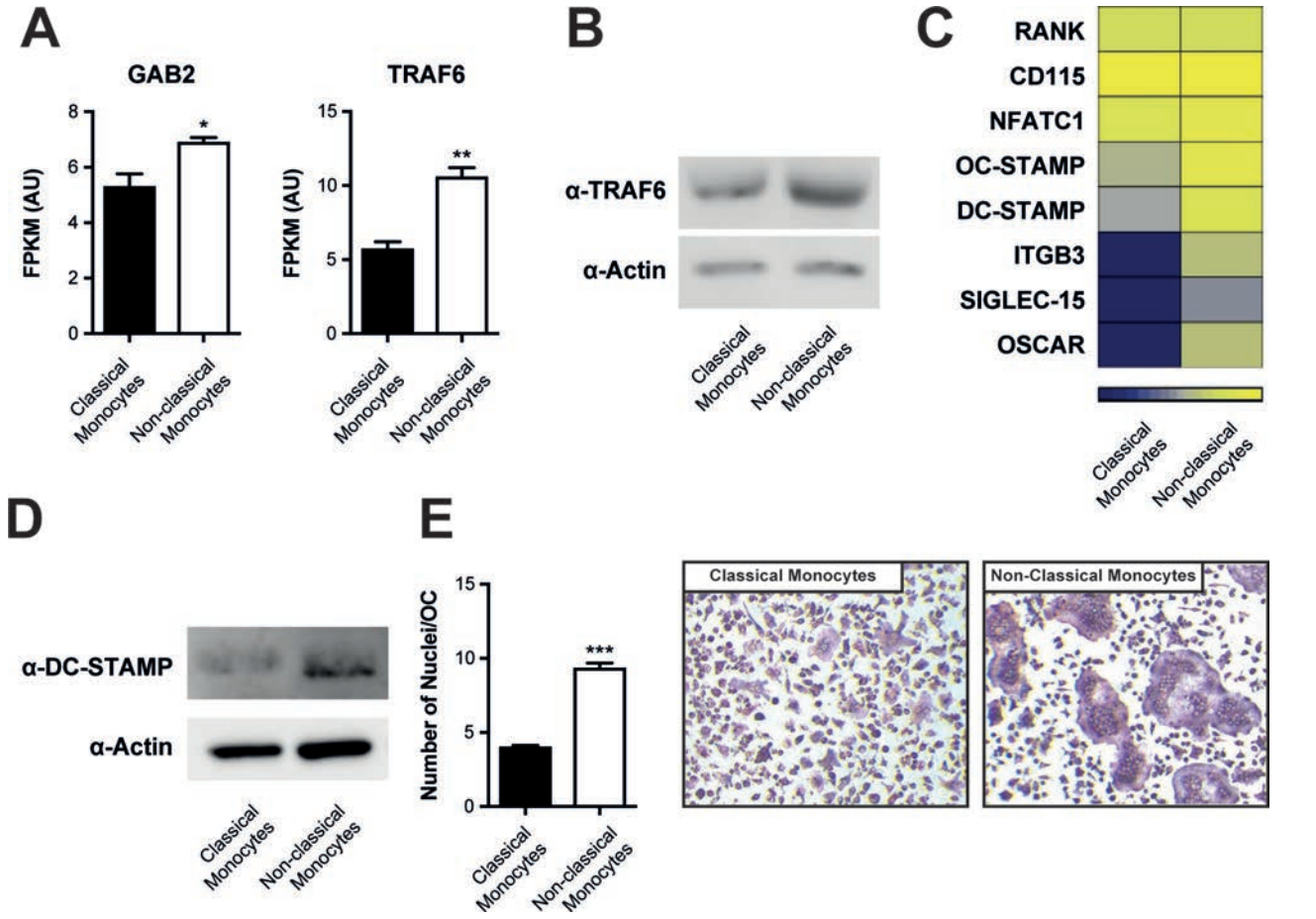

Figure 6 Increased expression of RANK-associated signal transduction molecules in non-classical monocytes. (A) Expression levels (FPKM values) of Gab2 and TRAF6 in RNA sequencing dataset of sorted Ly6C$^+$ classical and Ly6C$^-$ non-classical monocytes stimulated with MCSF (4d) and RANKL (1d) (n=3 each). Results are shown as mean±SEM (B) Western blot for the presence of TRAF6 in Ly6C$^+$ classical and Ly6C$^-$ non-classical monocytes after stimulation with MCSF for 3 days. Actin was used for loading control. Results are representative of three independent experiments. (C) Heat map of osteoclast-related genes of RNA sequencing of classical vs non-classical monocytes stimulated with MCSF (4d) and RANKL (1d) (n=3 each). (D) Western blot for the presence of DC-STAMP in Ly6C$^+$ classical and Ly6C$^-$ non-classical monocytes after stimulation with MCSF (4d) and RANKL (1d). (E) Quantification of nuclei/OC of classical and non-classical monocytes derived OCs. Results are shown as mean±SEM. (F) Representative pictures of TRAP staining to detect OCs generated from classical and non-classical monocytes. *P<0.05; **P<0.01; ***P<0.001. OCs, osteoclasts; TRAP, tartrate-resistant acid phosphatase.

we demonstrate that non-classical monocytes have a significantly increased potential to differentiate into OC. Analysis of the transcriptional response of these cells to RANKL revealed enrichment of genes involved in iron metabolism in non-classical monocytes. Intriguingly, osteoclastogenesis has been shown to depend on iron, and genes involved in iron metabolism have been shown to be essential in the generation of OC.[45] In general, RANKL-induced genes were enriched in non-classical monocytes, suggesting an increased responsiveness of non-classical monocytes to RANKL. It will be interesting to bioinformatically compare transcriptional signatures obtained by us with monocytes subpopulations.[46] In a more detailed analysis, we found components of the RANK-associated signal transduction machinery to be expressed at higher levels in non-classical monocytes versus classical monocytes, most importantly TRAF6 and Gab2. This observation offers a potential molecular explanation for the increased responsiveness to RANKL.

Of note, we also found increased levels of markers regulating fusion of OC, such as DC-STAMP and OC-STAMP. Indeed OCs derived from non-classical monocytes displayed increased numbers of nuclei/OC, suggesting increased potential to form multinucleated cells compared with non-classical monocytes. Interestingly, increased DC-STAMP expression has also been noted in human non-classical CD16$^+$ monocytes, and these cells have been found to be elevated in RA and psoriatic arthritis.[47–49]

Monocytes are of pivotal importance in arthritis. All effective therapies lead primarily to a reduction in synovial monocytes,[50] and most currently effective targeted therapies interfere with monocyte-derived cytokines, such as TNF and IL-6, their signal transduction or cell-cell interaction.[51 52] Recognising which subset of monocytes exhibits particularly pathogenic effects is of great importance to develop more specific therapies with potentially less safety issues. Our data identify non-classical monocytes to be both biomarkers of tissue damage as well as direct culprits due to their increased potential to differentiate into OCs. Therefore, these cells might be attractive therapeutic targets.

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Contributors SB, AP, JSS, MB, TK, MIK, BKP, SH and KR conceived and designed the study. AP, VS, MH, NBB, EG-A, HL, C-WS, BN, JSB, AF and ES performed the

experiments. YM and JO performed RNA sequencing analysis. SB, AP, SH, BN, MB, KR, MIK, BKP, TK, MC, VS and JM analysed the data. AP, JSB, MH, JSS and SB wrote the final draft. All authors read, revised and approved the final manuscript.

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EXTENDED REPORT

TLR4^{+}CXCR4^{+} plasma cells drive nephritis development in systemic lupus erythematosus

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ABSTRACT

Objectives In patients with systemic lupus erythematosus (SLE), immune tolerance breakdown leads to autoantibody production and immune-complex glomerulonephritis. This study aimed to identify pathogenic plasma cells (PC) in the development of lupus nephritis.

Methods PC subsets in peripheral blood and renal tissue of patients with SLE and lupus mice were examined by flow cytometry and confocal microscopy, respectively. Sorting-purified PCs from lupus mice were adoptively transferred into *Rag2*-deficient recipients, in which immune-complex deposition and renal pathology were investigated. In culture, PCs from lupus mice and patients with SLE were treated with a TLR4 inhibitor and examined for autoantibody secretion by enzyme-linked immunospot assay (ELISPOT). Moreover, lupus mice were treated with a TLR4 inhibitor, followed by the assessment of serum autoantibody levels and glomerulonephritis activity.

Results The frequencies of TLR4^{+}CXCR4^{+} PCs in peripheral blood and renal tissue were found significantly increased with the potent production of anti-dsDNA IgG, which were associated with severe renal damages in patients with SLE and mice with experimental lupus. Adoptive transfer of TLR4^{+}CXCR4^{+} PCs from lupus mice led to autoantibody production and glomerulonephritis development in *Rag2*-deficient recipients. In culture, TLR4^{+}CXCR4^{+} PCs from both lupus mice and patients with SLE showed markedly reduced anti-dsDNA IgG secretion on TLR4 blockade. Moreover, in vivo treatment with TLR4 inhibitor significantly attenuated autoantibody production and renal damages in lupus mice.

Conclusions These findings demonstrate a pathogenic role of TLR4^{+}CXCR4^{+} PCs in the development of lupus nephritis and may provide new therapeutic strategies for the treatment of SLE.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a multiple organ-involved autoimmune disease with severe inflammatory responses characterised by anti-nuclear autoantibody (ANA) production and immune-complex glomerulonephritis.[1] Among multiple contributors to the pathogenesis of SLE, autoreactive B cells and plasma cells (PC) play pivotal roles in systemic immune tolerance breakdown and local autoimmune inflammation during renal damages.[2–4] It has been shown that circulating and renal-infiltrated autoantibody-producing PCs are the major driver of lupus nephritis (LN), which are resistant to conventional and B cell depleting therapies.[2 5–8] Notably, recent findings that PC depletion with proteasome inhibitor bortezomib results in ameliorated LN in both murine lupus and patients with refractory SLE have indicated PCs as a promising target for the treatment of SLE and LN.[9 10]

Previous studies have reported the expansion of PCs in both peripheral blood and inflamed kidneys of patients with SLE with LN, which positively correlates with local inflammation and disease severity.[11–13] Although HLA-DR^{high}CD27^{high} plasmablasts are identified as an indicator of disease activity in patients with SLE,[14] long-lived PCs have been recognised as the major contributor to the maintenance of perpetuated autoantibody production and development of immune-complex glomerulonephritis. In animal studies, adoptive transfer of long-lived PCs from NZB/W mice drives immune-complex nephritis in *Rag1*$^{-/-}$ mice.[8] Currently, little is known about the phenotypic features of pathogenic PCs in driving nephritis in patients with SLE.

Recent studies have suggested that damaged kidney releases several endogenous products such as high mobility group box 1 (HMGB1) protein and heat shock proteins to maintain local inflammation.[15] Moreover, these endogenous products have been shown to activate PCs via Toll-like receptor 4 (TLR4)-mediated signalling transduction.[16–18] In lupus-prone B6$^{lpr/lpr}$ mice, TLR4 deficiency results in less severe autoantibody response and ameliorated renal damage.[19] In contrast, overexpression of TLR4 leads to autoimmune glomerulonephritis.[20] However, how TLR4 mediates autoreactive PC response and promotes LN development has remained largely unclear.

Both chemokines and their receptors are known to play an important role in PC homeostasis and migration.[21] Notably, overexpression of CXCR4 and CXCL12 has been shown to be critically involved in renal infiltration of B cells and nephritis development in lupus-prone B6.*Sle1Yaa*, BXSB and MRL/lpr mice.[21] This study aimed to characterise the functional subsets of pathogenic PCs and their contributions to the development of nephritis in SLE.

METHODS

Peripheral blood samples were collected from 37 patients with SLE and 15 healthy individuals.

Kidney biopsy samples were obtained from 22 patients with LN. Informed consents were obtained from all subjects who participated in the study. Patient characteristics are shown in the online supplementary materials (see online supplementary methods and supplementary tabless 1 and2). Female MRL/MPJ, MRL/lpr, C57BL/6 wild-type and *Rag2*$^{-/-}$ mice were maintained at the Laboratory Animal Unit of The University of Hong Kong according to standard operating procedures. Methods in all experiments, statistics and related references are described in online supplementary materials (online supplementary methods and supplementary tables 3 and4).

RESULTS

Increased circulating TLR4$^+$CXCR4$^+$ PCs correlate with disease activity and renal damages in patients with SLE

To examine the correlation of circulating PC frequency with disease activity, we divided patients with SLE into two groups with the inactive or active disease in the current cohort. According to the SLE Disease Activity Index (SLEDAI), patients diagnosed with active SLE had scores ranging from 6 to 30 and patients with inactive SLE showed scores ranging from 0 to 5 (see online supplementary table 1 for patient characteristics).[22] Flow cytometric analysis revealed that both frequencies and cell numbers of circulating Lin$^-$CD27^{high}CD38^{high}CD138$^+$ PCs among peripheral blood mononuclear cells (PBMC) were significantly increased by approximately threefold expansion in patients with SLE with active disease when compared with healthy individuals and patients with inactive disease (figure 1A, online supplementary figure 1). Within the compartment of PCs, a marked expansion of circulating TLR4$^+$CXCR4$^+$double-positive (DP) subset in both frequencies and cell numbers was further detected in patients with active lupus (figure 1B,C). Immunofluorescent staining of freshly isolated PBMCs from patients with active SLE confirmed the phenotype of DP PCs in patients with active SLE as revealed by confocal microscopy (figure 1D). Moreover, both frequencies and cell numbers of circulating DP PCs among PBMCs were positively correlated with SLEDAI scores, serum anti-dsDNA IgG titres and urine protein levels in patients with SLE (figure 1E).

To determine the autoreactive nature of PC subsets, both TLR4$^+$CXCR4$^+$ DP PCs and TLR4$^-$CXCR4$^-$double-negative (DN) PCs from PBMCs of patients with active SLE were sorting-purified for ELISPOT assay. As shown in figure 1F, the frequencies of anti-dsDNA IgG-producing PCs among DP subset were significantly higher than those among DN PCs in patients with SLE with active disease. Further flow cytometric analysis showed that circulating DP PCs were Ki-67$^-$ non-dividing cells whereas DN PCs had significantly higher frequencies of Ki-67$^+$ cells in patients with active SLE (figure 1F,G). Thus, these results indicated that DP PCs were terminally differentiated PCs with the potent production of anti-dsDNA IgG, suggesting a pathogenic role of DP PCs in lupus pathogenesis.

Enhanced renal infiltration of TLR4$^+$CXCR4$^+$ PCs correlates with nephritis severity in patients with SLE

Previous studies showed that the CXCR4/CXCR12 axis was critically involved in the leucocyte trafficking and nephritis progression in murine lupus.[21] Thus, we hypothesised that DP PCs may migrate to damaged kidneys and contribute to nephritis progression by autoantibody production and immune-complex deposition. As expected, we detected significant infiltration of TLR4$^+$CXCR4$^+$ PCs in renal biopsy samples from patients with LN by immunofluorescence microscopy (figure 2A, online supplementary table 2). Notably, the infiltration of DP PCs was much more prominent in patients with SLE with class IV nephritis than those in patients with lupus with class II and class III nephritis. However, no significant difference in frequencies of renal-infiltrated DP PCs was detected between patients with class II and class III LN (figure 2B). In this cohort study, the frequencies of renal-infiltrated TLR4$^+$CXCR4$^+$ PCs positively correlated with SLEDAI scores and urine protein levels in patients with LN (figure 2C), which suggests that renal-infiltrated PCs may contribute to nephritis development in SLE pathogenesis.

TLR4$^+$CXCR4$^+$ PCs directly drive nephritis development in murine lupus

To determine whether TLR4$^+$CXCR4$^+$ PCs are directly involved in the development of LN, we examined chromatin-immunised mice with experimental lupus and MPL/lpr mice with spontaneous lupus.[23] The chromatin-immunised mice displayed the hallmarks of lupus including high titres of ANA (including anti-dsDNA and antichromatin antibodies) and proteinuria associated with glomerular immune-complex deposition, which resembled the salient features of human SLE.[24 25] Compared with control mice, the frequencies and cell numbers of TLR4$^+$CXCR4$^+$ PCs in the blood, spleens and kidneys were significantly increased in chromatin-immunised mice with lupus (figure 3A–C). Consistent with the findings from patients with SLE, infiltrated TLR4$^+$CXCR4$^+$ PCs were detected in the renal tissue of lupus mice, which was closely correlated with elevated serum creatinine levels (figure 3D,E). Similarly, a marked expansion of DP PCs in the peripheral blood, spleen and kidney was also found associated with nephritis development in lupus-prone MPL/lpr mice (figure 3F–H). Remarkably, TLR4$^+$CXCR4$^+$ subsets from lupus mice contained 10-fold higher frequencies of anti-dsDNA IgG-producing PCs when compared with their DN counterparts (figure 4A). Additionally, DN PCs had significantly higher frequencies of Ki-67$^+$ cells in comparison with DP PC counterparts (figure 4B). Moreover, DP PCs expressed significantly higher levels of *prdm1* and *bcl-xL* transcripts than their DN counterparts (see online supplementary figure 2). Thus, these data confirmed the phenotype of DP PC subset as terminally differentiated PCs.[26] To detect the persistence of PC subsets, we performed BrdU labelling in chromatin-immunised mice and detected high frequency of BrdU$^+$ DP PCs at 14 weeks after in vivo labelling (figure 4C,D), indicating that DP PCs are long-lived PCs.

To ascertain whether TLR4$^+$CXCR4$^+$ PCs play a pathogenic role in LN development, we transferred sorting-purified DP PCs and DN PCs from chromatin-immunised lupus mice into immunodeficient *Rag2*$^{-/-}$ recipients. Compared with DN PCs, transfer of TLR4$^+$CXCR4$^+$ PCs led to markedly enhanced autoantibody responses and glomerulonephritis in recipient *Rag2*$^{-/-}$ mice, in which significantly elevated levels of serum ANA and creatinine were also detected (figure 4E,F). Moreover, elevated serum creatinine levels were observed in DP PC transferred recipients (see online supplementary figure 3) starting from 15 days after cell transfer. Consistently, transfer of DP PCs from MRL/lpr mice also triggered LN in *Rag2*$^{-/-}$ recipients (see online supplementary figure 4). Notably, the levels of *cxcl12* transcripts were markedly upregulated in the kidneys of lupus mice, other chemokines including *cxcl9*, *cxcl10* and *cxcl13* did not show any obvious change in their mRNA levels (see online supplementary figure 5). Together, these data demonstrate the direct involvement of TLR4$^+$CXCR4$^+$ PCs in autoantibody production and nephritis development in lupus mice.

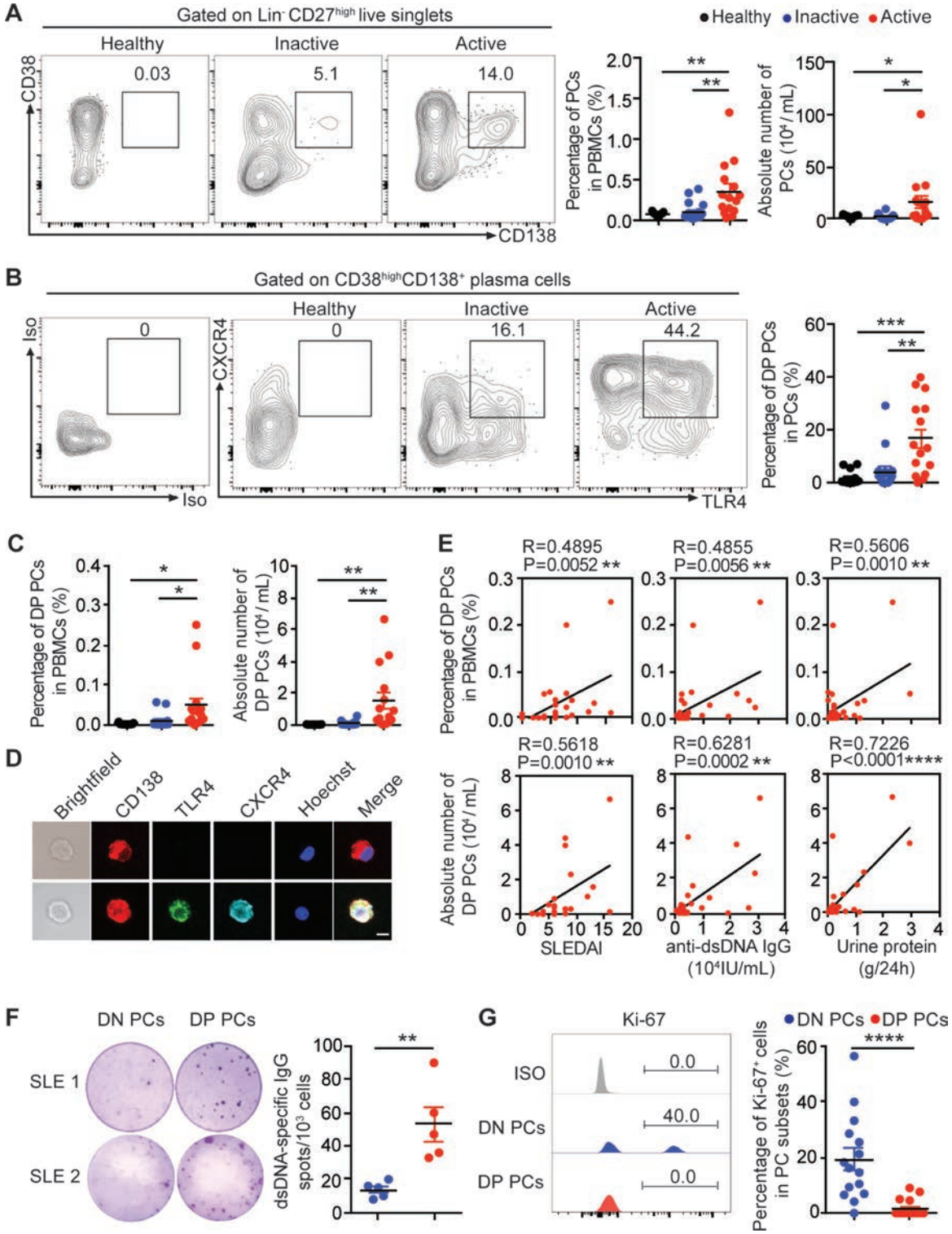

Figure 1 Increased circulating TLR4^{+}CXCR4^{+}plasma cells correlate with disease activities in patients with SLE. (A–C) Representative flow cytometric profiles and data plots show both frequencies and cell numbers of Lin^{-}CD27^{high}CD38^{high}CD138^{+}plasma cells (PC) and Lin^{-}CD27^{high}CD38^{high}CD138^{+}TLR4^{+}CXCR4^{+}double-positive PC subset (DP PCs) in PBMCs from patients with active SLE (SLEDAI ≥6; n=15), patients with inactive SLE (SLEDAI <6; n=16) and healthy controls (n=15). (D) Representative cytospin images show both morphology and immunostaining of TLR4^{-}CXCR4^{-}double-negative PCs (DN PCs, upper panel) and TLR4^{+}CXCR4^{+} PCs (lower panel) in PBMCs from patients with active SLE; original magnification: ×40; scale bar, 5 µm. (E) Data plots show the correlation of both frequencies (upper panels) and cell numbers (lower panels) of circulating DP PCs with SLEDAI scores, serum anti-dsDNA IgG and urine protein levels in patients with SLE (n=31). (F) Representative images (left panel) and data plot (right panel) show dsDNA-specific IgG spots among DP PCs and DN PCs purified from patients with active SLE as detected by enzyme-linked immunospot (ELISPOT) assay (n=5). (G) Representative flow cytometric histograms (left panel) and data plot (right panel) show the frequencies of Ki-67^{+} cells in DP PCs and DN PCs from patients with active SLE. The shaded histogram in grey represents isotype control staining (n=15). Both correlation coefficient R and p values were calculated by the Pearson's correlation test. Data are shown as mean±SEM; two-tailed Mann-Whitney U test. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. PBMC, peripheral blood mononuclear cell; SLE, systemic lupus erythematosus; SLEDAI, SLE Disease Activity Index.

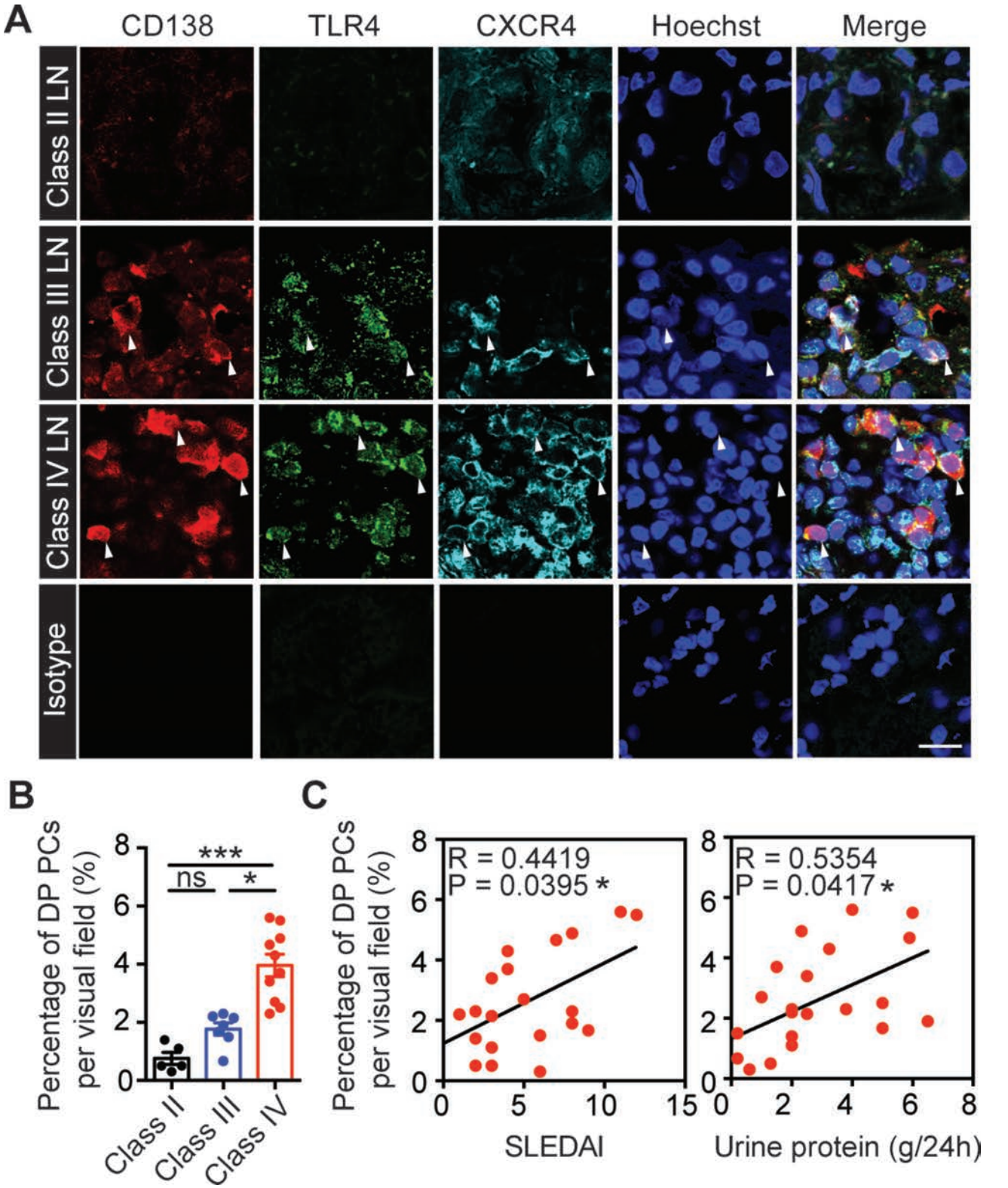

Figure 2 Renal infiltration of TLR4$^+$CXCR4$^+$plasma cells (PC) correlates with kidney pathology of patients with lupus nephritis (LN). (A) Representative confocal images show infiltration of TLR4$^+$CXCR4$^+$in kidney biopsies from patients with systemic lupus erythematosus (SLE) with LN. The upper panel shows renal tissue sample with class II LN whereas the middle panels display class III LN and class IV LN according to the criteria for classification of LN in SLE. The confocal images show immunostaining of FITC, PE and APC-conjugated antibodies as well as isotype controls (bottom panel) in renal biopsies from patients with LN; original magnification: ×40; scale bar, 20 µm. (B) The data show the frequencies of renal-infiltrated DP PCs in patients with class II LN (n=5) and class III LN (n=7) and class IV diffuse LN (n=10), respectively. (C) The data plots show the correlation analysis of renal-infiltrated DP PC frequency with SLEDAI scores and urine protein levels in patients with LN (n=22). Both correlation coefficient R and p values were calculated by the Pearson's correlation test. Each dot represents an individual patient with LN. Data are shown as mean±SEM; one-way analysis of variance (ANOVA) followed by the Newman-Keuls test. *P<0.05; ***P<0.001. APC, allophycocyanin; DP PCs, double-positive PC subset; FITC, fluorescein isothiocyanate; ns, not significant; PE, phycoerythrin; SLEDAI, SLE Disease Activity Index.

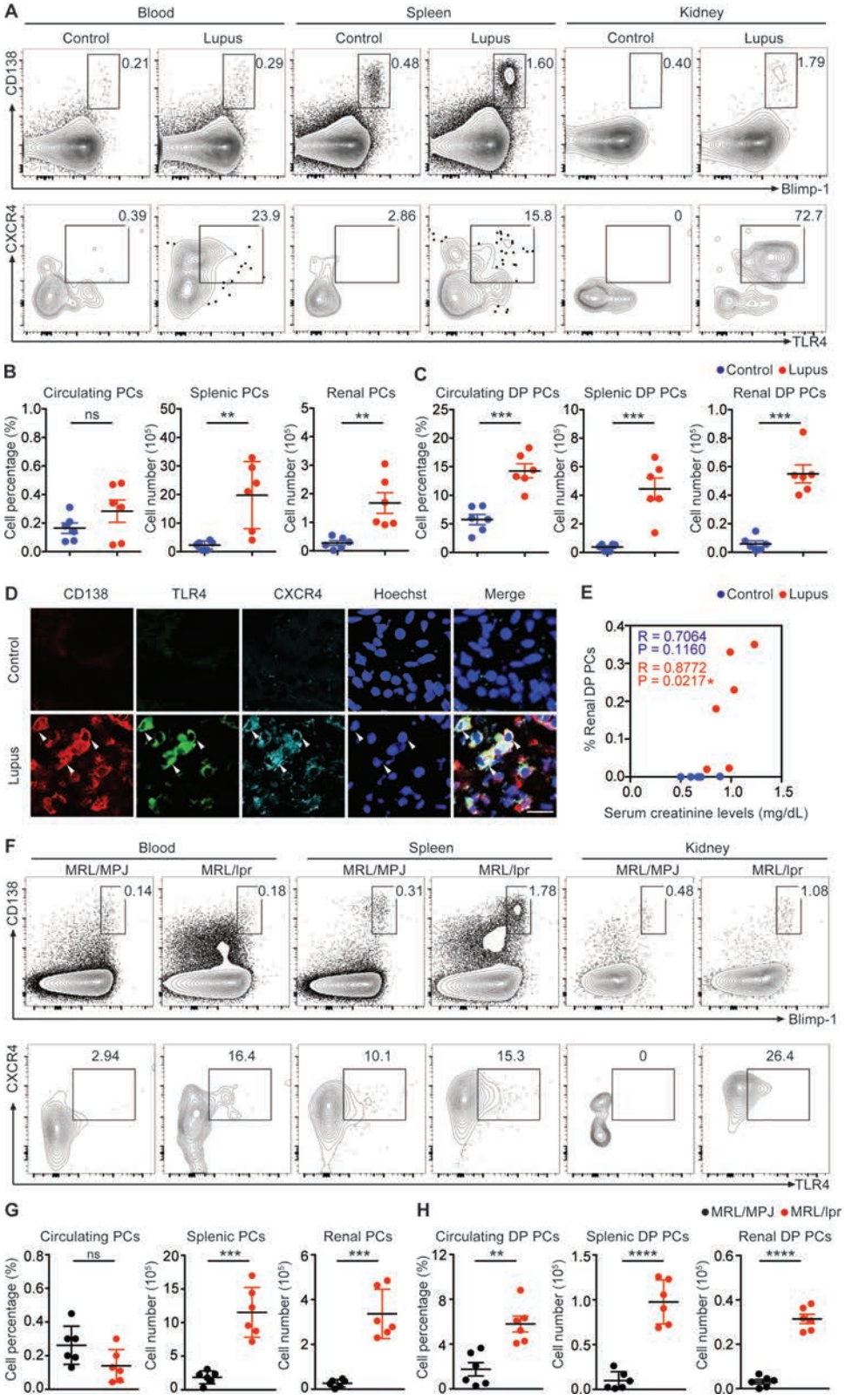

Figure 3 Systemic and local renal increased TLR4$^+$CXCR4$^+$plasma cells (PC) in lupus mice. (A) Representative flow cytometric profiles and data analysis show both frequencies and cell numbers of CD138$^+$Blimp-1$^+$ PCs (B) and CD138$^+$Blimp-1$^+$TLR4$^+$CXCR4$^+$ PCs (C) in peripheral blood, spleens and kidneys of chromatin-induced lupus mice and adjuvant-immunised control mice (n=6 per group). (D) Representative confocal images show renal-infiltrated DP PCs in kidney sections of lupus mice and control mice. Original magnification: ×40; scale bar, 20 µm. (E) Data plot shows the correlation analysis of renal-infiltrated DP PC frequencies with serum creatinine levels in control mice and lupus mice. Both correlation coefficient R and p values were calculated by the Pearson's correlation test. (F) Representative flow cytometry profiles and data plots show both frequencies and cell numbers of CD138$^+$Blimp-1$^+$ PCs (G) and CD138$^+$Blimp-1$^+$TLR4$^+$CXCR4$^+$ DP PCs (H) in peripheral blood, spleens and kidneys of 15-week-age MRL/MPJ and MRL/lpr mice (n=6 per group). Data are obtained from three independent experiments and shown as mean±SEM; unpaired two-tailed Student's t-test. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. DP PCs, double-positive PC subset; ns, not significant.

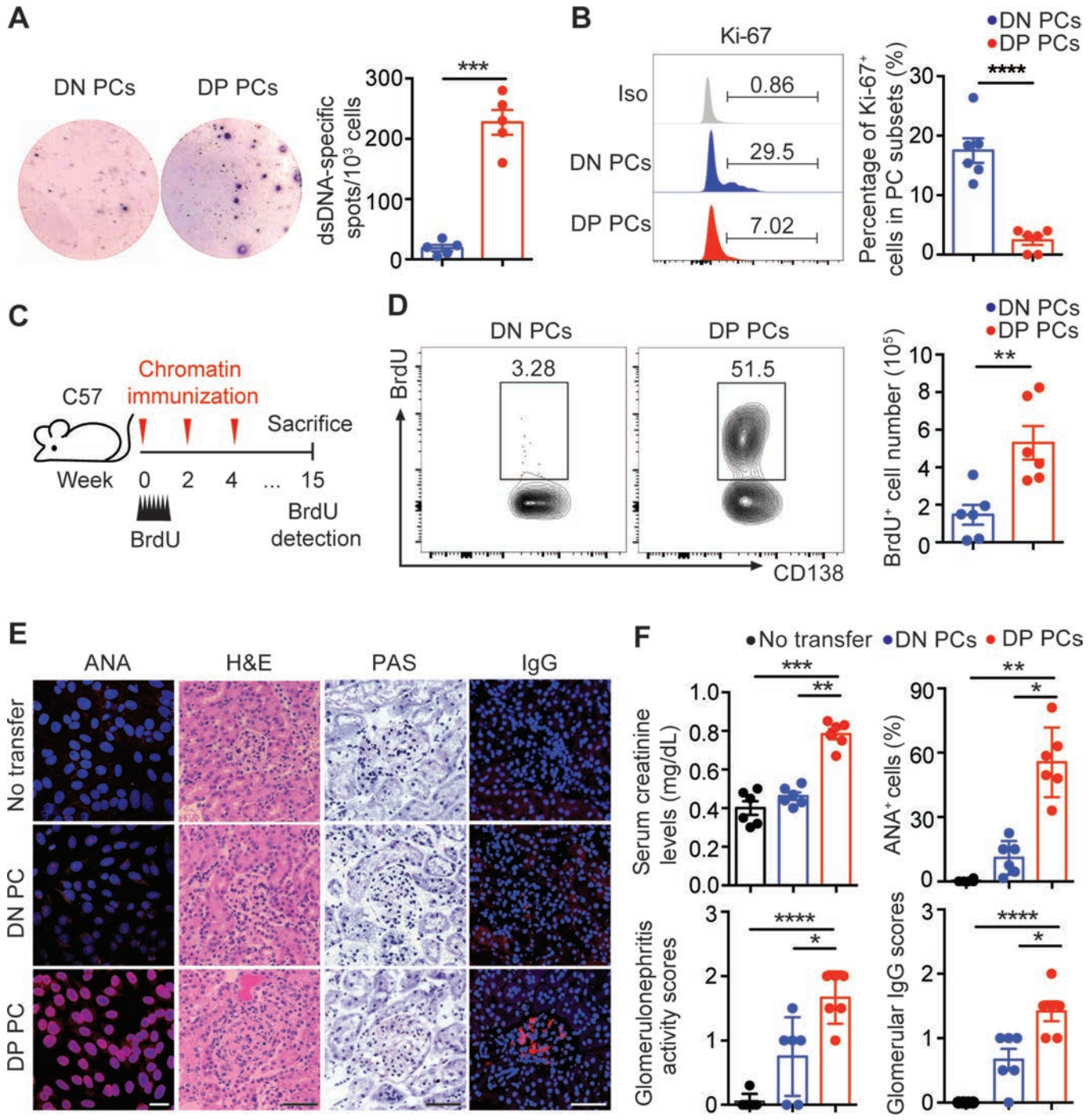

Figure 4 Adoptive transfer of TLR4$^+$CXCR4$^+$plasma cells (PC) drives lupus nephritis in Rag-2-deficient mice. (A) Representative images and data analysis show dsDNA-specific IgG spots among purified DP PCs and DN PCs from lupus mice as detected by enzyme-linked immunospot (ELISPOT) assay (n=5). (B) Representative flow cytometric histograms and data analysis show the frequencies of Ki-67$^+$ cells in DP PCs and DN PCs from lupus mice (n=6). The upper panel shows isotype control staining. (C) The scheme shows the protocol of in vivo BrdU incorporation assay in chromatin-immunised mice followed by flow cytometric analysis 14 weeks later (n=6). (D) Representative flow cytometric profiles show the frequencies and total numbers of BrdU$^+$ DP PCs and DN PCs in the spleens of chromatin-immunised mice. Data are obtained from at least three independent experiments and shown as mean±SEM; unpaired two-tailed Student's t-test. **P<0.01; ***P<0.001; ****P<0.0001. (E) Sorting-purified DN PCs or DP PCs from lupus mice were adoptively transferred into *Rag2*$^{-/-}$ mice, representative ANA staining with serum samples and kidney sections with H&E staining, PAS staining and immunofluorescent staining of IgG in recipient *Rag2*$^{-/-}$ mice with adoptive transfer of DP PCs or DN PCs on day 30 after cell transfer (n=6 per group). Original magnification: ×40; scale bars, 20 µm. (F) Data analysis of serum creatinine levels, quantitative analysis of ANA$^+$ cells and renal damages in *Rag2*$^{-/-}$ mice with transfer of DN PCs or DP PCs. The *Rag2*$^{-/-}$ mice without cell transfer were used as controls. Data are obtained from two independent experiments and shown as mean±SEM; one-way analysis of variance (ANOVA) followed by the Newman-Keuls test. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. ANA, antinuclear autoantibodies; DN PCs, double-negative PC subset; DP PCs, double-positive PC subset; PAS, Periodic acid-Schiff.

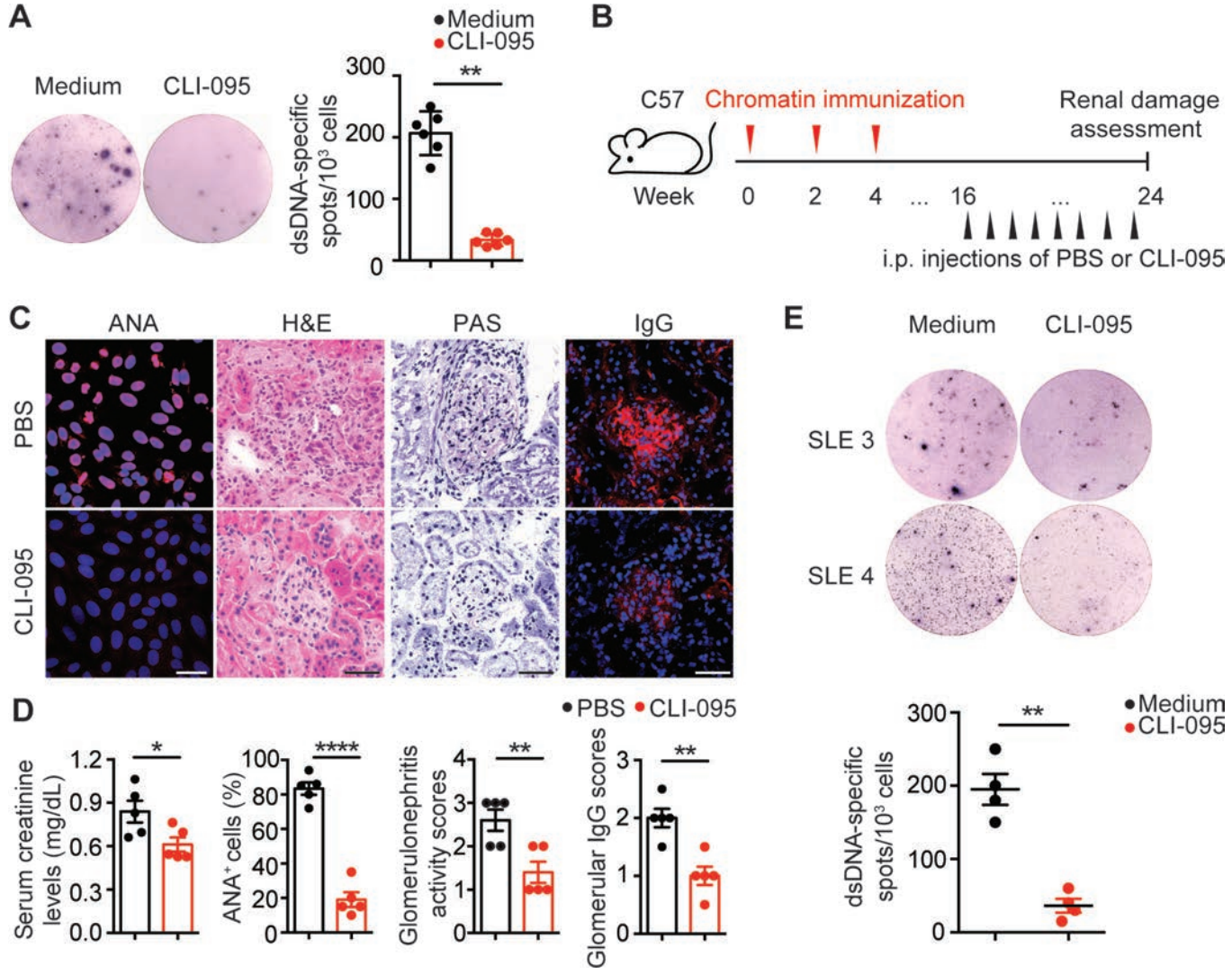

Figure 5 Treatment with TLR4 inhibitor attenuates autoantibody production and ameliorates renal damage. (A) Representative images and data analysis show dsDNA-specific IgG spots among sorting-purified double-positive PC subset (DP PCs) from lupus mice with or without CLI-095 treatment as detected by enzyme-linked immunospot (ELISPOT) assay (n=6). (B) The scheme shows the protocol of chromatin-immunised mice with or without treatment of TLR4 inhibitor CLI-095 (n=5 per group). Representative images (C) and quantitative measures (D) of ANA staining with serum samples and kidney sections with H&E staining, PAS staining and immunofluorescent staining of IgG in lupus mice with or without CLI-095 treatment (20 mg/kg/week) for 8 weeks. Original magnification: ×40; scale bars, 20 µm. Serum creatinine levels were also measured in lupus mice. Data are obtained from at least three independent experiments and shown as mean±SEM; unpaired two-tailed Student's t-test in (A) and (D). *P<0.05; **P<0.01; ****P<0.0001. (E) Representative images and data analysis show dsDNA-specific IgG spots among sorting-purified DP PCs from patients with active SLE with or without CLI-095 treatment as detected by ELISPOT assay (n=4). Data are shown as mean±SEM; Mann-Whitney U test. **P<0.01. ANA, antinuclear autoantibodies; PAS, Periodic acid-Schiff; PBS, phosphate buffered saline; SLE, systemic lupus erythematosus.

Blockade of TLR4 signal inhibits autoantibody production by TLR4$^+$CXCR4$^+$ PCs and ameliorates nephritis development in murine lupus

To determine the role of TLR4 signal in regulating PC function, we treated sorting-purified DP PCs from lupus mice with the TLR4 blocker CLI-095. Interestingly, the frequencies of anti-dsDNA IgG-secreting cells among DP PCs showed a marked reduction by more than fivefold on the treatment with CLI-095 (figure 5A).

To verify whether the blockade of TLR4 signal affects autoreactive PC response and disease progression in vivo, we found that CLI-095-treated lupus mice exhibited markedly reduced renal immune-complex deposition and significantly decreased serum levels of ANA and creatinine. Moreover, histological analysis confirmed significantly ameliorated LN in CLI-095-treated lupus mice (figure 5B–D). To further verify the function of TLR4 signal in human PCs, we cultured DP PCs purified from PBMCs of patients with active SLE in the presence or absence of CLI-095. Consistently, DP PCs from patients with SLE showed a marked fourfold decrease in the frequencies of anti-dsDNA IgG-secreting cells when treated with TLR4 inhibitor as detected by ELISPOT assay (figure 5E). Together, these data suggest the therapeutic potential of blocking TLR4 signal in PCs for lupus treatment.

DISCUSSION

In this study, we have identified a distinct subset of pathogenic TLR4$^+$CXCR4$^+$ PCs that positively correlates with disease activity in patients with SLE. Moreover, the infiltration of

TLR4$^+$CXCR4$^+$ PCs is detected in the renal tissue of patients with SLE, especially in those with class IV nephritis. In addition, we have observed the expansion of both circulating and renal-infiltrated TLR4$^+$CXCR4$^+$ PCs in mice with experimental lupus. Importantly, adoptive transfer of TLR4$^+$CXCR4$^+$ PCs from lupus mice can significantly enhance autoantibody response and immune-complex glomerulonephritis in *Rag-2*-deficient recipients. Together, these results have demonstrated a pathogenic role of TLR4$^+$CXCR4$^+$ PCs in the development of LN.

Numerous studies have focused on elucidating the developmental regulation of B cell differentiation and activation, but much less information is available for the phenotypic features of autoreactive PCs and their functional dysregulations in autoimmune pathogenesis.[27–33] To identify new phenotypes of PC subsets, we have found that TLR4$^+$CXCR4$^+$ PCs are significantly increased in the circulation and renal tissue of both patients with SLE with active disease and lupus mice. Compared with their DN PC counterparts, TLR4$^+$CXCR4$^+$ PCs are potent producers of anti-dsDNA IgG. Notably, TLR4$^+$CXCR4$^+$ PCs show salient features of terminally differentiated PCs since they express high levels of *prdm1* gene that encodes Blimp-1. Moreover, majority of TLR4$^+$CXCR4$^+$ PCs exhibit negative staining for Ki-67 as a non-dividing cell population. In human, naïve B cells lack TLR4 expression while PCs are found to express high levels of TLRs including TLR4.[34] Early studies have reported the expansions of TLR9-expressing PCs and memory B cells and their correlation with the presence of anti-dsDNA antibodies, suggesting that endogenous nucleic acids released by apoptotic cells may contribute to B cell overactivation and autoantibody production via TLR9 stimulation.[35] It has become clear that both exogenous and endogenous TLR4 ligands such as lipopolysaccharide and HMGB1 can activate B cells via TLR4/MD2 signalosome.[36 37] Consistent with a role of TLR4 in PC function and lupus development, recent studies have shown that mice deficient for *tlr4* exhibit ameliorated lupus progression with pristane induction.[20 38] Here, we show that blocking TLR4 signalling with CLI-095 markedly inhibits autoantibody secretion by PCs from both patients with SLE and lupus mice. Moreover, we have observed significantly reduced autoantibody response and ameliorated disease severity in lupus mice treated with CLI-095. Although it is currently unclear which signals or factors account for the expansion of TLR4$^+$CXCR4$^+$ PC subset, it is plausible that endogenous TLR4 ligands such as HMGB1 may be involved in activating this distinct PC subpopulation and promoting autoantibody production. Nevertheless, further studies are needed to elucidate the molecular mechanisms underlying TLR4-mediated signalling pathways in promoting antibody production by PCs in SLE.

Recent studies have shown that adoptive transfer of long-lived PCs from NZB/W mice can migrate to the spleen and bone marrow of immunodeficient *Rag-1* mice and continuously generate autoantibody and immune complex.[8] In this study, we have identified TLR4$^+$CXCR4$^+$ PCs as long-lived PCs by in vivo BrdU labelling. Remarkably, TLR4$^+$CXCR4$^+$ PCs purified from lupus mice, on adoptive cell transfer, can efficiently migrate to the renal tissue (data not shown) and drive nephritis in recipient *Rag2*$^{-/-}$ mice, which is consistent with previous findings that overexpression of CXCR4 and CXCL12 is critically involved in renal infiltration of B cells and nephritis development in lupus-prone B6.*Sle1Yaa*, BXSB and MRL/lpr mice.[21] Together, these data further suggest an important role of the CXCR4/CXCL12 axis in the migration of pathogenic PCs into inflamed organs such as kidney in SLE.[21]

There is compelling evidence that newly generated plasmablasts are short lived while long-lived PCs contribute to the maintenance of immunological memory. In particular, autoreactive long-lived PCs continuously produce pathogenic autoantibodies without antigen stimulation and often reside in the bone marrow and inflamed organs. Recent studies have shown that non-proliferating PCs are resistant to conventional cytotoxic drugs and B cell depletion therapies for SLE.[7 39] Thus, it remains a promising challenge to target long-lived PCs for achieving better efficacies in the treatment of SLE. To correlate our findings from murine lupus with translational studies, we have shown that blocking TLR4 signalling with CLI-095 treatment markedly inhibits autoantibody secretion by PCs from patients with SLE, which further support a critical role of TLR4 signal in mediating autoantibody production and LN.[40] Current therapies that target PCs such as treatment with proteasome inhibitors have proved to be effective for treating autoimmune diseases in both human and mouse, but there is increasing evidence that proteasome inhibitors can also affect T cells.[41] Therefore, our present findings suggest that inhibition of TLR4 signal may represent a selective strategy to target a distinct PC subset for the effective treatment of SLE.

In conclusion, our results have identified TLR4$^+$CXCR4$^+$ PCs as a pathogenic subset in autoantibody response and nephritis development in human and murine lupus. Thus, further validation of these findings may facilitate the development of new approaches to target autoreactive PCs for the treatment of SLE.

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Contributors KM, XW, XL, WD and XY designed and performed mouse experiments and statistical analysis. JL, FM, YF and YZ performed experiments with human samples. KWC, XH, DL, XZ and LS participated in the design of the study and helped in analysing data. KM, LS and LL supervised the study and wrote the manuscript.

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Competing interests None declared.

Patient consent Not required.

Ethics approval All protocols using human samples were approved by Ethics Committee of the Second Affiliated Hospital of Jinan University, Ethics Committee of the First Hospital Affiliated to Army Medical University and Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong Western Cluster (HKU/HA HKW IRB). All protocols involving live animals were approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR) at The University of Hong Kong.

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EXTENDED REPORT

Apoptosis-derived membrane vesicles drive the cGAS–STING pathway and enhance type I IFN production in systemic lupus erythematosus

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ABSTRACT

Objective Despite the importance of type I interferon (IFN-I) in systemic lupus erythematosus (SLE) pathogenesis, the mechanisms of IFN-I production have not been fully elucidated. Recognition of nucleic acids by DNA sensors induces IFN-I and interferon-stimulated genes (ISGs), but the involvement of cyclic guanosine monophosphate (GMP)–AMP synthase (cGAS) and stimulator of interferon genes (STING) in SLE remains unclear. We studied the role of the cGAS–STING pathway in the IFN-I-producing cascade driven by SLE serum.

Methods We collected sera from patients with SLE (n=64), patients with other autoimmune diseases (n=31) and healthy controls (n=35), and assayed them using a cell-based reporter system that enables highly sensitive detection of IFN-I and ISG-inducing activity. We used Toll-like receptor-specific reporter cells and reporter cells harbouring knockouts of cGAS, STING and IFNAR2 to evaluate signalling pathway-dependent ISG induction.

Results IFN-I bioactivity and ISG-inducing activities of serum were higher in patients with SLE than in patients with other autoimmune diseases or healthy controls. ISG-inducing activity of SLE sera was significantly reduced in STING-knockout reporter cells, and STING-dependent ISG-inducing activity correlated with disease activity. Double-stranded DNA levels were elevated in SLE. Apoptosis-derived membrane vesicles (AdMVs) from SLE sera had high ISG-inducing activity, which was diminished in cGAS-knockout or STING-knockout reporter cells.

Conclusions AdMVs in SLE serum induce IFN-I production through activation of the cGAS–STING pathway. Thus, blockade of the cGAS–STING axis represents a promising therapeutic target for SLE. Moreover, our cell-based reporter system may be useful for stratifying patients with SLE with high ISG-inducing activity.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease in which pathogenic autoantibodies are produced against nucleic acids and their interacting proteins, resulting in inflammation and tissue damage. SLE is characterised by flares and remissions, with multiple organ damage ranging from mild rash to severe nephritis, and has a poor prognosis.[1 2]

Although the exact cause of SLE remains unclear, type I interferon (IFN-I) plays important roles in its pathogenesis and progression.[3] Serum IFN-I and expression of multiple IFN-I-stimulated genes (ISGs) in peripheral blood mononuclear cells (PBMCs) are elevated in SLE and associated with SLE disease activity.[4–6] Also, clinical lupus occasionally occurs after treatment of chronic hepatitis with recombinant IFN-α.[7] Furthermore, although SLE is heterogeneous, over 80% of patients exhibit a prevalent IFN signature.[8] Clinical trials revealed that IFN-I-targeting therapies are more effective in patients with high ISG expression.[9 10]

Activation of pattern-recognition receptors (PRRs) is important for IFN-I production. Two major PRR signalling pathways recognise DNA: Toll-like receptor 9 (TLR9) and cyclic GMP–AMP synthase (cGAS)–stimulator of interferon genes (STING).[11 12] These DNA-sensing pathways are crucial for SLE onset. A recent genome-wide association study demonstrated that several alleles of genes related to nucleic acid degradation and IFN-I signalling are associated with the risk of SLE.[13–17] In humans and mice, defective DNA degradation leads to excessive DNA and systemic inflammation via overproduction of IFN-I.[18–21] Also, stimulation of TLR9 by double-stranded DNA (dsDNA) and immune complexes (ICs) from patients with SLE results in IFN-I overproduction.[22 23] TLR9 is important in SLE pathogenesis, but other molecular mechanisms also contribute to IFN-I production.[24 25] Given that cGAS and STING are involved in DNA recognition,[26–28] the cGAS–STING pathway may play a role in SLE.

Although *DNaseII*$^{-/-}$ mice die during development due to overproduction of IFN-I, both *Sting*$^{-/-}$*DNaseII*$^{-/-}$ and *cGAS*$^{-/-}$*DNaseII*$^{-/-}$ prevent embryonic lethality and reduce lupus-like manifestation, despite intact production of autoantibodies.[29–31] In three prime repair exonuclease 1 (*Trex1*)-deficient mice, development of lupus-like symptoms is ameliorated by knockout of cGAS or STING.[30 32] In humans, gain-of-function mutation in STING causes SLE-like disease.[33 34] Furthermore, PBMCs of SLE express higher levels of cGAS, and approximately 15% of patients with SLE express detectable levels of cyclic GMP–AMP (cGAMP) in their PBMCs.[35] However, the role of the cGAS–STING pathway in SLE pathogenesis remains

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unclear. To address this issue, we used patient samples and a cell-based reporter system that enables highly sensitive measurement of serum IFN-I bioactivity and ISG-inducing activity.

MATERIALS AND METHODS

Clinical samples

Subjects were Japanese patients admitted to the Department of Clinical Immunology, Osaka University, from 2012 to 2017. SLE (n=75), systemic sclerosis (SSc) (n=24) and primary Sjögren syndrome (SjS) (n=11) were diagnosed based on the 1997 American College of Rheumatology (ACR) revised criteria for the classification of SLE,[36] the 1980 ACR criteria for the classification of SSc[37] and the American-European Consensus Group 2002 criteria for SjS,[38] respectively. Healthy control (HC) samples were obtained from 31 volunteer donors. Eleven patients were excluded from the study according to the criteria described in online supplementary figure S1. Disease activity of SLE was assessed according to Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) score.[39]

Reporter cell lines

THP1-Blue ISG (THP1-ISG), HEK-Blue IFN-α/β, THP1-Dual, THP1-Dual-KOSTING and THP1-Dual-KOcGAS cells were purchased from InvivoGen. Knockout lines THP1-ISG-KOSTING and THP1-ISG-KOIFNAR2 were generated from THP1-ISG using the CRISPR-Cas9 gene-editing system.[40] Briefly, THP1-ISG was lentivirally transduced with lentiCas9-Blast and lentiGuide-Puro (Addgene) fused with sgRNA. Monoclonal knockout cells were confirmed by western blotting and DNA sequencing.

Cell-based reporter assay

For measurement of IFN-I, HEK-Blue IFN-α/β was incubated with 20% serum. For measurement of ISG-inducing activity, phorbol 12-myristate 13-acetate (PMA)-treated THP1-ISG was cultured with 20% serum. Secreted embryonic alkaline phosphatase (SEAP) in supernatant was detected with QUANTI-Blue (InvivoGen) and quantified by optical density at 620 nm. PBMCs from healthy donors were isolated with Ficoll-Paque Plus (GE Healthcare). PBMCs were incubated with 20% serum, and IFN-I levels in culture supernatant were measured with HEK-Blue IFN-α/β. For isolation of apoptosis-derived membrane vesicles (AdMVs), serum was centrifuged at 1700 g for 15 min to remove cell debris, then centrifuged twice at 16 000 g for 20 min at 4°C, and pellets were reconstituted in culture medium or phosphate buffered saline (PBS).

Extraction and measurement of dsDNA

Extracellular dsDNA in serum or AdMVs was extracted using DNA Extractor SP Kit (Wako) and quantified using the Quant-iT dsDNA Assay Kit (Invitrogen).

Statistical analysis

All statistical analyses were conducted using JMP Pro V.12.2.0. Non-parametric Mann-Whitney U test was used for comparisons between two groups and Steel test was used for comparisons between more than three groups. P values <0.05 were considered statistically significant. Correlations between clinical parameters and ISG-inducing activity or IFN-I level were determined using Spearman's rank correlation coefficient (ρ).

Table 1 Characteristics of patients with SLE and HC

Characteristics and clinical manifestations	SLE (n=64)	HC (n=31)
Age (years)	41 (34–51)	23 (22–25)
Sex (female)	60 (93.7)	6 (19.3)
Duration of disease (years)	7 (0.625–15)	
SLEDAI-2K	11.5 (8–20)	
PSL (mg/day)	11.25 (0.5–25)	
WBC (x 10^9/L)	5.840 (4.327–8.947)	
Hb (g/dL)	11.1 (9.025–12.375)	
Plt (/μL)	204 (107.25–262.5)	
dsDNA antibody (IU/mL)	40 (8.8–140)	
C3 (mg/dL)	69 (53–94.5)	
C4 (mg/dL)	11 (7–16.5)	
CH50 (CH50/mL)	30.3 (13.7–45.85)	
C1q (μg/mL)	2.8 (1.5–6.75)	
g-Cre	1.15 (0.217–3.063)	
CRP (mg/dL)	0.12 (0.04–0.6075)	
Ferritin (ng/dL)	140 (68–377)	
Rash	14 (21.8)	
Oral ulcers	7 (10.9)	
Arthritis	23 (35.9)	
Serositis	15 (23.4)	
Nephritis	35 (54.6)	
NPSLE	5 (7.8)	

Data are presented as n (%) or median (IQR).
CRP, C-reactive protein; dsDNA, double-stranded DNA; Hb, hemoglobin; HC, healthy control; NPSLE, neuropsychiatric systemic lupus erythematosus; Plt, platelet; PSL, prednisolone; SLE, systemic lupus erythematosus; WBC, white blood cell.

RESULTS

Elevated IFN-I bioactivity in SLE serum

In the Osaka University Hospital database, we searched for patients with SLE who satisfied the ACR revised criteria for classification of SLE.[36] Sixty-four patients with SLE were enrolled in this study (see online supplementary figure S1). The clinical characteristics of patients and HCs are summarised in table 1. For comparison, 24 patients with SSc[37] and 11 patients with SjS[38] were also enrolled (see online supplementary table S1). To measure the bioactivity of IFN-I in serum, we used HEK-Blue IFN-α/β, which produces SEAP in response to IFN-I (see online supplementary figure S2A,B). IFN-I bioactivity was significantly higher in SLE sera than in sera from HC or patients with other connective tissue diseases (figure 1A).

Induction of ISGs by SLE serum

Progression of SLE is associated with upregulation of IFN-I and ISGs.[4–6] Using THP1-ISG cells, we investigated whether SLE serum could promote further IFN-I production. These cells secrete SEAP in response to various ISG-inducing stimuli, including IFN-I, 2'3'-cGAMP, cyclic-diAMP (c-diAMP) and lipopolysaccharide (see online supplementary figure S2C,D). ISG-inducing activity was considerably higher in SLE sera than in sera from HC, SjS or SSc (figure 1B). Although ISGs are upregulated in the PBMCs of SLE, SjS and SSc,[41 42] only SLE sera had significant ISG-inducing activity.

IFN-I-independent ISG-inducing activity in SLE serum

We next examined the contribution of IFN-I in ISG induction by blocking IFN-I with antibodies against IFN-α, IFN-β and

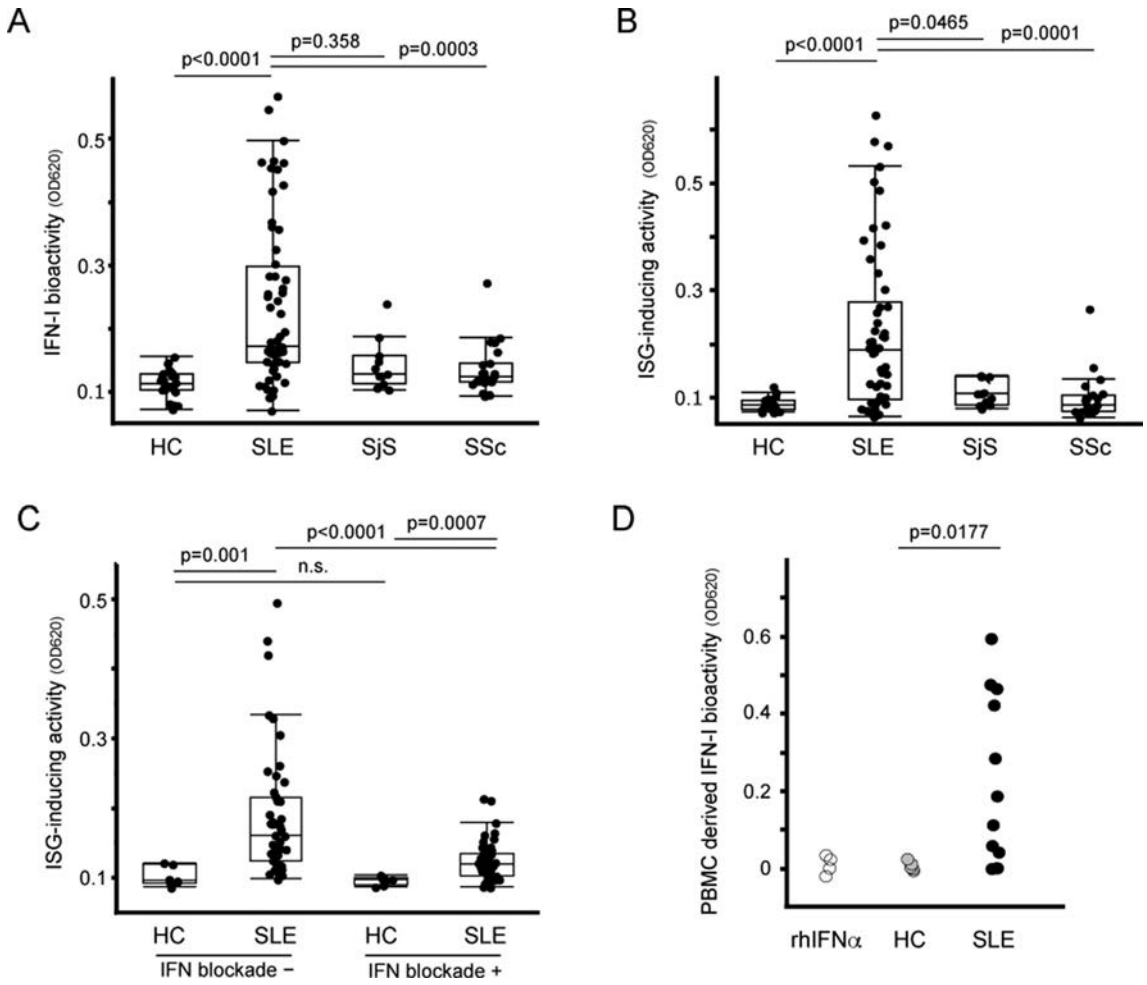

Figure 1 High IFN-I levels and ISG-inducing activities of SLE serum. (A) IFN-I bioactivities in sera. IFN-I bioactivities in SLE (n=54), SjS (n=11), SSc (n=24) and HC (n=24) were measured using HEK-Blue IFN-α/β reporter cells. (B) ISG-inducing activities in sera. Levels of ISG-inducing activity in SLE (n=60), SjS (n=11), SSc (n=24) and HC (n=24) were measured using THP1-ISG reporter cells. (C) ISG-inducing activity of SLE sera with IFN-I signalling blockade. THP1-ISG reporter cells were stimulated with sera from SLE (n=48) and HC (n=7) in the absence (IFN blockade –) or presence (IFN blockade +) of antibody cocktail (anti-IFN-α, anti-IFN-β and anti-IFNAR2). (D) IFN-I production in PBMCs induced by SLE sera. Recombinant human IFN-α(rhIFNα), SLE sera (n=12) or HC sera (n=5) were incubated for 24 hours with or without PBMCs from healthy donors. Levels of IFN-I in the culture supernatants were measured using HEK-Blue IFN-α/β reporter cells. PBMC-derived IFN-I levels were calculated by subtracting the IFN-I levels in PBMC-absent medium from the IFN-I levels in PBMC-present medium. The box chart indicates the 75th percentile (upper), median (middle) and 25th percentile (lower) (A–C). Overall statistical differences between SLE and other groups were evaluated by Steel test (A,B). Overall differences between the groups were evaluated by Mann-Whitney U test (C,D). HC, healthy control; IFN-I, type I interferon; ISG, IFN-I-stimulated genes; PBMC, peripheral blood mononuclear cells; SjS, Sjögren syndrome; SLE, systemic lupus erythematosus; SSc, systemic sclerosis.

IFNAR2 (see online supplementary figure S3A). Although ISG induction was reduced by IFN-I signalling blockade, ISG-inducing activity still remained in SLE sera (figure 1C). To confirm this, we established IFNAR2-knockout THP1-ISG (THP1-ISG-KOIFNAR2) (see online supplementary figure S3B,C).[40] Even in these cells, ISG-inducing activity was observed with SLE sera (see online supplementary figure S3D). We then evaluated the effect of SLE serum in human PBMCs. PBMCs from healthy donors did not produce additional IFN-I on stimulation with recombinant IFN-α/β (see online supplementary figure S3E). However, SLE sera induced significant IFN-I production in the PBMCs (figure 1D). Thus, ISG induction by SLE serum is driven by not only IFN-I but also other factors.

Association of ISG-inducing activity and TLR9

Loss of tolerance to nucleic acids and impaired DNA degradation are thought to be important in SLE pathogenesis.[18 19] We purified and quantified extracellular DNA in sera and found that the amount of dsDNA was higher in SLE than in HC (figure 2A). Because TLR9 is a major dsDNA sensor,[11] we investigated its involvement in SLE serum-mediated inflammation. THP1-ISG was unresponsive to agonists of TLR9 (figure 2B and online supplementary figure S4A), TLR7 and TLR8 (see online supplementary figure S4B). To assess the involvement of TLR9, we used HEK-Blue TLR9 (see online supplementary figure S4C,D). Some SLE sera activated HEK-Blue TLR9, but this activation did not correlate with the levels of IFN-I-independent ISG-inducing activity (figure 2C). Because TLR7 plays important roles in SLE pathogenesis,[43] we examined the involvement of TLR7 and TLR8 (see online supplementary figure S4E,F). Although sera from several patients activated TLR7 and TLR8, the levels of activation did not correlate with IFN-I-independent ISG-inducing activity (see online supplementary figure S5A,B). Therefore, pathways other than TLR7, TLR8 or TLR9 mediated the ISG induction by SLE sera.

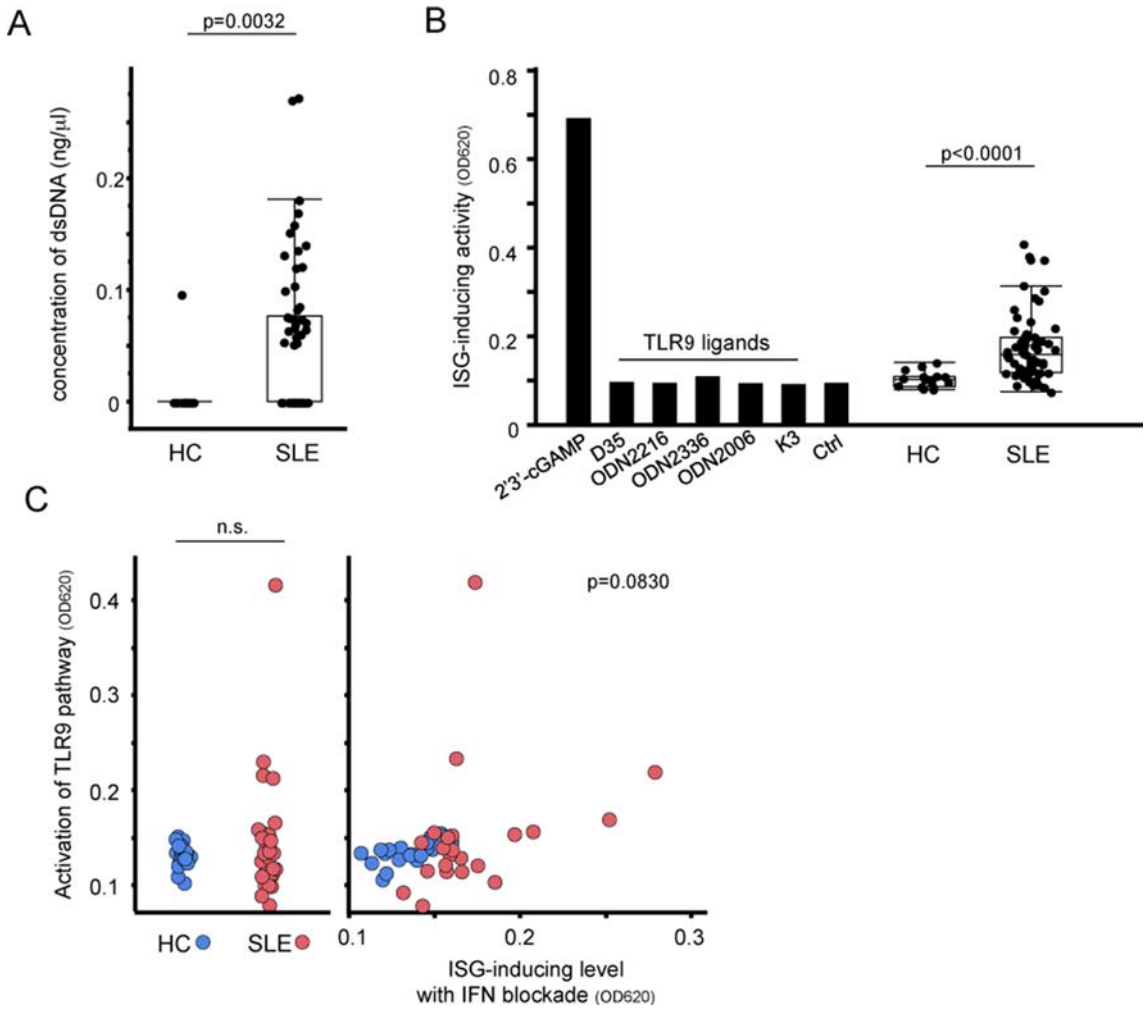

Figure 2 Elevated levels of dsDNA in SLE serum and involvement of TLR9. (A) dsDNA in sera. DNA was purified from SLE (n=64) and HC (n=18) sera and quantified. (B) THP1-ISG stimulated with TLR9 agonists. THP1-ISG cells were stimulated for 24 hours with STING agonist (5 µg/mL 2'3'-cGAMP), TLR9 agonist (10 µg/mL D35, 500 nM ODN2216, 10 µg/mL ODN2336, 10 µg/mL ODN2006, 10 µg/mL K3), control medium (Ctrl) or sera from SLE (n=64) and HC (n=17). (C) IFN-I-independent ISG-inducing activities and correlation with activation of TLR9. HEK-Blue TLR9 cells were stimulated with sera from SLE (n=22) and HC (n=20) for 24 hours. Levels of TLR9 pathway activation induced by sera from SLE (red circle) or HC (blue circle) are shown in the left panel. Correlation between TLR9 activation (y-axis) and ISG-inducing activity following IFN-I blockade (x-axis) is shown in the right panel. The box chart indicates the 75th percentile (upper), median (middle) and 25th percentile (lower) (A,B). Statistical analysis was performed by Mann-Whitney U test (A, B and left panel of C) and Hoeffding's D statistic (right panel of C). cGAMP, cyclic GMP–AMP; dsDNA, double-stranded DNA; HC, healthy control; IFN-I, type I interferon; ISG, IFN-I-stimulated genes; SLE, systemic lupus erythematosus; STING, stimulator of interferon genes; TLR9, Toll-like receptor 9.

Induction of ISGs by SLE serum via the STING pathway

The cGAS–STING pathway is another dsDNA sensor.[12] To evaluate its contribution in SLE serum-mediated inflammation, we established STING-knockout THP1-ISG (ISG-KOSTING) (figure 3A). When these cells were stimulated with SLE sera, most samples exhibited reduced ISG-inducing activity (figure 3B), suggesting that ISG induction was at least partially dependent on STING pathway. STING-dependent ISG-inducing activity correlated with the IFN-I-independent ISG-inducing activity of SLE sera (figure 3C). Thus, activation of the STING pathway may be responsible for high IFN-I levels in SLE.

Activation of the cGAS–STING pathway by AdMVs in SLE serum

The STING agonist 2'3'-cGAMP is synthesised by cGAS after dsDNA recognition.[12] Hence, we investigate whether STING agonists were present in serum. Serum was analysed using triple quadrupole-type liquid Chromatography-Mass spectrometry (LCMS), which was capable of detecting 2'3'-cGAMP and c-diAMP at concentrations as low as 100 fmol/mL (see online supplementary figure S6A). Eight samples with STING-dependent ISG induction were analysed; however, neither 2'3'-cGAMP nor c-diAMP was detected (see online supplementary figure S6B). Consistent with this, ISG induction by SLE sera was not reduced even after cGAMP degradation with ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) (see online supplementary figure S6C).[44] Thus, 2'3'-cGAMP and c-diAMP were not present in SLE serum.

Next, we examined the involvement of dsDNA in ISG induction. However, ISG-inducing activity was not diminished by DNase I treatment (figure 4A). We also investigated the involvement of ICs, but neither anti-FcγRI (CD64) antibody nor non-specific polyclonal IgG suppressed ISG induction by SLE sera (see online supplementary figure S7A,B). Hence, we examined the role of extracellular membrane vesicles in serum. To this end, we isolated AdMVs from serum (see online supplementary figure

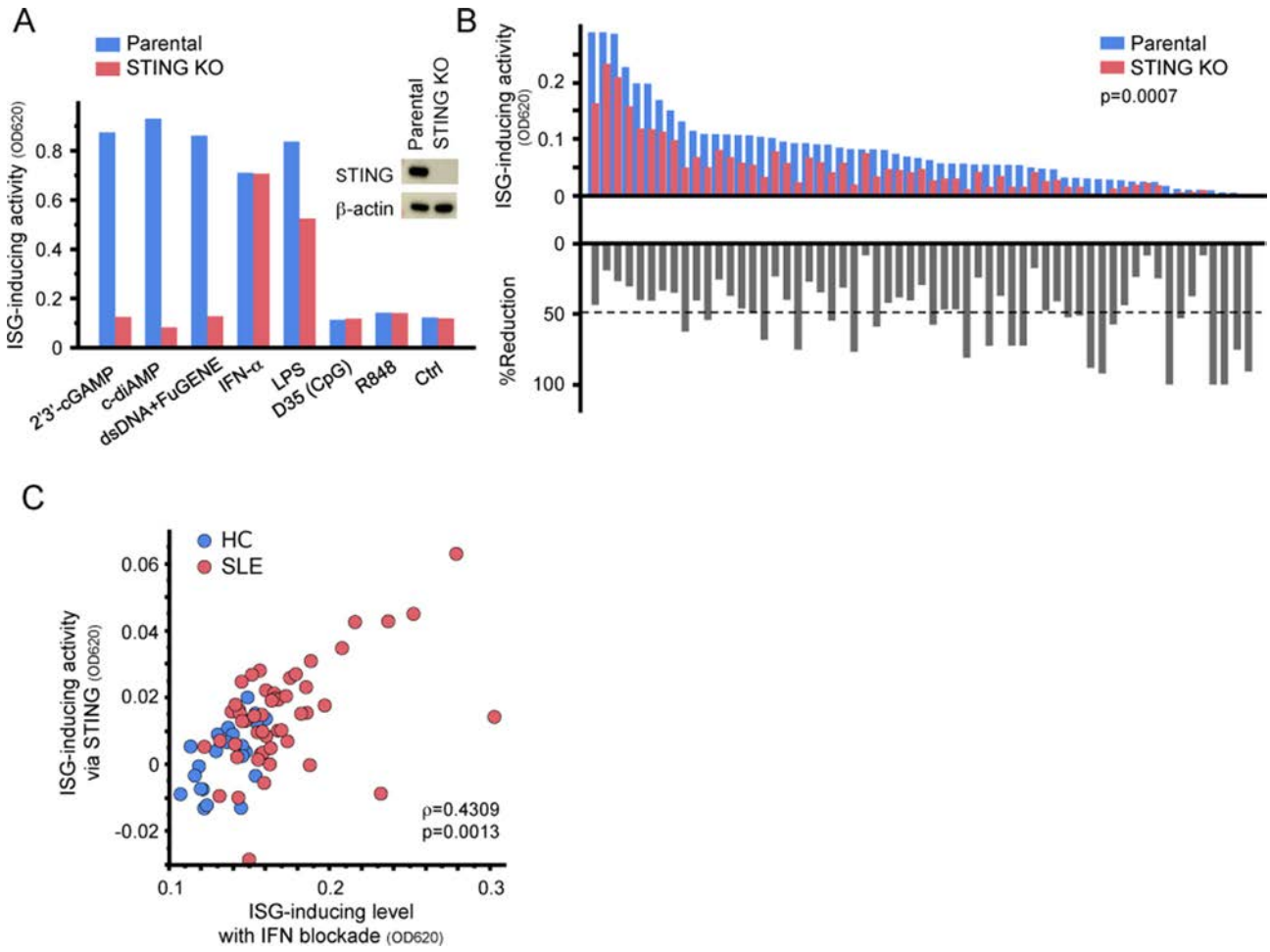

Figure 3 Serum-induced ISG expression via STING. (A) Establishment of STING-knockout reporter cells (ISG-KOSTING). Expression of STING was evaluated by western blotting. ISG-KOSTING cells did not respond to STING agonists (bar graph). Parental THP1-ISG (Parental) and ISG-KOSTING cells were stimulated with STING agonists (5 µg/mL 2'3'-cGAMP, 10 µg/mL c-diAMP), cGAS agonists (dsDNA+FuGENE), cytokine (1000 U/mL IFN-α), TLR agonists (500 ng/mL LPS, 10 µg/mL D35, or 10 µg/mL R848) or control medium (Ctrl) for 24 hours. Statistical analysis was performed by Mann-Whitney U test. (B) ISG-inducing activity in THP1-ISG-KOSTING. Parental THP1-ISG and THP1-ISG-KOSTING cells were stimulated with SLE sera (n=59) for 24 hours (upper bars). Each bar represents serum from an individual patient. %Reduction indicates the rate of reduction in reporter activity of THP1-ISG-KOSTING relative to that of parental THP1-ISG (lower bars). Dashed line indicates the mean of %reduction (C) Correlation between IFN-I-independent ISG-inducing activity and STING-dependent ISG-inducing activity. STING-dependent ISG-inducing activity was calculated by subtracting the absorbance of SEAP in THP1-ISG-KOSTING from that in parental THP1-ISG. (B). Statistical analysis was performed by Mann-Whitney U test (B) and non-parametric Spearman's rank test (C). c-diAMP, cyclic-diAMP; cGAMP, cyclic GMP–AMP; cGAS, cyclic GMP–AMP synthase; dsDNA, double-stranded DNA; HC, healthy control; IFN-I, type I interferon; ISG, IFN-I-stimulated genes; LPS, lipopolysaccharide; SEAP, secreted embryonic alkaline phosphatase; SLE, systemic lupus erythematosus; STING, stimulator of interferon genes; TLR, Toll-like receptor.

S8A–C). dsDNA was contained in AdMVs and protected from DNase I degradation (figure 4B,C). We then cultured PBMCs with the isolated AdMVs and measured IFN-I in the culture supernatant. AdMVs from SLE sera facilitated IFN-I production, whereas those from HC did not (figure 4D). In addition, AdMV-induced ISG expression in THP1-Dual, a THP1-ISG reporter with luciferase activity instead of SEAP production, was decreased by knockout of cGAS or STING (figure 4E and online supplementary figure S9A,B). These results indicate that AdMVs in SLE serum promote IFN-I production and ISG induction through the cGAS–STING pathway.

Clinical features and ISG-inducing activity of SLE serum

Finally, we investigated the relationship between clinical features and the IFN-I bioactivity or ISG-inducing activity of SLE serum. As shown in figure 5A, the ISG-inducing activity of SLE sera correlated with the SLEDAI-2K score.[39] Likewise, the IFN-I bioactivity, STING-dependent ISG-inducing activity and IFN-I-independent ISG-inducing activity also individually correlated with SLEDAI-2K (see online supplementary figure S10A). Patients with high ISG-inducing activity manifested lymphocytopaenia, low serum C4 levels and high anti-dsDNA antibody titres (figure 5B–D and online supplementary figure S10B). The presence of lupus nephritis did not affect the levels of IFN-I or ISG-inducing activity (figure 5E and online supplementary figure S10C). These results suggested that the ISG-inducing activity of serum measured by reporter cells could be a useful biomarker for assessing SLE disease activity.

DISCUSSION

In this study, we showed that SLE serum induced ISGs, in part, through cGAS–STING pathway. In addition, we found that dsDNA was present in SLE serum, and that AdMVs in SLE serum facilitated ISG expression via the cGAS–STING pathway. Using

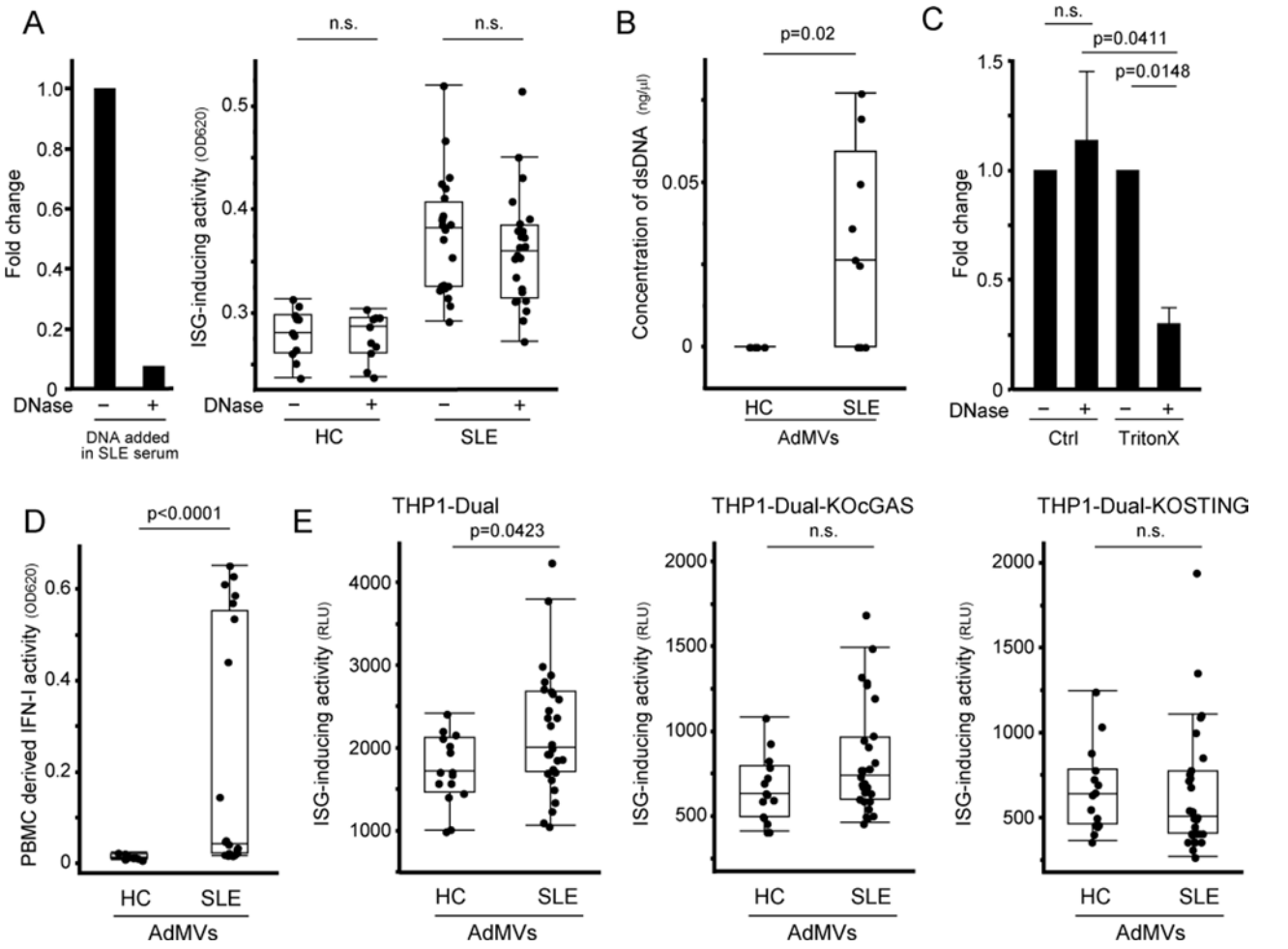

Figure 4 Induction of IFN-I production and ISG expression by apoptosis-derived membrane vesicles from SLE serum. (A) ISG-inducing activity of SLE sera after DNase I treatment. DNase I activity in serum was confirmed by degradation of DNA added in SLE serum (left). After sera from SLE (n=24) and HC (n=11) were pretreated with or without DNase I, THP1-ISG was stimulated with pretreated sera for 24 hours (right). (B) Concentration of dsDNA in AdMVs from sera. DNA was purified from AdMVs of SLE (n=9) and HC (n=6) and quantified. (C) Degradation of dsDNA in AdMVs from SLE (n=6) using DNase I with or without membrane-rupturing treatment. Membrane of AdMVs was permeabilised with Triton X-100 prior to DNase I treatment. (D) Induction of IFN-I production in PBMCs by AdMVs from SLE sera. AdMVs isolated from SLE (n=21) and HC (n=11) sera were cocultured with PBMCs from healthy donors for 24 hours. IFN-I bioactivity in the culture supernatants was measured using HEK-Blue IFN-α/β. (E) cGAS and STING-dependent ISG induction by AdMVs. THP1-Dual, THP1-Dual-KOcGAS and Dual-KOSTING cells were cultured with AdMVs from SLE (n=29) or HC (n=15) for 24 hours. ISG-inducing activity was evaluated by measuring luciferase activity in culture supernatants. The box chart indicates the 75th percentile (upper), median (middle) and 25th percentile (lower) (right panel of A, B, D, E). Values are mean±SEM (C). Statistical analysis was performed by Mann-Whitney U test (A, B, D, E) or Steel test (C). AdMV, apoptosis-derived membrane vesicles; cGAS, cyclic GMP–AMP synthase; dsDNA, double-stranded DNA; HC, healthy control; IFN-I, type I interferon; ISG, interferon-stimulated genes; PBMC, peripheral blood mononuclear cells; SLE, systemic lupus erythematosus; STING, stimulator of interferon genes.

a cell-based reporter system, we demonstrated that the levels of IFN-I bioactivity and ISG-inducing activity were associated with SLE disease activity. Together, our results elucidate a possible pathological mechanism of SLE in which AdMVs drive IFN-I production through the cGAS–STING pathway (see online supplementary figure S11).

TLRs are major receptors in innate immunity that recognise nucleic acids. Endosomal TLRs are important in pathogenic overexpression of IFN-I and ISGs in SLE.[15 17 22 43 45 46] However, the identification of cGAS and STING[26–28] implicated their roles in the onset of SLE. In murine models, the role of cGAS–STING seems to be controversial. For instance, *Sting*$^{-/-}$MRL/*Fas*^{lpr} mice exhibit more severe disease phenotype,[47] while deficiency of cGAS or STING in *DNaseII*$^{-/-}$ mice results in reduced lupus-like symptoms.[30 32 35] In humans, constitutively active mutations of STING are associated with SLE-like diseases.[33 34] Although these studies suggest that the cGAS–STING pathway contributes to lupus pathophysiology, it remains unclear whether cell intrinsic or extrinsic factors activate this pathway. Recent studies reported that cGAMP is present in SLE-derived PBMCs,[35] and that cGAMP is released into the extracellular space upon stimulation.[48–50] We studied this phenomenon using ultra-high-sensitivity LCMS, but detected neither 2'3'-cGAMP nor c-diAMP in the SLE sera. However these experiments were limited by the small sample size. Here, we showed that the amount of dsDNA in SLE sera was high. SLE serum-induced ISG expression was mediated by cGAS and STING, suggesting that cell-extrinsic dsDNA activated the cGAS–STING pathway. Consistent with our findings, oxidised mitochondrial DNA released by NETosis can drive the cGAS–STING pathway.[51 52] Our study did not address the characteristics of dsDNA in serum, such as fragment size, physicochemical properties, methylation status and histone

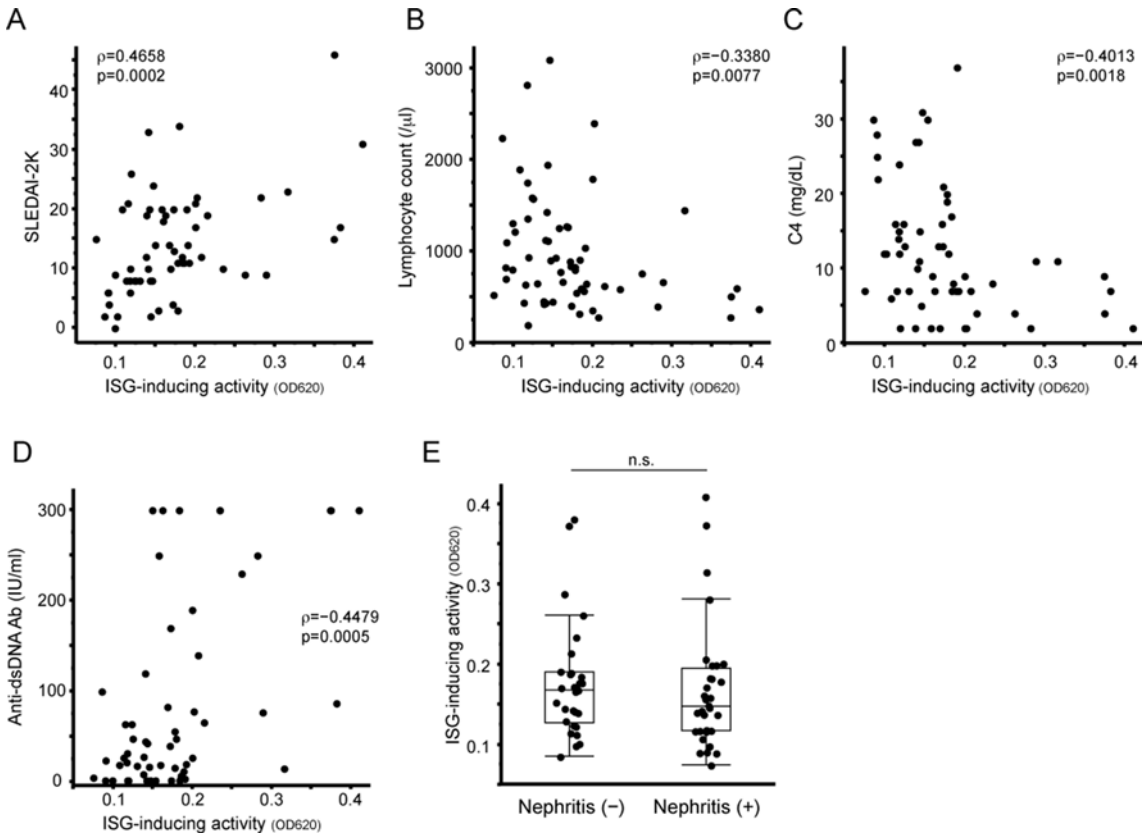

Figure 5 Relationships between ISG-inducing activity of SLE serum and clinical features of SLE. (A) Correlation between ISG-inducing activity and SLE disease activity, assessed according to SLEDAI-2K. (B–D) Association of ISG-inducing activity and clinical features of SLE. Patients with high levels of ISG-inducing activity manifested lymphocytopaenia (B), low serum C4 levels (C) and high anti-dsDNA antibody titres (D). All clinical data were obtained from the medical records of Osaka University Hospital. (E) ISG-inducing activity and lupus nephritis. Patients with SLE were divided into two groups according to the presence (n=35) or absence (n=29) of lupus nephritis. ISG-inducing activity of SLE sera (n=64) was evaluated using THP1-ISG cells (A–E). The box chart indicates the 75th percentile (upper), median (middle) and 25th percentile (lower) (E). Statistical analyses were performed by non-parametric Spearman's rank test (A–D) or Mann-Whitney U test (E). dsDNA, double-stranded DNA; IFN-I, type I interferon; ISG, IFN-I-stimulated genes; SLE, systemic lupus erythematosus.

modification. Therefore, further investigation of these features is necessary.

One possible delivery mechanism for extracellular dsDNA into the cytosol is engulfment of ICs.[53] We assessed the contribution of ICs in SLE serum-mediated ISG induction by blocking FcγR. However, blocking engulfment of ICs did not affect ISG induction, suggesting that ICs did not mediate ISG-inducing activity in our experimental setting.

We observed that AdMVs isolated from SLE serum contain dsDNA and induce ISG expression. Nucleic acids present in extracellular vesicles are protected from enzymatic degradation.[54] In accordance with this, dsDNA in AdMVs was resistant to DNase I and SLE serum-mediated ISG induction was unchanged by DNase I treatment. Consistent with our findings, SLE-derived AdMVs can activate immune cells, resulting in enhanced IFN-I secretion.[55–57] In most studies, AdMVs are harvested from culture supernatant of PBMCs in which apoptosis has been artificially induced by ultraviolet radiation. By contrast, using a cell-based reporter system, we directly showed that AdMVs in SLE sera induced IFN-I production. Additionally, we demonstrated that AdMV-mediated ISG induction was cGAS-dependent and STING-dependent. In regard to the difference of AdMVs from SLE and those from HC, there are several possibilities: increased amount of AdMVs in SLE serum, increased amount of dsDNA in AdMVs, unique property of dsDNA in AdMVs, the nature of the membrane of AdMVs which may allow efficient engulfment by scavenger cells and so on. In our results, the amount of dsDNA in AdMVs was higher in SLE than that in HC. However, we could not address other possibilities. Further studies are needed to clarify the characteristics of AdMVs and their association with clinical manifestations.

Human TLR9 is highly expressed in certain immune cells, but at low levels in monocytes, macrophages and non-haematopoietic cells.[58 59] Nonetheless, high ISG expression has been observed in these cell types in patients with SLE. Several studies showed that TLR9 expression is downregulated in the PBMCs of SLE.[16 60] Additionally, the TLR9 response of plasmacytoid dendritic cells is transient and decreases with continuous exposure to ligands,[24 25] suggesting that TLR9-independent mediators are important for chronic and durable IFN-I production. The THP1 cells we used were unresponsive to agonists of TLR7, TLR8 and TLR9. Therefore, THP1 is suitable for evaluating the involvement of cytosolic nucleic acid sensors other than TLRs. Various cytosolic DNA sensors (eg, TLR9, cGAS, AIM2 and others) recognise dsDNA,[11 12] but functionally dominant sensors may depend on the cell type and inflammatory condition. In cells expressing low levels of TLR9, the cGAS–STING pathway may be the major sensor of extracellular DNA that ultimately leads to sustained IFN-I production in SLE.

In some patients with SLE, the dependence of IFN-I production on the cGAS–STING pathway was partial, suggesting the involvement of other factors. Cytosolic RNA can activate

IFN-I production.[11] Some SLE sera activated reporter cells for TLR7 and TLR8, suggesting that RNA may also be present in SLE serum. It is possible that RNA-sensing pathways might be involved in the SLE serum-induced IFN-I production. Therefore, further investigation of the roles of RNA sensors in SLE is warranted.

In terms of clinical indicators, we demonstrated that both IFN-I and ISG-inducing activity were higher in sera of SLE than other autoimmune diseases. The levels of IFN-I and ISG induction correlated with SLE disease activity. Stratification of patients with SLE based on molecular profiles is important because the clinical symptoms and gene expression profiles are heterogeneous.[1 2 8] The expression levels of ISGs are thought to be useful biomarkers for SLE stratification. Accordingly, treatments that target IFN-I have been shown to be effective in patients with high ISG expression.[9 10] However, the current method for evaluating ISG expression is still complicated,[9 10 41] and the sensitivity of ELISA is insufficient.[6 24] Because our cell-based reporter system enables easy and highly sensitive evaluation of both IFN-I and ISG induction, it may be useful for stratifying patients with SLE.

It should be noted that the number of samples in this pilot study might be insufficient to definitively determine the clinical significance of serum ISG-inducing activity. Also, the possibility of sampling bias cannot be excluded. Moreover, several issues still require clarification: whether the ISG-inducing activity of serum depends on the clinical features and whether measurement of ISG-inducing activity is useful for evaluating or predicting clinical progression and efficacy of treatment. Multicentre and prospective studies are needed to answer these questions.

Collectively, we have shown that SLE serum significantly induces ISG expression and IFN-I production. In mechanistic terms, AdMVs facilitate the IFN-I-producing cascade in a cGAS–STING-dependent manner. Accordingly, we propose the following model of SLE pathophysiology. First, defective clearance of apoptotic cells produces AdMVs containing dsDNA, which in turn induces IFN-I production via the cGAS–STING pathway. Next, IFN-I activates immune responses that lead to tissue damage in various organs, resulting in further generation of AdMVs, triggering a positive-feedback loop of IFN-I production and further tissue damage (see online supplementary figure S11). In this context, it may be promising to target the cGAS–STING pathway for SLE treatment, and our cell-based reporter system may be useful for stratifying patients with SLE with high ISG-inducing activity.

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Contributors HT and YKa designed the project. YKa and JP performed most experiments. YKa analysed the data. HT, YKa and JP wrote the manuscript. SA and WA performed the MS analysis. HK assisted with the experiments. SK, YH and YKi participated in the discussions. MNi, TH, YS and MNa collected clinical samples and provided critical suggestions. MU provided critical collaborative suggestions regarding the MS analysis. AK oversaw the study.

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Competing interests None declared.

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Ethics approval Enrolment of all participants was approved by the institutional review boards of Osaka University. Informed consent was obtained from all subjects in accordance with the Declaration of Helsinki and with approval of the ethical review board of Osaka University (no 12456-3 and 11122-4).

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EXTENDED REPORT

Cereblon modulator iberdomide induces degradation of the transcription factors Ikaros and Aiolos: immunomodulation in healthy volunteers and relevance to systemic lupus erythematosus

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ABSTRACT

Objectives *IKZF1* and *IKZF3 (*encoding transcription factors Ikaros and Aiolos) are susceptibility loci for systemic lupus erythematosus (SLE). The pharmacology of iberdomide (CC-220), a cereblon (*CRBN*) modulator targeting Ikaros and Aiolos, was studied in SLE patient cells and in a phase 1 healthy volunteer study.

Methods *CRBN*, *IKZF1* and *IKZF3* gene expression was measured in peripheral blood mononuclear cells (PBMC) from patients with SLE and healthy volunteers. Ikaros and Aiolos protein levels were measured by Western blot and flow cytometry. Anti-dsDNA and anti-phospholipid autoantibodies were measured in SLE PBMC cultures treated for 7 days with iberdomide. Fifty-six healthy volunteers were randomised to a single dose of iberdomide (0.03–6 mg, n=6 across seven cohorts) or placebo (n=2/cohort). CD19+ B cells, CD3+ T cells and intracellular Aiolos were measured by flow cytometry. Interleukin (IL)-2 and IL-1β production was stimulated with anti-CD3 and lipopolysaccharide, respectively, in an ex vivo whole blood assay.

Results SLE patient PBMCs expressed significantly higher *CRBN* (1.5-fold), *IKZF1* (2.1-fold) and *IKZF3* (4.1-fold) mRNA levels compared with healthy volunteers. Iberdomide significantly reduced Ikaros and Aiolos protein levels in B cells, T cells and monocytes. In SLE PBMC cultures, iberdomide inhibited anti-dsDNA and anti-phospholipid autoantibody production (IC_{50} ≈10 nM). Single doses of iberdomide (0.3–6 mg) in healthy volunteers decreased intracellular Aiolos (minimum mean per cent of baseline: ≈12%–28% (B cells); ≈0%–33% (T cells)), decreased absolute CD19+ B cells, increased IL-2 and decreased IL-1β production ex vivo.

Conclusions These findings demonstrate pharmacodynamic activity of iberdomide and support its further clinical development for the treatment of SLE.

Trial registration number NCT01733875; Results.

INTRODUCTION

Ikaros and Aiolos, encoded by the *IKZF1* and *IKZF3* genes, respectively, are members of the Ikaros family of zinc finger transcription factors which regulate immune cell development and homeostasis.[1] Polymorphisms in the *IKZF1* and *IKZF3* loci are associated with an increased risk of systemic lupus erythematosus (SLE).[2–11] While the roles of Ikaros and Aiolos in the development of the autoimmune responses characteristic of SLE are still being studied, it is clear that Ikaros is widely expressed in haematopoietic precursors and involved in both lymphoid and myeloid cell development, with Ikaros knockout mice lacking B cells, and Ikaros dominant negative mice lacking T cells. Aiolos expression is more restricted, found in pre-B cells and mature peripheral B cells, and is required for the generation of long-lived, high-affinity plasma cells.[1] Evidence suggests that Ikaros regulates key pathways such as the signal transducers and activators of transcription 4 and type 1 interferon (IFN), including the development of plasmacytoid dendritic cells, which are important producers of IFN-α.[11] Notably, the *IKZF1* polymorphism rs4917014 has been identified as an expression quantitative trait locus, causing the upregulation of type 1 IFN genes and the downregulation of complement genes, both of which are hallmarks of SLE pathophysiology.[12] While the *IKZF1* variant rs4917014*T strongly increases the 3′-UTR expression levels of *IKZF1*, as measured by RNA sequencing,[12] the effects of the *IKZF3* variants have not been clearly defined. Nonetheless, there is a compelling genetic association between these transcription factors and the immune dysfunction associated with lupus.[2–6]

Iberdomide (CC-220) is an orally available immunomodulatory compound under development for the treatment of SLE and relapsed/refractory multiple myeloma. This compound has been identified as a novel high-affinity ligand of cereblon (*CRBN*), part of the cullin-ring finger ligase-4 cereblon ($CRL4^{CRBN}$) E3 ubiquitin ligase complex that includes cullin 4A (*CUL4A*), DNA damage-binding protein 1 (*DDB1*) and regulator of cullin 1 (*ROC1*).[13 14] Earlier studies identified cereblon as a molecular-binding target of thalidomide, lenalidomide and pomalidomide; binding of these drugs to cereblon modulates the E3 ubiquitin ligase activity of the complex, resulting in the recruitment of the zinc finger transcription factors Ikaros and Aiolos to the complex, increasing their polyubiquitination and proteasome-dependent degradation.[14–16] The degradation of Ikaros and Aiolos is necessary and sufficient for inhibition of the proliferation of myeloma tumour cells, as well as for the immunomodulatory effects such as increased interleukin (IL)-2 expression by T cells.[17–20] Knockdown of

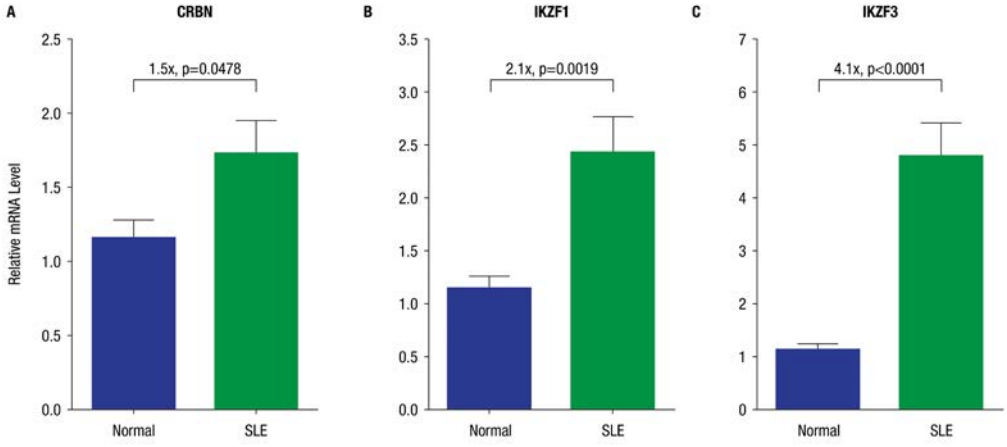

Figure 1 Overexpression of mRNA for cereblon (*CRBN)*, Ikaros (*IKZF1)* and Aiolos (*IKZF3)* mRNA in systemic lupus erythematosus (SLE) peripheral blood mononuclear cells (PBMC). Shown are relative mRNA levels of (A) *CRBN*, (B) *IKZF1* and (C) *IKZF3* in the PBMCs of normal donors (n=10) and of patients with SLE (n=11). The p values were generated using an unpaired t-test.

either *IKZF1* or *IKZF3* with shRNA in myeloma tumour cell lines markedly downregulates IRF4 expression and decreases cellular fitness.[18]

In previous biochemical and structural studies, it was demonstrated that CC-220 binds to cereblon with a higher affinity than lenalidomide or pomalidomide.[13] This results in CC-220 having a more potent and deeper degradation of the cereblon substrates Ikaros and Aiolos. The crystal structure of CC-220 in complex with cereblon and DDB1 reveals that the increase in potency correlates with increased contacts with cereblon away from the modelled binding site for Ikaros/Aiolos.[13] Thus, the structure of CC-220 enables greater Ikaros and Aiolos degradation via tighter binding to the cereblon E3 ligase complex, enabling the use of lower doses, thereby reducing the potential for off-target effects.

In the current set of preclinical studies assessing the immunomodulatory effects of iberdomide, we explored the expression of *CRBN*, *IKZF1* and *IKZF3* in peripheral blood mononuclear cells (PBMC) from patients with SLE compared with healthy volunteers, as well as the effect of iberdomide on Ikaros and Aiolos protein levels and SLE autoantibody production in vitro. In addition, we report the impact of iberdomide on immunological parameters in a phase 1, double-blinded,

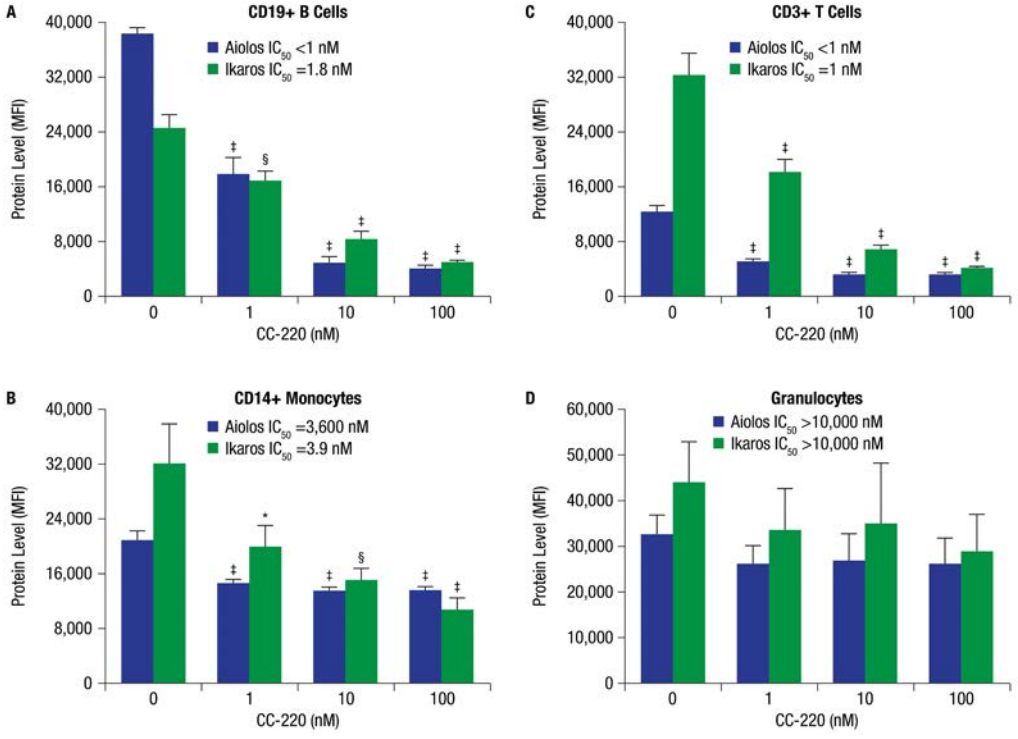

Figure 2 Iberdomide (CC-220) reduces Ikaros and Aiolos protein levels in whole blood leucocyte subsets: (A) CD19+ B cells, (B) CD14+ monocytes, (C) CD3+ T cells and (D) granulocytes. Whole blood from healthy donors was treated with CC-220 for 18 hours, lysed, fixed, permeabilised, stained with antibodies and analysed by flow cytometry. *P≤0.05; §P≤0.01; ‡P≤0.001 versus dimethyl sulfoxide (DMSO) control (n=3), by one-way analysis of variance/Dunnett's multiple comparison test. For representative flow cytometric data, see online supplementary figures S1 and S2. IC_{50}, half maximal inhibitory concentration; MFI, mean fluorescence intensity.

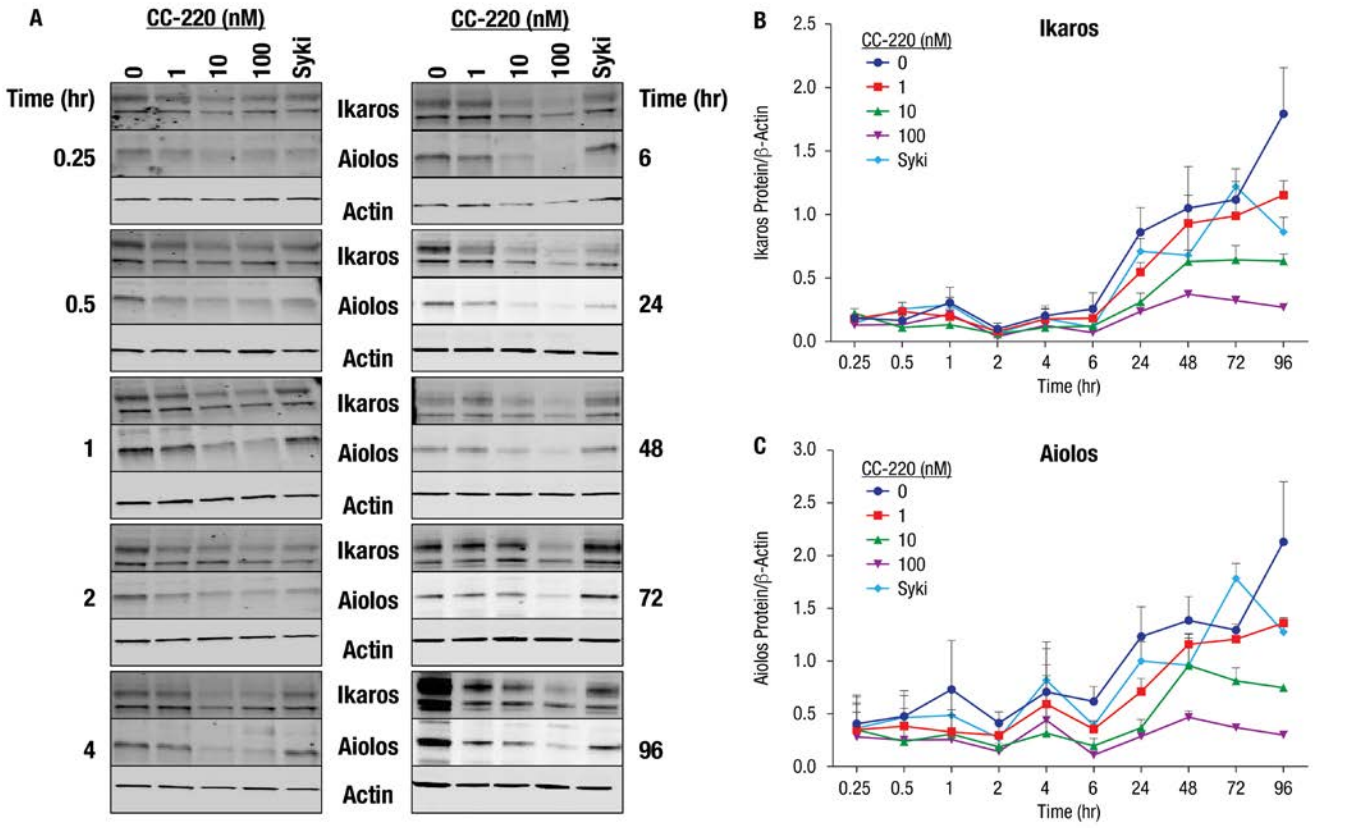

Figure 3 Effect of B cell stimulation and iberdomide (CC-220) on Ikaros and Aiolos protein levels over time. (A) CD19+ B cells were isolated from the peripheral blood mononuclear cells of normal donors and stimulated in the presence of CC-220 or a Syk inhibitor. (B, C) Ikaros and Aiolos were measured by Western blot analysis. Representative blots are shown; graphs represent the mean and SE of the mean (n=3).

placebo-controlled, single-ascending dose study in healthy volunteers.

METHODS

Gene expression studies

Viably frozen PBMCs from 11 patients with SLE and 10 healthy volunteers were obtained from Conversant Bio (Huntsville, Alabama, USA). Informed consent was collected in accordance with institutional review board approval, HIPAA compliance, FDA Good Clinical Practice and all regulatory requirements (see online supplementary methods for details).

Determining Ikaros and Aiolos protein levels in human whole blood by flow cytometry

Human heparinised whole blood from normal healthy volunteers (Bioreclamation, Westbury, New York, USA) was treated with 0.1% dimethyl sulfoxide (DMSO) and iberdomide 1, 10 or 100 nM for 18 hours at 37°C, 5% CO_2. After 18 hours, the blood was lysed and fixed for flow cytometric analysis (see online supplementary methods for details).

Iberdomide effect on Ikaros and Aiolos protein levels during CD19+ B cell culture

PBMCs were generated from human buffy coats and CD19+ B cells were isolated from PBMC using the EasySep Human B Cell Enrichment Cocktail protocol (STEMCELL Technologies, Vancouver, British Columbia, Canada). The human primary B cell differentiation protocol was adapted from previously described methods.[21 22] See online supplementary methods for details.

Iberdomide effect on SLE autoantibody production in vitro

PBMCs were purified from heparinised whole blood from donors with SLE, treated with a B cell differentiation cocktail, as described above, and incubated with increasing concentrations of iberdomide or DMSO (0.001% final concentration of DMSO) (see online supplementary methods for details).

Phase 1 clinical trial in healthy volunteers

In the phase 1, double-blind, placebo-controlled, single-ascending dose study, 56 healthy volunteers were randomised and enrolled in seven cohorts, with six subjects per cohort receiving a single oral dose of iberdomide 0.03–6 mg and two subjects per cohort receiving placebo. This study was conducted in Covance Clinical Research Unit (Madison, WI, USA). Approximately 4 mL of blood for lymphocyte phenotyping was collected on days −1, 1 (predose), 2 (24 hours postdose) and 3 (48 hours postdose) and at the follow-up visit (day 5). Blood was also collected predose and at 3, 12 and 24 hours postdose for ex vivo cytokine production assessment and transcription factor quantification. For cohorts administered the 4 and 6 mg doses of iberdomide, additional blood samples were taken on days 5 and 7, and at the follow-up visit (day 9). CD19+ B cells, CD3+ T cells and intracellular Aiolos were measured by flow cytometry. IL-2 and IL-1β production was stimulated with anti-CD3 and lipopolysaccharide (LPS), respectively, in the TruCulture ex vivo

whole blood assay system (Myriad RBM, Austin, Texas). Results were reported as per cent of baseline at each time point. The SAS Enterprise Guide V.4.1 software was used for data analysis (SAS Institute). All statistical tests were conducted with a two-sided significance level of 0.05. For parameters before and after iberdomide treatment, a Wilcoxon signed-rank test was used to compare raw values and per cent of baseline at each postbaseline time point with baseline values.

RESULTS

Compared with PBMC from normal donors, PBMCs from patients with SLE expressed significantly higher levels of mRNA for *CRBN* (1.5-fold), *IKZF1* (2.1-fold) and *IKZF3)* (4.1-fold) (figure 1A–C). Treatment of whole blood from healthy donors with increasing concentrations of iberdomide 1–100 nM significantly reduced Ikaros and Aiolos protein levels in B cells, T cells and monocytes, but not granulocytes (figure 2A–D and figure 3A–C). Reductions in Ikaros and Aiolos protein levels were significant versus control at the 1 nM concentration of iberdomide in B cells, T cells and monocytes (figure 2A–C and online supplementary figures S1 and S2); the IC_{50} for Aiolos was <1 nM in B cells and T cells, and the IC_{50} for Ikaros was 1.8 nM in B cells and 1 nM in T cells.

To address the specificity of the iberdomide-induced cereblon substrate degradation, other proteins with homology to Ikaros and Aiolos were measured in B cells and T cells using microcapillary electrophoresis western blotting. Treatment of B cells with iberdomide (10 nM for 24 hours) resulted in a significant decrease in the protein levels of Ikaros (66 kDa isoform) and Aiolos (58 kDa isoform), but not the other Ikaros family zinc finger factors Helios (*IKZF2*) or Pegasus (*IKZF5*) (see online supplementary figure S3). Expression of Eos (*IKZF4*) was not detected in B cells. Similarly, treatment of T cells with iberdomide (10 nM for 24 hours) resulted in a significant decrease in the protein levels of Ikaros (58 and 66 kDa isoforms) and Aiolos (58 and 80 kDa isoforms), but not Helios, Eos or Pegasus. Another zinc finger protein with sequence homology to Ikaros and Aiolos, Zinc Finger Protein 91 (*ZFP91*), was significantly reduced by iberdomide (10 nM for 24 hours) in B cells (see online supplementary figure S3).

In stimulated B cells, Ikaros and Aiolos protein levels were reduced by iberdomide treatment as early as 0.25 hour after treatment initiation (figure 3A). Compared with protein expression levels at time zero (cell culture initiation), Ikaros and Aiolos protein levels rose noticeably after 24–96 hours in the culture as a result of B cell activation. Under these B cell-activating conditions, iberdomide suppressed the Ikaros and Aiolos protein expression in a concentration-dependent manner (figure 3B,C).

In cultures of PBMCs from patients with SLE (n=10), iberdomide inhibited anti-dsDNA and anti-phospholipid IgM autoantibody production with an IC_{50} of approximately 10 nM in each case (figure 4A,B). Greater than 50% inhibition of anti-dsDNA and anti-phospholipid autoantibody production was observed in most donors at the 100 nM concentration of iberdomide, and at least 40%–50% inhibition at the 10 nM concentration in five of the 10 donors (see online supplementary table S1).

The pharmacodynamics of iberdomide were studied in a single-ascending dose phase 1 trial in healthy volunteers. In these subjects, a treatment-related decrease from baseline in intracellular Aiolos levels was observed following administration of iberdomide at 0.3–6 mg doses, with a mean per cent of baseline of 24.8% in B cells and 0% in T cells at 24 hours after administration of the 0.3 mg dose (figure 5A,B). In B cells at 12 hours postdose, Aiolos levels were 28.2% of baseline in the iberdomide

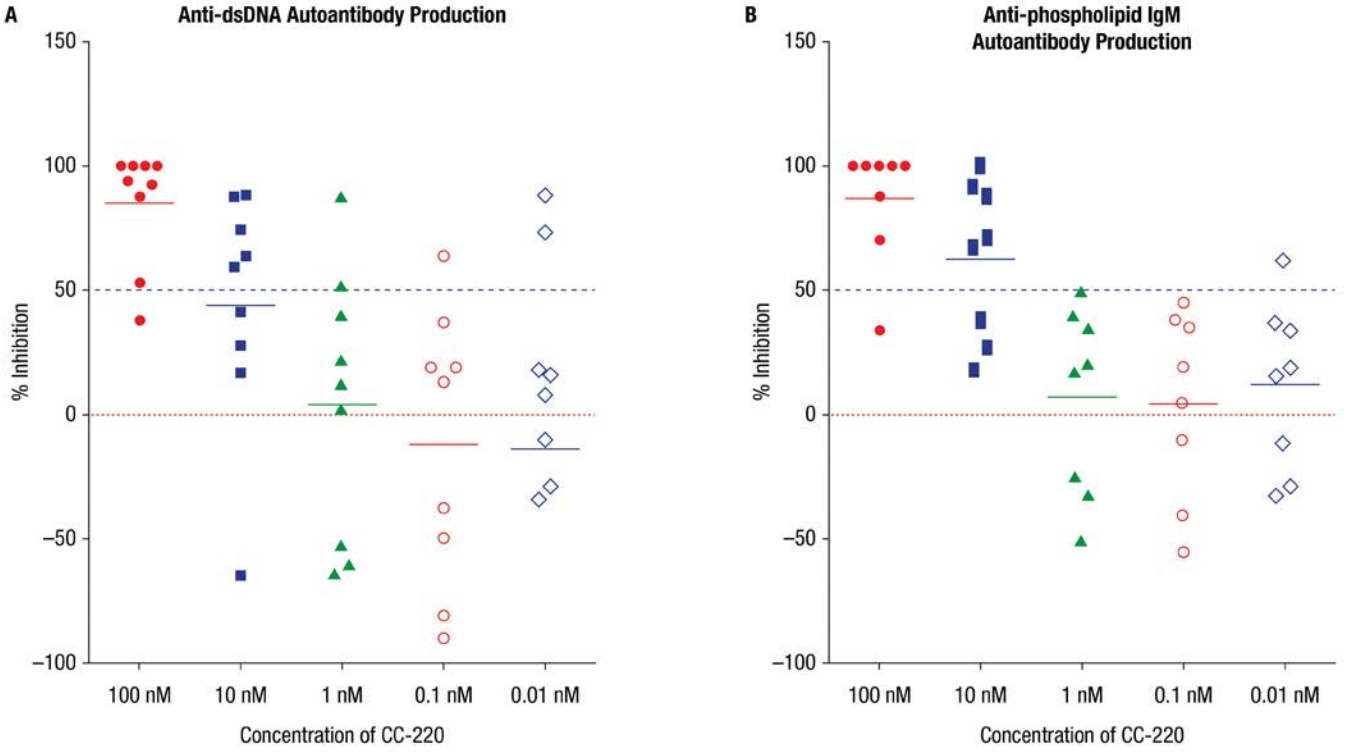

Figure 4 Iberdomide (CC-220) inhibits (A) anti-dsDNA and (B) anti-phospholipid IgM (systemic lupus erythematosus) autoantibody production in vitro. In cultures of peripheral blood mononuclear cells from patients with systemic lupus erythematosus, CC-220 inhibited production of anti-dsDNA autoantibodies (n=9) and anti-phospholipid autoantibodies (n=8) with a half maximal inhibitory concentration of approximately 10 nM.

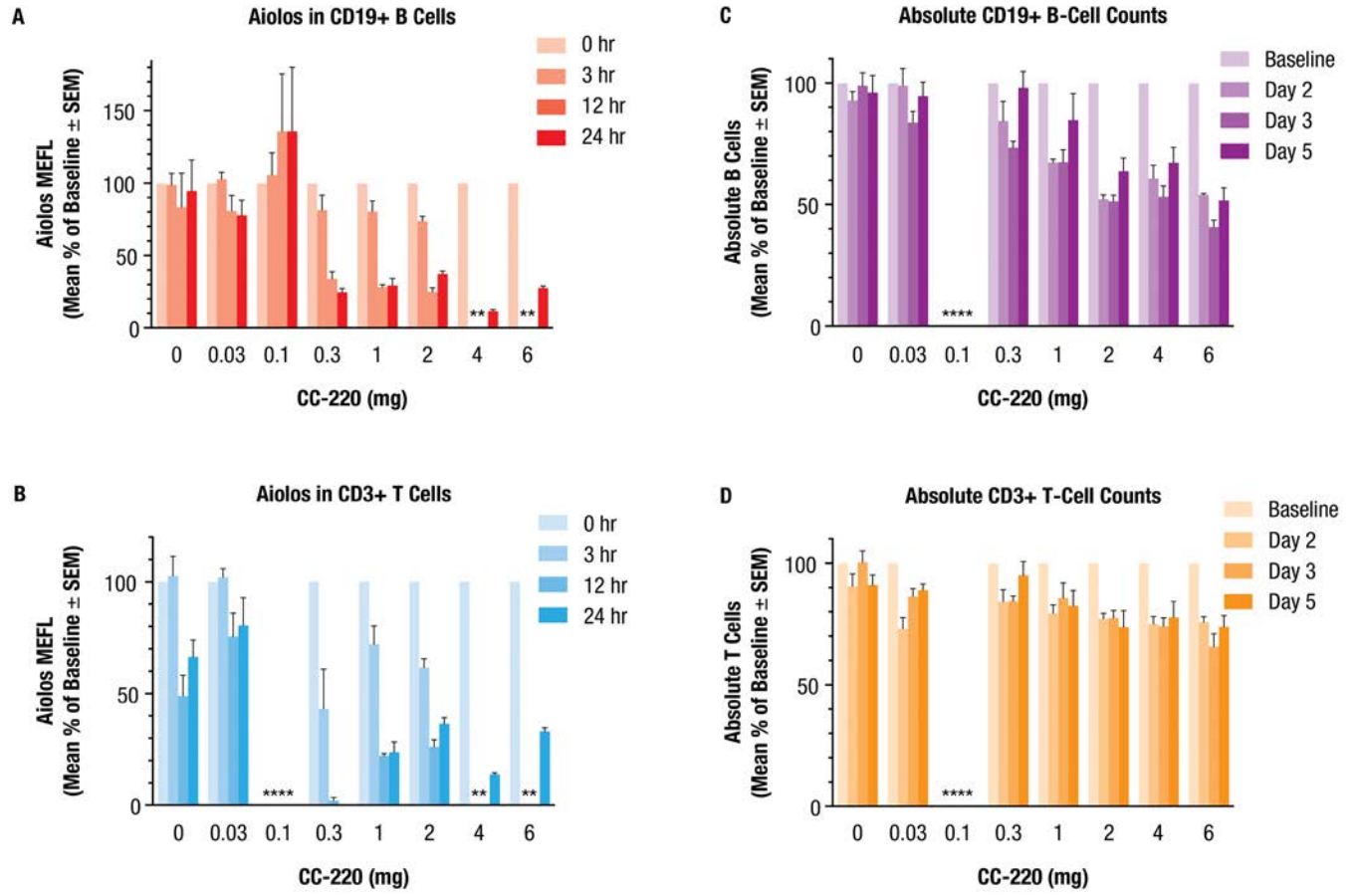

Figure 5 Effect of a single dose of iberdomide (CC-220) on Aiolos expression in (A) CD19+ B cells and (B) CD3+ T cells, and effect on (C) peripheral blood CD19+ B cell counts and (D) CD3+ T cell counts in healthy volunteers. Subjects were randomised to single doses of placebo (0) or CC-220 0.03 mg (cohort A), 0.1 mg (cohort B), 0.3 mg (cohort C), 1 mg (cohort D) or 2 mg (cohort E). Aiolos expression was measured as per cent of baseline at 3, 12 and 24 hours postdose. Peripheral blood B cell and T cell counts were measured as per cent of baseline at day 2, day 3 and day 5 postdose. n=6 per CC-220 treatment group, n=14 for pooled placebo groups. *Data not available. (Due to inclement weather at the clinical site during the dosing of the 0.1 mg cohort, some flow cytometric samples were not received for analysis within the stability window.) MEFL, molecules equivalent fluorescence.

1 mg group and 25.0% of baseline in the iberdomide 2 mg group. In B cells at 24 hours postdose, Aiolos levels were 11.5% of baseline in the iberdomide 4 mg group and 27.7% of baseline in the iberdomide 6 mg group (data not shown). In T cells at 12 hours postdose, Aiolos levels were 22.3% of baseline in the iberdomide 1 mg group and 26.1% of baseline in the iberdomide 2 mg group. In T cells at 24 hours postdose, Aiolos levels were 13.8% of baseline in the iberdomide 4 mg group and 33.1% of baseline in the iberdomide 6 mg group (data not shown). In B cells and T cells, the mean per cent of baseline values compared with baseline was statistically significant at 3 hours postdose for the iberdomide 2 mg group, at 12 hours postdose for the iberdomide 0.3 mg group and at 24 hours postdose for the iberdomide 0.3 and 2 mg groups (all $p<0.05$).

An iberdomide treatment-related decrease was noted in absolute CD19+ B cell counts, and a more modest effect on CD3+ T cell counts in healthy volunteers for doses between 0.3 and 6 mg, with minimum mean per cent of baseline values of 73.5% for B cells on day 3, and 84.1% for T cells on day 2 after administration of the 0.3 mg dose (figure 5C,D). For absolute CD19+ B cell counts, the effect of iberdomide appeared to be dose related: the mean per cent of baseline values was 67.3% on day 2 in the iberdomide 1 mg group ($p<0.05$) and 51.3% on day 3 in the iberdomide 2 mg group ($p<0.05$); on day 3, the mean per cent of baseline values was 53.3% in the iberdomide 4 mg group and 40.8% in the iberdomide 6 mg group ($p<0.05$). For absolute CD3+ T cell counts, the mean per cent of baseline was 79.4% on day 2 in the iberdomide 1 mg group (not significant), and 73.8% on day 5 in the iberdomide 2 mg group ($p<0.05$); on day 3, the mean per cent of baseline was 74.0% in the iberdomide 4 mg group and 65.7% in the iberdomide 6 mg group (both $p<0.05$).

Furthermore, iberdomide administration resulted in increased IL-2 production and decreased IL-1β production in whole blood ex vivo (figure 6A,B). For anti-CD3-stimulated IL-2 production, the mean per cent of baseline was 315%, 973% and 915% at 12 hours after administration of the iberdomide 0.3, 1 and 2 mg doses (all not significant), respectively (figure 6A), and 779% and 1896% at 24 hours for the iberdomide 4 and 6 mg groups, respectively (both not significant). Following anti-CD3 stimulation, there was also increased production of IFN-γ from 1 to 6 mg iberdomide, with a maximum mean per cent of baseline of approximately 304% for IFN-γ after administration of 1 mg iberdomide. There was also increased production of the cytokine granulocyte-macrophage colony-stimulating factor at the highest dose levels (4 and 6 mg). For LPS-stimulated IL-1β production, the mean per cent of baseline was 49.5% ($p<0.05$), 21.8% (not significant) and 16.3% ($p<0.05$) at 12 hours after administration

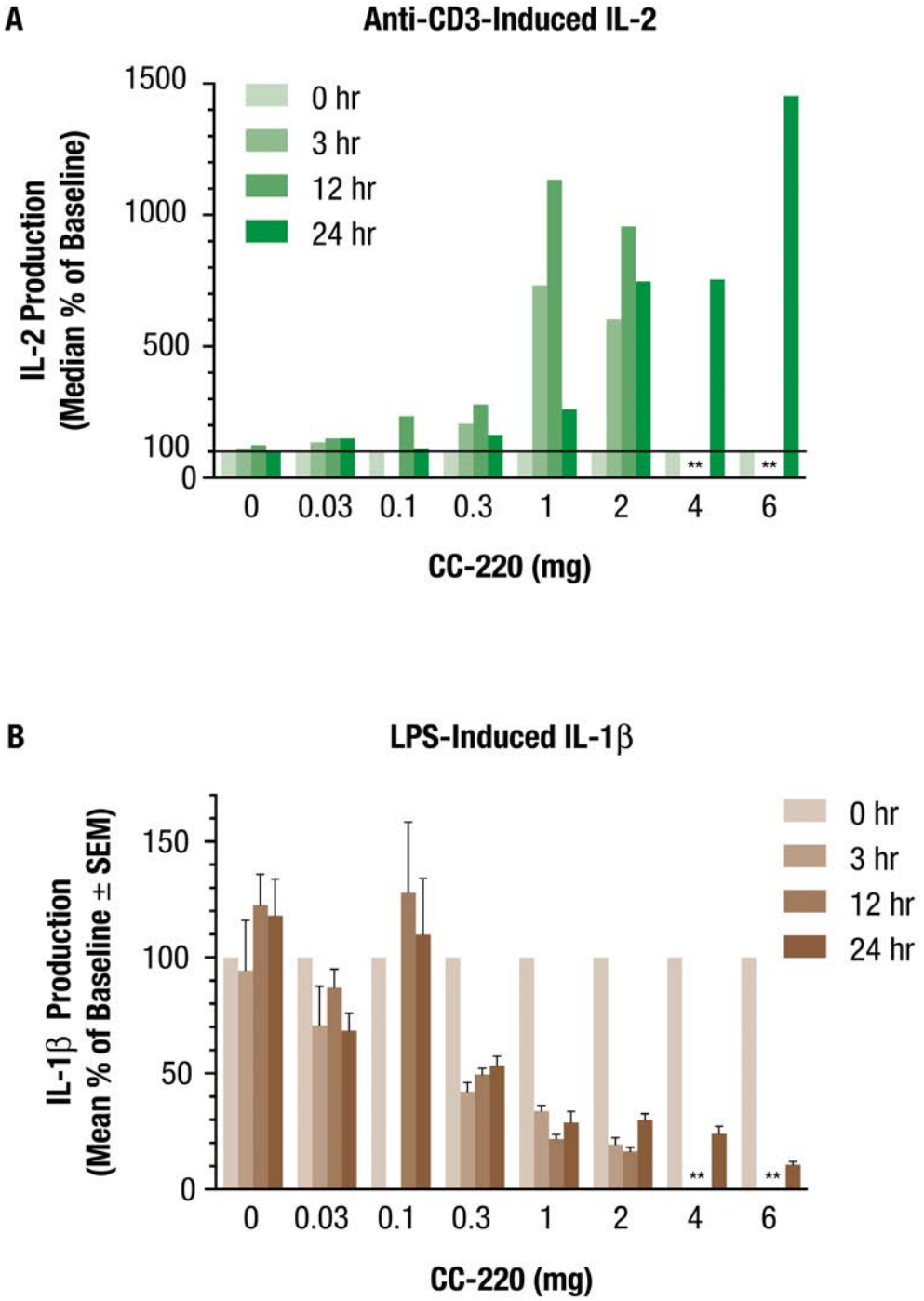

Figure 6 A single dose of iberdomide (CC-220) increased interleukin (IL)-2 production in (A) anti-CD3-stimulated whole blood and (B) decreases IL-1β production by lipopolysaccharide (LPS)-stimulated whole blood ex vivo in healthy volunteers. Subjects were randomised to single doses of placebo (0) or CC-220 0.03 mg (cohort A), 0.1 mg (cohort B), 0.3 mg (cohort C), 1 mg (cohort D) or 2 mg (cohort E). IL-2 (pg/mL) and IL-1β production by ex vivo anti-CD3 or LPS-stimulated whole blood, respectively, was measured as per cent of baseline at 3, 12 and 24 hours postdose. n=6 per CC-220 treatment group, n=10 for pooled placebo groups. *Data not available.

of the iberdomide 0.3, 1 and 2 mg doses, respectively (figure 6B), and 24.1% ($p<0.05$) and 10.7% (not significant) at 24 hours for the iberdomide 4 and 6 mg doses, respectively. Following LPS stimulation, other proinflammatory cytokines IL-1α, IL-6 and tumour necrosis factor (TNF)-α were also inhibited in a generally dose-related manner across the 0.3–6 mg iberdomide dose range. The minimum pharmacologically active dose, 0.3 mg iberdomide, had minimum mean per cent of baseline values of approximately 40%, 43% and 44% for IL-1α, IL-6 and TNF-α, respectively.

In this single-ascending dose study in healthy volunteers, there were no serious adverse events or severe treatment-emergent adverse events (TEAE) reported, and no subjects withdrew from the study due to a TEAE. All TEAEs were mild in severity with the exception of one case of moderate urticaria in the 4 mg dose group. There were no clinically significant or

apparent drug or dose-related trend changes or findings noted in clinical laboratory evaluations, vital sign measurements, physical examinations or neurological examinations for this study. Overall, there were no remarkable clinical safety findings during the study. Iberdomide was well tolerated when administered as single oral doses up to 6 mg in this group of healthy subjects.

DISCUSSION

Iberdomide (CC-220) is a novel oral immunomodulatory compound that targets cereblon, part of the $CRL4^{CRBN}$ E3 ubiquitin ligase complex. We and others have shown that binding of thalidomide and its analogues to cereblon results in recruitment and increased degradation of the transcription factors Ikaros and Aiolos. Furthermore, genetic studies have associated polymorphisms in the genes encoding Ikaros (*IKZF1*) and Aiolos (*IKZF3*) with the risk of developing SLE, and these transcription factors may play a role in the pathogenesis of the disease. The first set of in vitro experiments reported here was conducted to assess expression levels of these genes of interest in patients with SLE, and indeed confirmed that *CRBN*, *IKZF1* and *IKZF3* mRNA are overexpressed in the PBMCs of patients with SLE relative to healthy controls.

Further in vitro experiments were conducted to more clearly define the immunomodulatory effects of iberdomide. These experiments demonstrated that the addition of iberdomide at concentrations as low as 1 nM resulted in a potent reduction in Ikaros and Aiolos protein levels in B cells, T cells and monocytes from healthy donors. The other Ikaros family zinc finger transcription factors, namely Helios, Eos and Pegasus, were not significantly reduced by treatment of B cells or T cells with iberdomide, illustrating that there is selectivity for cereblon substrates even within this group of related proteins. However, another zinc finger protein with sequence homology to Ikaros and Aiolos, ZFP91, was also degraded in iberdomide-treated B cells, demonstrating that the effects of iberdomide on other cereblon substrates cannot be ruled out.

The reduction in Ikaros and Aiolos in B cells was associated with inhibition of anti-dsDNA and anti-phospholipid IgM autoantibody production in the PBMCs of patients with SLE with an IC_{50} of approximately 10 nM. This inhibitory effect on immunoglobulin production is not specific to autoantibodies, since iberdomide inhibited B cell differentiation to plasmablast differentiation, and production of IgG and IgM, to a similar extent whether the B cells were isolated from patients with SLE or from healthy donors.[23]

Iberdomide effects on the immune system were also confirmed in pharmacodynamic results from a phase 1, placebo-controlled, single-ascending dose study in healthy volunteers. In this study, administration of single doses of iberdomide 0.3–6 mg reduced intracellular Aiolos protein expression in B cells and T cells, and reduced absolute B cell counts, and to a lesser degree T cell counts in the peripheral blood. Iberdomide administration also increased T cell-derived IL-2 production and decreased LPS-induced IL-1β production in whole blood ex vivo. The pharmacologic effects of iberdomide are therefore at least tripartite in nature: (1) reduction of absolute B cell counts; (2) augmentation of IL-2 production by T cells; and (3) inhibition of IL-1β production in response to a proinflammatory stimulus. This constellation of effects provides a unique opportunity for the treatment of autoimmune conditions such as SLE, which are characterised by autoantibody production, deficiencies in regulatory T cells and a proinflammatory cytokine milieu.

Taken together, these findings support the further development of iberdomide for the treatment of SLE and other autoimmune diseases. A pilot, phase 2a, randomised, double-blind, placebo-controlled study of iberdomide is ongoing (ClinicalTrials.gov Identifier: NCT02185040)[24] to evaluate its effectiveness in patients with active SLE.

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Contributors PHS, YY, LW, JK, GR, MT, MP and RC conceptualised and designed the study. LW and JK conducted the preclinical experiments. PHS, YY, MT, MP and RC planned and conducted the clinical trial. All authors were integral in the interpretation of the results. ZY and LL performed the programming and statistical analysis of the clinical data. PHS, YY, LW, JK and GR prepared the data and the first draft of the manuscript. All authors were involved in critical review of the data as well as drafting and revising the manuscript, and all have approved the final version of the paper to be published.

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Ethics approval Schulman Associates IRB

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EXTENDED REPORT

Synovial macrophage M1 polarisation exacerbates experimental osteoarthritis partially through R-spondin-2

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ABSTRACT

Objectives To investigate the roles and regulatory mechanisms of synovial macrophages and their polarisation in the development of osteoarthritis (OA).

Methods Synovial tissues from normal patients and patients with OA were collected. M1 or M2-polarised macrophages in synovial tissues of patients with OA and OA mice were analysed by immunofluorescence and immunohistochemical staining. Mice with tuberous sclerosis complex 1 (TSC1) or Rheb deletion specifically in the myeloid lineage were generated and subjected to intra-articular injection of collagenase (collagenase-induced osteoarthritis, CIOA) and destabilisation of the medial meniscus (DMM) surgery to induce OA. Cartilage damage and osteophyte size were measured by Osteoarthritis Research Society International score and micro-CT, respectively. mRNA sequencing was performed in M1 and control macrophages. Mice and ATDC5 cells were treated with R-spondin-2 (Rspo2) or anti-Rspo2 to investigate the role of Rspo2 in OA.

Results M1 but not M2-polarised macrophages accumulated in human and mouse OA synovial tissue. TSC1 deletion in the myeloid lineage constitutively activated mechanistic target of rapamycin complex 1 (mTORC1), increased M1 polarisation in synovial macrophages and exacerbated experimental OA in both CIOA and DMM models, while Rheb deletion inhibited mTORC1, enhanced M2 polarisation and alleviated CIOA in mice. The results show that promoting the macrophage M1 polarisation leads to exacerbation of experimental OA partially through secretion of Rspo2 and activation of β-catenin signalling in chondrocytes.

Conclusions Synovial macrophage M1 polarisation exacerbates experimental CIOA partially through Rspo2. M1 macrophages and Rspo2 are potential therapeutic targets for OA treatment.

INTRODUCTION

Osteoarthritis (OA) is a highly prevalent and degenerative joint disorder, causing pain and functional disability, and representing an enormous clinical and financial burden.[1] OA is traditionally characterised by erosion of cartilage and subchondral bone sclerosis. It is now well established that OA is a disease which affects all joint tissues, characterised by progressive degeneration of the articular cartilage, vascular invasion of articular surface, subchondral bone remodelling, osteophyte formation and synovial inflammation (synovitis).[2] Accumulating evidence suggests that synovial inflammation is correlative with the pathogenesis and progression of OA.[3–5] Synovitis is significantly associated with OA severity,[6] and 38% of patients with OA of grades 2–3 according to the Kellgren-Lawrence (KL) classification have infrapatellar synovitis, as do up to 83% with KL grade 4.[7]

Normal synovium consists of two distinct tissue layers. One is the intimal lining layer with two to three layers of macrophages and fibroblast-like synoviocytes; the other is the synovial sublining layer, composed of fibrous connective tissue and blood vessels, with few lymphocytes or macrophages.[8] Macrophage accumulation in the intimal lining, reflecting mostly proliferative synovial tissue, is the principal morphological characteristic of synovitis.[9] Macrophages can be broadly classified into classically activated M1 macrophages and alternatively activated M2 macrophages, in response to stimuli from their microenvironment.[10] M1 macrophages are activated by interferon-γ and lipopolysaccharide (LPS) or tumour necrosis factor alpha (TNF-α), secreting large amounts of proinflammatory cytokines and mediators, such as TNF-α, interleukin (IL)-1, IL-6 and IL-12.[11] M2 macrophages, also known as wound-healing macrophages, have been further divided into specific subtypes and have anti-inflammatory activity.[12 13] Earlier studies have shown that macrophages accumulate and become polarised (M1 or M2) in the synovium and articular cavity during OA development, implying a correlation between macrophages and OA.[14 15] However, a recent study demonstrated that macrophage depletion (with significantly fewer macrophages of both M1 and M2) increased inflammation and did not attenuate the severity of OA in obese macrophage Fas-induced apoptosis-transgenic mice.[16] These studies indicate that OA is a complex disease that encompasses many pathways leading to degenerative conditions that progress to a common final outcome of end-stage OA, and some of these pathways involve inflammation especially macrophages. However, the role of macrophages, their polarisation in OA development and the underlying mechanisms are still unknown.

Emerging evidence reveals that the mammalian target of rapamycin (mTOR) pathway plays key roles in macrophage polarisation.[17–19] mTOR is a central regulator of cellular metabolism and forms

two distinct complexes, mTORC1 and mTORC2. Tuberous sclerosis complex 1/2 (TSC1/2) exhibits a selective guanosine triphosphatase-activating protein activity towards the small GTPase, Ras homologue enriched in brain (Rheb), which binds to and activates mTORC1.[20 21] Several reports have shown that mice with mTORC1 constitutive activation in the myeloid lineage by deletion of TSC1 are refractory to IL-4-induced M2 polarisation, but mount a hyperinflammatory response to LPS, while mice with mTORC1 abruption have reduced M1 polarisation.[17 18] Such divergence in M1 versus M2 activation is also observed in a model of TSC-deficient bone marrow-derived macrophages.[22] Thus, mice with mTORC1 activation or inhibition in the myeloid lineage are desirable models to explore the role of macrophages and their polarisation in OA.

In this study, we used mice with *Tsc1* or *Rheb1* deletion in the myeloid lineage to generate collagenase-induced osteoarthritis (CIOA), a high synovial activation OA model.[23 24] Destabilisation of the medial meniscus (DMM) surgery, a low synovial activation OA model, was also performed in mice with constitutive mTORC1 activation in the myeloid lineage (TSC1KO). We found that TSC1KO mice exhibited enhanced M1 macrophage polarisation and exacerbated cartilage degeneration and osteophyte formation in experimental OA. Conversely, deletion of Rheb1 in the myeloid lineage (Rheb1KO) enhanced synovial macrophage M2 polarisation and attenuated OA. We further identified R-spondin-2 (Rspo2) as a protein secreted by M1 macrophages which exhibits strong effects in OA development. Thus, M1 macrophages or Rspo2 represents novel therapeutic targets for OA treatment.

MATERIALS AND METHODS

Detailed experimental procedures are described in supplemetary materials and methods (see online supplemetary file 9).

RESULTS

M1 but not M2-polarised macrophages accumulate in synovial tissue of patients with OA and OA mice

To explore the role of synovial macrophages in OA development, we first examined synovial inflammation and macrophage phenotypes in human OA synovial tissue. Consistent with previous results,[25 26] high levels of synovial hyperplasia and abundant cell infiltration were observed in human OA synovial tissue, combined with significantly higher synovitis scores than that of normal controls (figure 1A,B). We further identified the accumulation and phenotypic characterisation of macrophages in OA synovial tissue. Compared with controls, marked elevation of F4/80 (macrophage marker)-positive cells was detected in both layers of OA synovial tissue, together with a significant increase of iNOS (M1-like macrophage marker)-positive cells predominantly in the intimal lining layer. In contrast, the proportion of cells positive for the M2-like macrophage marker CD206 in OA synovium showed a slight decrease (figure 1A,C). These results were further confirmed by qPCR analysis of digested synovial tissue samples from patients with OA and controls (see online supplementary figure 1A). These findings demonstrate that M1 but not M2-polarised macrophages accumulate in the synovium of OA joints from older patients and patients with OA as compared with young and normal controls.

We then assessed the accumulation of macrophages in synovium and carried out phenotypic characterisation of synovium near the patellofemoral junction in the CIOA mouse model (intra-articular injection of collagenase) (see online supplementary figure 1B). Similarly, little proliferation was observed in the synovium of control knees, but the synovium became hyperplastic and hypertrophic accompanied with degeneration and loss of structure of the cartilage of OA knees (figure 1D,E). Of note, collagenase injection consistently increased the number of macrophages in both lining layers of synovial tissue. The percentage of M1-like macrophages was increased at both 7 and 28 days after surgery. M2-like macrophages were present in both layers at 7 days after surgery, but existed only in some areas of the sublining layer at 28 days, with a significant decrease in number compared with controls (figure 1D,F). Taken together, these findings demonstrate that macrophages accumulate in OA synovial tissue, with enhanced M1-like and reduced M2-like polarisation, suggesting their potential role in the pathogenesis and development of OA.

Constitutive activation of mTORC1 in macrophages enhances their M1 polarisation in OA synovium

Mice with myeloid lineage specific activation of mTORC1, which present an enhanced M1 response and spontaneously develop M1-related inflammatory disorders, have been used as a model to investigate the role of polarised macrophages.[17] However, the activity of mTORC1 in macrophages and its contribution to macrophage polarisation in OA synovial tissue are unknown. Interestingly, enhanced phosphorylation of S6 (S235/236) (a downstream effecter of mTORC1) and its colocalisation with F4/80 was detected in human OA synovial tissue (figure 2A,B). The polarised macrophages and activation of mTORC1 signalling were also detected in synovium of young and aged mice (see online supplementary figure 2). Furthermore, in the CIOA mouse model, macrophage mTORC1 activity was significantly enhanced when synovial hyperplasia was florid, while p-S6-positive macrophages were almost undetectable in control synovium (figure 2C). These data suggest that along with macrophage accumulation, synovial macrophage mTORC1 was activated during OA.

In order to determine whether mTORC1 activation contributes to macrophage polarisation in synovium, we generated mice with conditional ablation of the *Tsc1* gene in myeloid cells, using a Cre expression cassette under the control of the lysozyme proximal promoter (TSC1KO), in which the target gene was specifically ablated from macrophages and neutrophils. In gross appearance, TSC1KO mice exhibited a slightly decreased body weight and length but no significant difference compared with control. No discernible differences in the morphology or organisation of the articular cartilage or growth plates were apparent between the TSC1KO mice and the littermate control mice at 8 weeks of age (see online supplementary figure 3A–G). Notably, synovial macrophages demonstrated high activation of mTORC1, a significant increase of M1-like polarisation and a decrease of M2-like polarisation in synovium of TSC1KO mice 4 weeks after CIOA surgery compared with control mice (figure 2D).

Synovial macrophage M1 polarisation exacerbates cartilage damage and osteophyte formation in collagenase-induced OA

We further examined the functional role of accumulated synovial M1 macrophages in the development of OA. In the CIOA mouse model, the anterior cruciate ligament (ACL) still existed with serious damage at the end of the experiment and

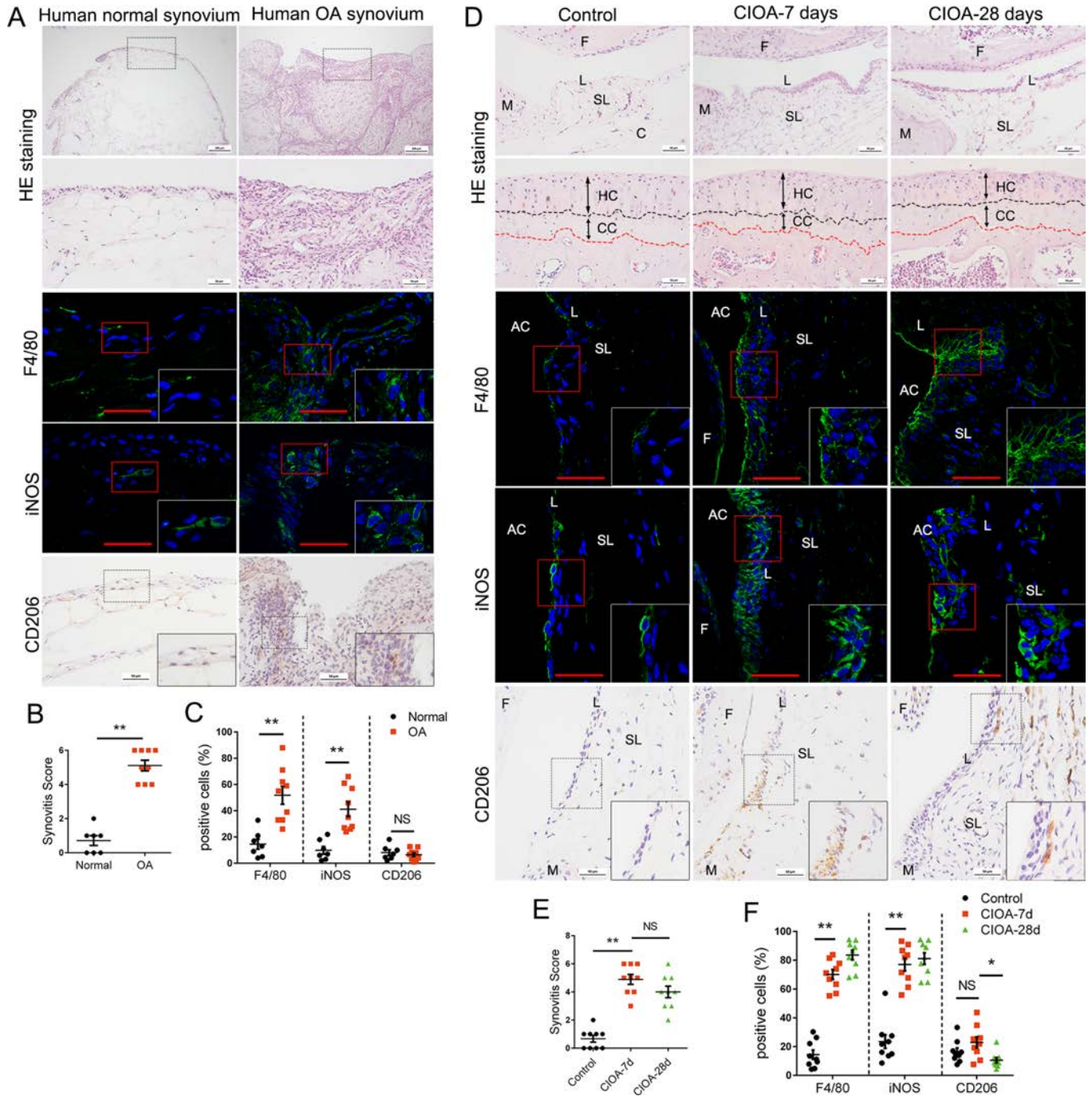

Figure 1 Macrophage polarisation in synovial tissue from patients with OA and OA mice. (A) Representative images of H&E staining (top), immunofluorescence of F4/80 and iNOS (middle), immunohistochemistry of CD206 (bottom) in normal and OA human synovial tissues. Scale bars: 50 µm, 200 µm. (B) Quantification of synovitis score in normal (n=7) and OA (n=9) human synovial tissues. Student's t-test. **P<0.01. (C) Quantification of F4/80, iNOS and CD206-positive macrophages as a proportion of total macrophages in normal and OA human synovial tissues. Student's t-test. **P<0.01. (D) Representative images of H&E staining (top), immunofluorescence of F4/80 and iNOS (middle), immunohistochemistry of CD206 (bottom) in synovium of controls and mice at 7 and 28 days after intra-articular injection of collagenase (CIOA surgery). Scale bar: 50 µm. (E) Quantification of synovitis score in synovium of CIOA mice. n=9 per group. One-way analysis of variance (ANOVA) and Tukey's multiple comparison test. **P<0.01. (F) Quantification of F4/80, iNOS and CD206-positive macrophages as a proportion of total macrophages in synovium of CIOA mice. One-way ANOVA and Tukey's multiple comparison test. *P<0.05; **P<0.01. Boxed area is enlarged in the bottom right corner. F4/80: green; iNOS: green; DNA: blue. Data are shown as mean±SEM. AC, articular cavity; CC, calcified cartilage; CIOA, collagenase-induced osteoarthritis; F, femur; HC, hyaline cartilage; L, synovial lining macrophages; M, meniscus; NS, not significant; OA, osteoarthritis; SL, synovial sublining macrophages.

TSC1KO mice showed increased synovitis score compared with control mice at both 4 and 8 weeks after surgery (see online supplementary figure 3H–J). Notably, TSC1KO mice demonstrated a significant higher OA score than controls. Four weeks after surgery, in TSC1KO mice the articular cartilage was substantially thinner, the area of surface fibrillation was larger and the chondrocytes showed an abnormal distribution compared with control mice. At 8 weeks after surgery,

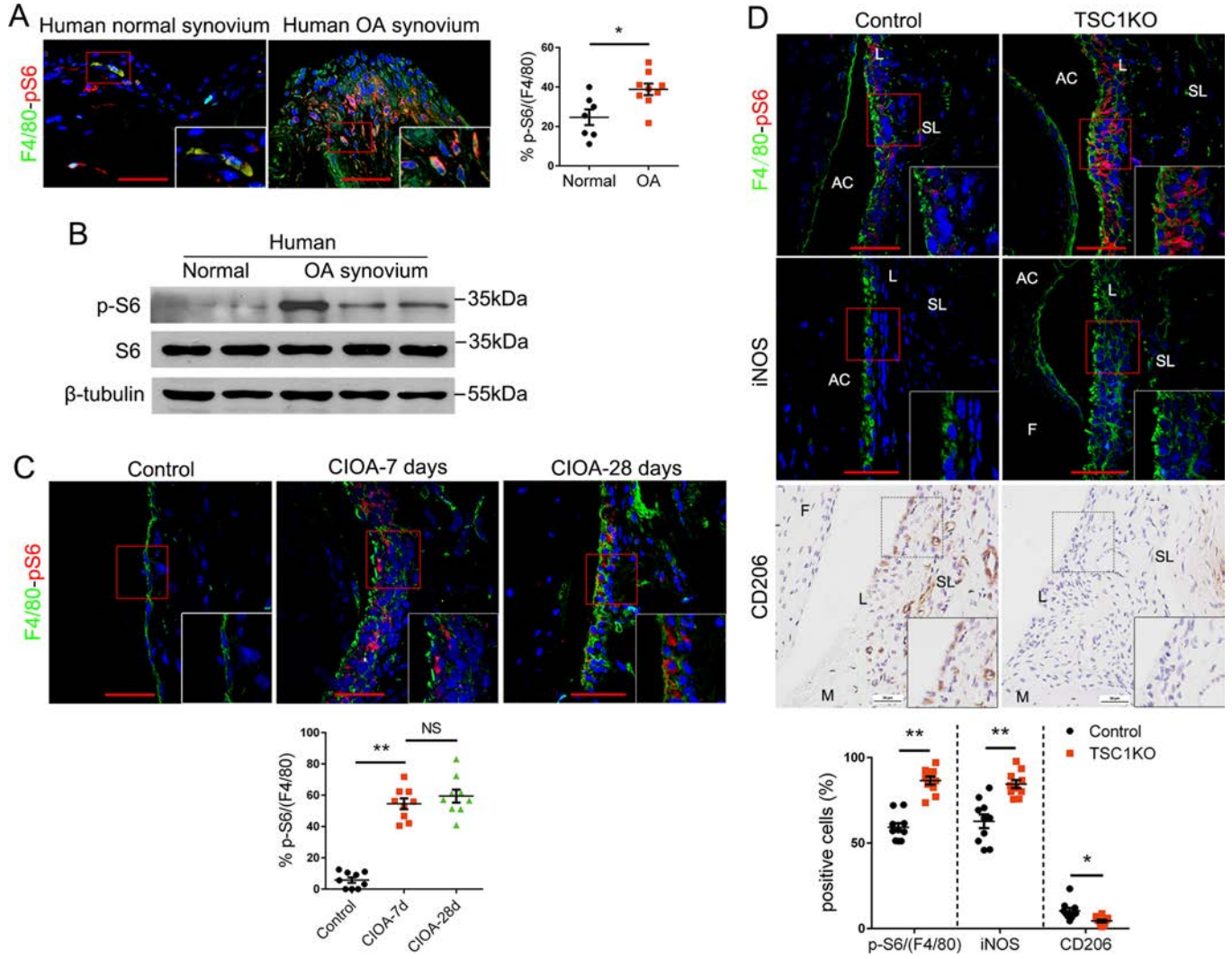

Figure 2 mTORC1 signalling is activated in M1-polarised synovial macrophages from patients with OA and CIOA mice. (A) Representative images of coimmunostaining of F4/80 and pS6 in human synovium and quantitative analysis of pS6-positive macrophages compared with total macrophages. n=7 for human normal synovium, n=9 for human OA synovium. Student's t-test. *P<0.05. Scale bar: 50 µm. (B) Western blot of pS6 in human synovial tissue. (C) Representative images of coimmunostaining of F4/80 and pS6 in synovium of controls and mice at 7 and 28 days after CIOA surgery, and quantitative analysis of pS6-positive macrophages compared with total macrophages. n=9 per group. Scale bar: 50 µm. One-way analysis of variance (ANOVA) and Tukey's multiple comparison test. **P<0.01. (D) Representative images of coimmunostaining of F4/80 and pS6, immunofluorescence of iNOS and immunohistochemistry of CD206 in synovium of TSC1KO and control mice at 4 weeks after CIOA surgery, and quantitative analysis of pS6, iNOS and CD206-positive macrophages compared with total macrophages. Scale bar: 50 µm; n=10 per group. Boxed area is enlarged in the bottom right corner. Student's t-test. *P<0.05; **P<0.01. F4/80: green; pS6: red; iNOS: green; DNA: blue. Data are shown as mean±SEM. AC, articular cavity; CIOA, collagenase-induced osteoarthritis; F, femur; L, synovial lining macrophages; M, meniscus; NS, not significant; OA, osteoarthritis; SL, synovial sublining macrophages.

more severe cartilage degeneration (figure 3A) and increased MMP13 expression (figure 3B) were observed in TSC1KO mice compared with their littermate controls. Furthermore, TSC1KO mice also exhibited accelerated osteophyte formation at 8 weeks after surgery. We employed micro-CT analysis and three-dimensional (3D) modelling of knee joints to comprehensively evaluate the osteophytosis. Compared with controls, TSC1KO mice developed larger periarticular osteophytes with significantly increased volume and surface area of osteophytes at the end stage of OA (figure 3C). The acceleration of OA development in TSC1KO mice was further verified by DMM surgery (see online supplementary figure 4). These findings suggest that macrophage M1 polarisation exacerbates cartilage degeneration and osteophyte formation during OA progression in mice.

Synovial macrophage M2 polarisation prevents OA development

We next examined the role of M2 macrophages in OA development. Mice with conditional ablation of the *Rheb1* gene, an upstream activator of mTORC1, in myeloid cells (Rheb1KO) were generated, in which M2 polarisation was enhanced while M1 polarisation was reduced in alveolar macrophages as we reported previously.[27] There were no discernible differences either in gross appearance or in the organisation of the articular cartilage and growth plates between the Rheb1KO mice and littermate controls (see online supplementary figure 5A–G). As expected, Rheb1KO mice displayed inhibition of mTORC1 activity in synovial macrophages. In addition, there were far more CD206-positive cells in the synovium of

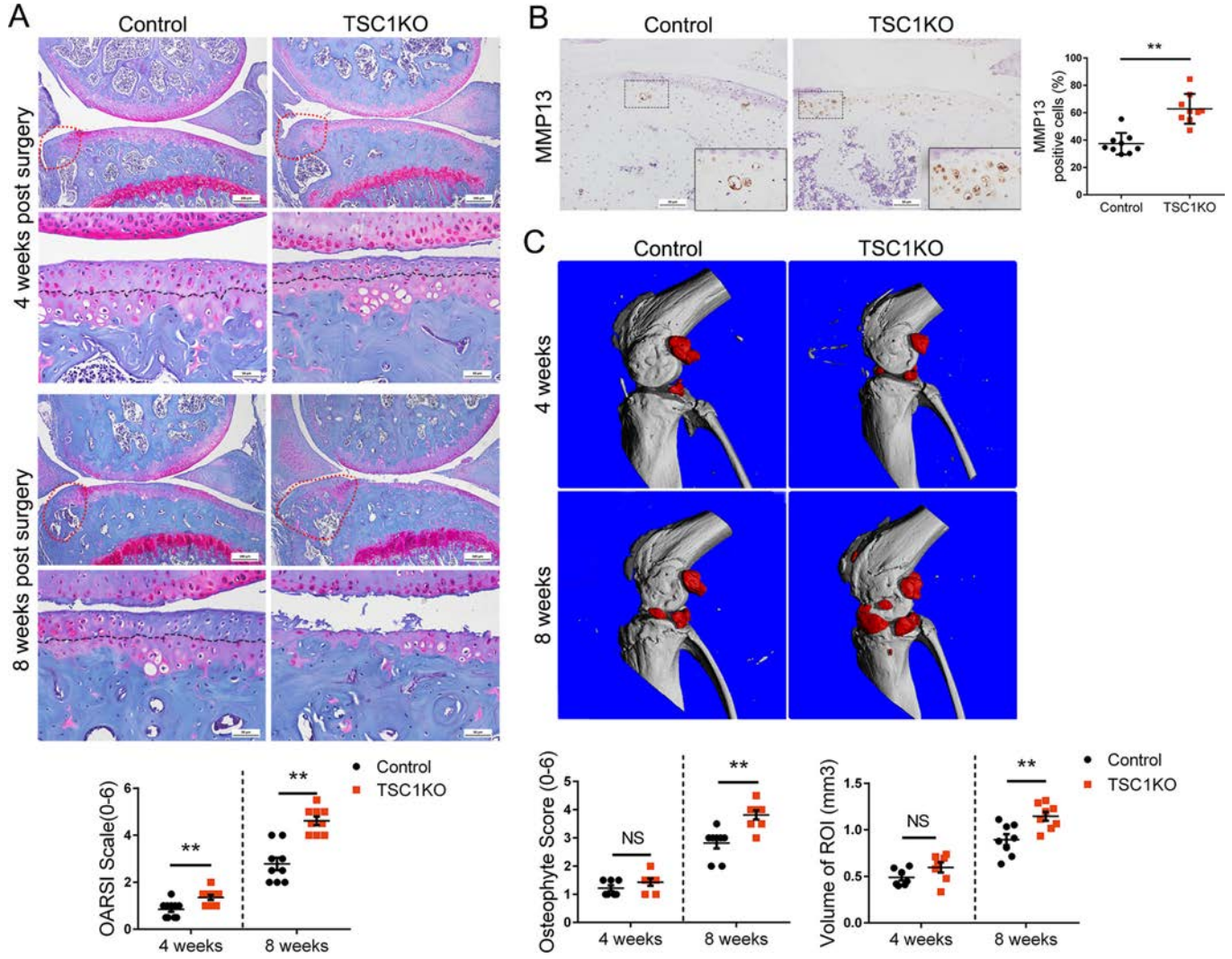

Figure 3 Synovial macrophage M1 polarisation promotes osteoarthritis (OA) development in mice. (A) Safranin O/fast green staining of joints from TSC1KO and control mice at 4 (n=10) and 8 weeks (n=9) after intra-articular injection of collagenase, and quantitative analysis of Osteoarthritis Research Society International (OARSI) scale. Red boxed area on the left of the tibial plateau represents osteophyte formation. Scale bars: 50 µm, 100 µm; Student's t-test. **P<0.01. (B) Representative images of immunohistochemical staining of MMP13 in articular chondrocytes of TSC1KO and control mice at 8 weeks after collagenase-induced osteoarthritis (CIOA) surgery, and quantitative analysis of MMP13-positive chondrocytes compared with total chondrocytes. Scale bar: 50 µm; n=9. Boxed area is enlarged in the bottom right corner. Student's t-test. **P<0.01. (C) Micro-CT scan and three-dimensional reconstruction of the knee joint from TSC1KO and control mice at 4 weeks (n=7) and 8 weeks (n=8) after CIOA surgery, and quantitative analysis of osteophyte score and volume of region of interest (ROI). The ROI is marked in red for periarticular osteophytes; Student's t-test. **P<0.01. Data are shown as mean±SEM. NS, not significant.

Rheb1KO mice than in controls, together with a decreased proportion of iNOS-positive cells (figure 4A). Thus, disruption of Rheb1 increases synovial macrophage M2 polarisation but decreases M1 polarisation in mice.

CIOA surgery was further performed on 8-week-old Rheb1KO mice and their littermate controls. Rheb1KO mice showed decreased synovial inflammation at 4 and 8 weeks after CIOA surgery along with injured ACL (see online supplementary figure 5H–J). No significant difference in knee OA Osteoarthritis Research Society International (OARSI) score between Rheb1KO and control mice was observed at 4 weeks after surgery. By 8 weeks after surgery, however, the OARSI score was significantly reduced in Rheb1KO mice (figure 4B). Accordingly, the expression of MMP13 in the cartilage of Rheb1KO mice was dramatically reduced compared with that of controls (figure 4C). Moreover, Rheb1KO mice developed much smaller osteophytes at 8 weeks after surgery (figure 4B). Together, these data suggest that synovial macrophage M2 polarisation prevents the development of collagenase-induced OA in mice.

M1-polarised macrophages produce inflammatory cytokines/enzymes and promote hypertrophic chondrocyte differentiation and maturation in vitro

To explore the mechanisms through which M1 macrophages promote OA progression, we performed mRNA sequencing to identify cytokines/enzymes involved in synovitis and cartilage degeneration by comparing the cultured macrophages from TSC1KO mice and their littermate controls (RNA sequencing data have been deposited in the NCBI SRA database under accession codes SRR6660735 and SRR6660734). Among 2279 mRNAs identified, 1153 mRNAs were upregulated and 1126 were downregulated in macrophages from TSC1KO mice

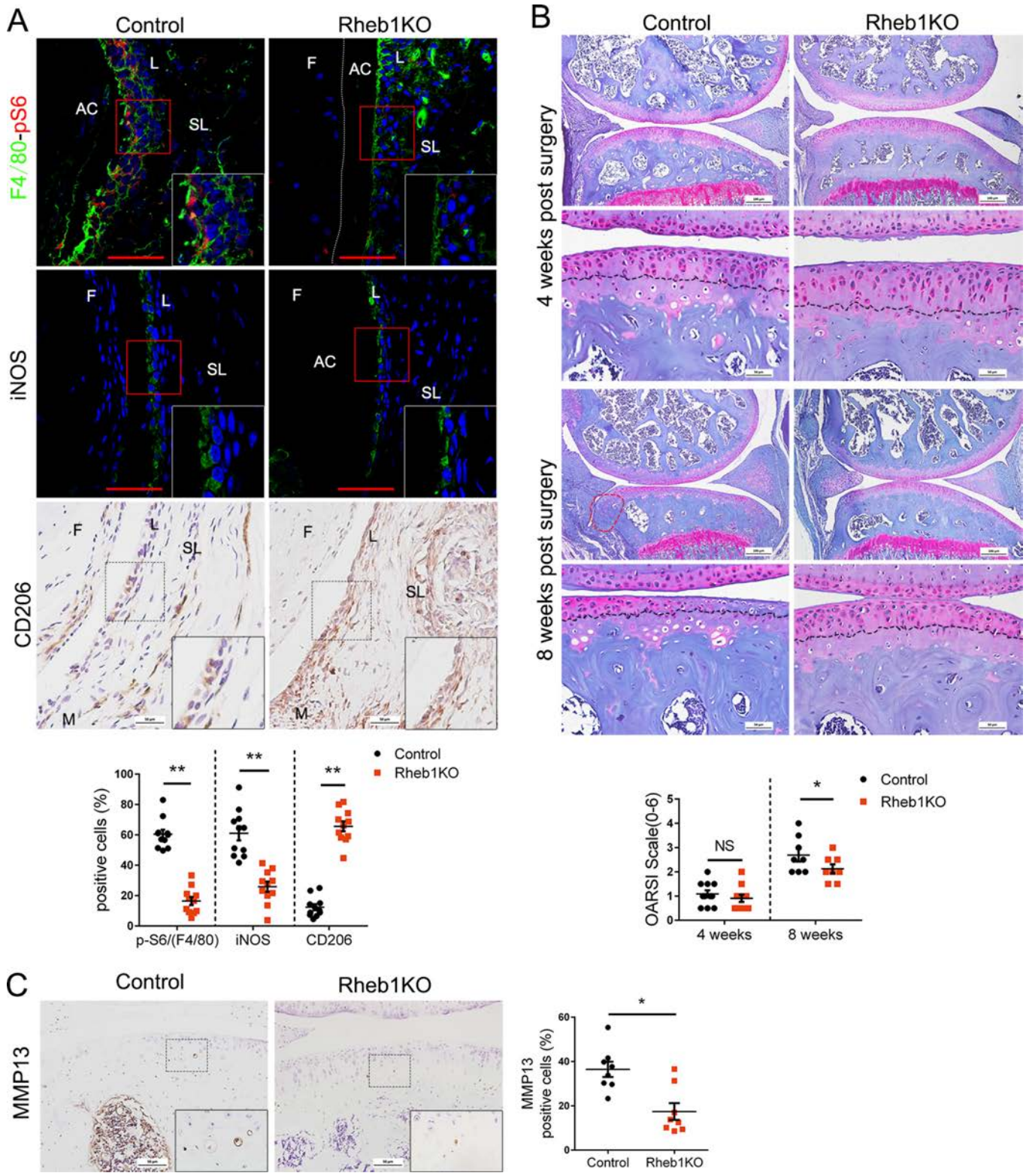

Figure 4 Synovial macrophage M2 polarisation prevents osteoarthritis (OA) development in mice. (A) Representative images of coimmunostaining of F4/80 and pS6, immunofluorescence of iNOS and immunohistochemistry of CD206 in synovium of Rheb1KO and control mice at 4 weeks after collagenase-induced osteoarthritis (CIOA) surgery, and quantitative analysis of pS6, iNOS and CD206-positive macrophages compared with total macrophages. Scale bar: 50 µm; n=11 per group. Student's t-test. **P<0.01. (B) Safranin O/fast green staining of joints from Rheb1KO and control mice at 4 (n=11) and 8 (n=8) weeks after intra-articular injection of collagenase, and quantitative analysis of OARSI scale. Red boxed area on the left of the tibial plateau represents osteophyte formation. Scale bars: 50 µm, 100 µm; Student's t-test. *P<0.05. (C) Representative images of immunohistochemical staining of MMP13 in articular chondrocytes of Rheb1KO mice at 8 weeks after CIOA surgery (n=8) and quantitative analysis of MMP13-positive chondrocytes compared with total chondrocytes. Scale bar: 50 µm; Student's t-test. *P<0.05. Boxed area is enlarged in the bottom right corner. F4/80: green; pS6: red; iNOS: green; DNA: blue. Data are shown as mean±SEM. AC, articular cavity; F, femur; L, synovial lining macrophages; M, meniscus; NS, not significant; OARSI, Osteoarthritis Research Society International; SL, synovial sublining macrophages.

compared with their littermate controls (see online supplementary table 1). The cytokines IL-1, IL-6 and TNF-α were upregulated in TSC1KO macrophages in response to LPS and have been previously shown to be produced by M1 macrophages and to contribute to OA development.[28] The upregulation of these inflammatory cytokines was further confirmed by Q-PCR and ELISA (see online supplementary figure 6A,B).

Articular chondrocyte activation, involving aberrant proliferation and hypertrophic differentiation, is essential for OA initiation and progression.[29 30] Therefore, we examined the effect of M1 macrophages on chondrogenesis and differentiation. ATDC5 chondroprogenitor cells were treated with conditioned medium (CM) from cultured macrophages from TSC1KO or control mice together with insulin, transferrin, selenium (ITS) to induce chondrogenesis. Decreased expression of Sox9, a master regulator of chondrogenesis, as well as the chondrocyte markers Col2a1 and Acan, was found in TSC1KO CM-treated ATDC5 cells. In contrast, CM from TSC1KO macrophages promoted terminal differentiation of chondrocytes, as evidenced by enhanced Col10a1 and Runx2 expression at day 14 after ITS treatment (figure 5A). Furthermore, toluidine blue staining to show matrix mineralisation further confirmed that TSC1KO macrophage CM promoted chondrocyte maturation (figure 5B). Taken together, these findings demonstrate that M1-polarised macrophages induced by mTORC1 produce high levels of inflammatory cytokines and promote hypertrophic chondrocyte differentiation and maturation, suggesting that it plays a critical role in cartilage degeneration during OA.

M1-polarised macrophages produce Rspo2 to promote OA development in mice

We next sought to identify the M1 macrophage-derived cytokines/enzymes responsible for cartilage degradation and osteoblast formation during OA. In addition to IL-1, IL-6 and TNF-α, Rspo2, a cytokine upregulated 33.6-fold in TSC1KO mice, and further analysed by Q-PCR and ELISA (see online supplementary figure 6C,D), captured our attention. Rspo2 has been identified as an agonist for Wnt signalling and synergises with Wnt to activate β-catenin and facilitate chondrocytes and osteoblast differentiation,[31] but its role in macrophage and OA development has not been reported. We confirmed that Rspo2 was expressed in synovial tissue (figure 5C) and serum (figure 5D) of TSC1KO mice. Furthermore, Lgr5, a receptor of Rspo2, was highly expressed in articular cartilage chondrocytes (figure 5E). Although no significant difference in the number of Lgr5-positive cells was detected between the articular cartilage of TSC1KO and control mice, TSC1KO macrophages produced much higher levels of Rspo2 than controls, manifested by a marked increase of Rspo2 mRNA in TSC1KO macrophages and Rspo2 protein in TSC1KO mouse serum. Importantly, β-catenin-positive cells were significantly enhanced in articular cartilage of TSC1KO mice compared with controls (figure 5E).

We then treated ATDC5 cells with or without anti-Rspo2 antibody to neutralise endogenous Rspo2 after stimulation with ITS and CM from TSC1KO macrophage culture. As expected, the effects of M1 macrophage CM on expression of Sox9, Col2a1 and Acan were rescued by Rspo2 antibody on day 5, and the upregulation of Col10a1 and Runx2 was significantly reduced on day 14. The stimulatory effect of M1 macrophages on matrix mineralisation was also abolished by treatment with anti-Rspo2 (see online supplementary figure 7A,B).

To further identify the role of Rspo2 in development of the OA phenotype induced by M1 macrophages in vivo, anti-Rspo2 antibody was injected intra-articularly to TSC1KO mice to neutralise endogenous Rspo2 after CIOA surgery. Interestingly, the antibody against Rspo2 significantly reduced the destruction of the cartilage and the OARSI score compared with the vehicle-treated mice. Micro-CT analysis and 3D modelling of knee joints revealed that anti-Rspo2 attenuated osteophytosis (figure 6A). Moreover, intra-articular injection of rhRspo2 in C57 mice promoted cartilage degradation and osteophyte formation (figure 6B). Consistently, articular cartilage β-catenin-positive cells were enhanced by rhRspo2 and reduced by anti-Rspo2 antibody injection (figure 6C and online supplementary figure 7C). These data suggest that M1 macrophages promote cartilage degeneration and osteophyte formation partly through secretion of Rspo2.

DISCUSSION

Using mouse models with enhanced M1 or M2-polarised macrophages, we identified for the first time the critical role of synovial macrophage M1 polarisation in the development of OA. We showed that synovial M1 macrophage polarisation was stimulated by mTORC1 activation and in turn exacerbated cartilage degeneration and osteophyte formation in experimental OA, while M2 polarisation was enhanced by mTORC1 inhibition and attenuated OA development. Furthermore, we found that M1 macrophages secreted Rspo2 to exacerbate experimental OA progression. Downregulating M1 macrophage polarisation or abolishing Rspo2 is thus a potential therapeutic target for OA treatment.

Recent studies have shown the essential role of synovial macrophages in the pathogenesis of rheumatoid arthritis (RA).[32 33] Several studies have reported that depletion of macrophages attenuates RA,[34 35] but the exact role of M1 and M2 macrophages in RA remains to be identified. M1 macrophage polarisation can be enhanced by activation of Notch, JNK and ERK1/2 signalling pathways, resulting in enhanced release of inflammatory factors such as TNF-α, IL-1β, IL-6 and IL-23 during RA development,[36 37] while M2 macrophages exhibit an anti-inflammatory effect by releasing IL-10 and transforming growth factor-β1 during RA.[38] However, little is known about the role of macrophages and their polarisation in OA. Macrophages are accumulated and polarised (M1 or M2) in the synovium and articular cavity during OA development, suggesting a correlation between macrophages and OA.[25] A recent in vitro study by Utomo *et al* revealed that M1 but not M2 macrophages exert prominent effects on inflammation and degeneration of cultured cartilage explants.[39] Furthermore, systemic depletion of macrophages reduces osteophyte formation in a collagen-induced arthritis model.[40] However, in another recent study conditional macrophage depletion increased inflammation and did not inhibit the development of OA in obese mice.[16] The role of macrophage polarisation and its regulatory mechanism in the pathogenesis and progression of OA remains unknown. Using mice with mTORC1 activation or inhibition in the myeloid lineage, this study establishes that macrophage M1 polarisation potentiates, while M2 polarisation attenuates, OA development in mice. We show that mTORC1 signalling and M1 macrophages are activated in the hyperplastic synovium of patients with OA and experimental OA mice. We also found that enhancement of M1 macrophages by mTORC1 activation in synovium efficiently increased the severity of pathology in CIOA, while M2 macrophages stimulated by mTORC1 inhibition protected mice

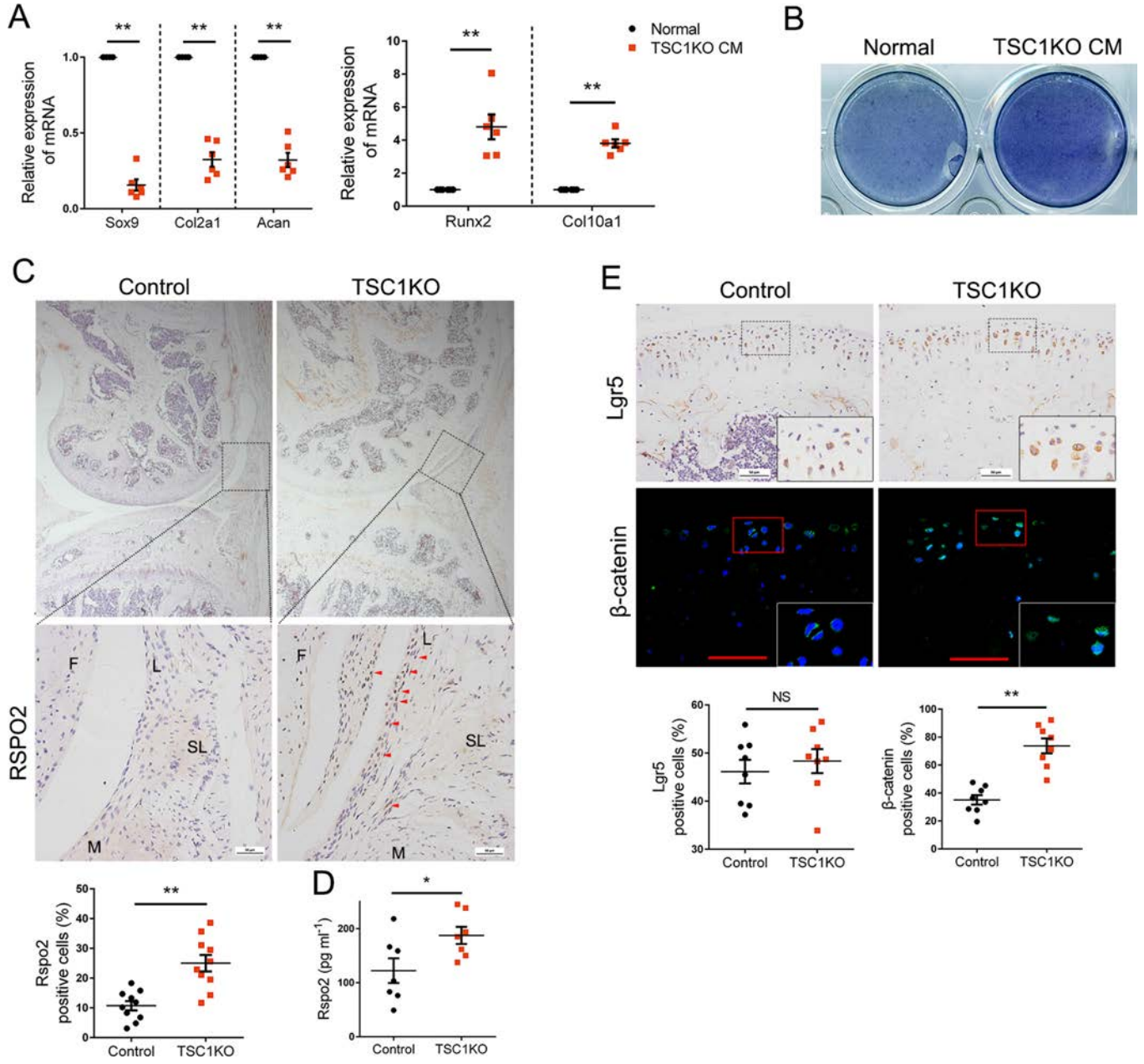

Figure 5 M1-polarised macrophages produce Rspo2 and promote hypertrophic chondrocyte terminal differentiation and maturation. (A) Quantitative PCR analysis of Sox9, Col2a1, Acan, Col10a1 and Runx2 in ATDC5 cells treated with conditioned medium (CM) from macrophage cultures from TSC1KO or control mice. n=6 per group. Student's t-test. **P<0.01. (B) Representative images of toluidine blue staining in ATDC5 cells treated with CM from macrophage cultures from TSC1KO or control mice. (C) Representative images of immunohistochemistry of Rspo2 in synovium of TSC1KO and control mice at 4 weeks after collagenase-induced osteoarthritis (CIOA) surgery, with quantification of Rspo2-positive cells as a proportion of total cells. Scale bar: 50 µm; n=10 per group. Student's t-test. **P<0.01. (D) Rspo2 concentrations assessed by ELISA in serum of TSC1KO and control mice; n=7 per group. Student's t-test. *P<0.05. (E) Representative images of immunohistochemical staining of Lgr5, and immunofluorescence of β-catenin in articular chondrocytes of TSC1KO and control mice, with quantification of Lgr5 and β-catenin-positive chondrocytes as a proportion of total articular chondrocytes. Scale bar: 50 µm; n=8 per group. Student's t-test. **P<0.01. Boxed area is enlarged in the bottom right corner. β-catenin: green; DNA: blue. Data are shown as mean±SEM. F, femur; L, synovial lining macrophages; M, meniscus; NS, not significant; SL, synovial sublining macrophages.

against collagenase-induced cartilage degradation and osteophyte formation. Although TSC1KO mice also exhibited a significant acceleration of OA development in DMM model, we found that the effect of M1 macrophages was more prominent in an OA model with high synovial activation (CIOA), compared with low (DMM). These results suggest that the role of M1 macrophage in OA was related to synovitis. Nevertheless, our results showed that M1 and M2 macrophages always coexist in synovium and the imbalance of macrophage polarisation with a significant increase of M1 macrophages occurs during OA development. In these models, when M1 or M2 macrophages are stimulated, the proportion of opposite phenotypes is reduced. Enhancement of M2 macrophage numbers by mTORC1 inhibition attenuated OA along with a significant reduction in M1 macrophages. Moreover, mRNA sequence screening revealed that M1 macrophage-produced inflammatory cytokines and Rspo2 mediate its

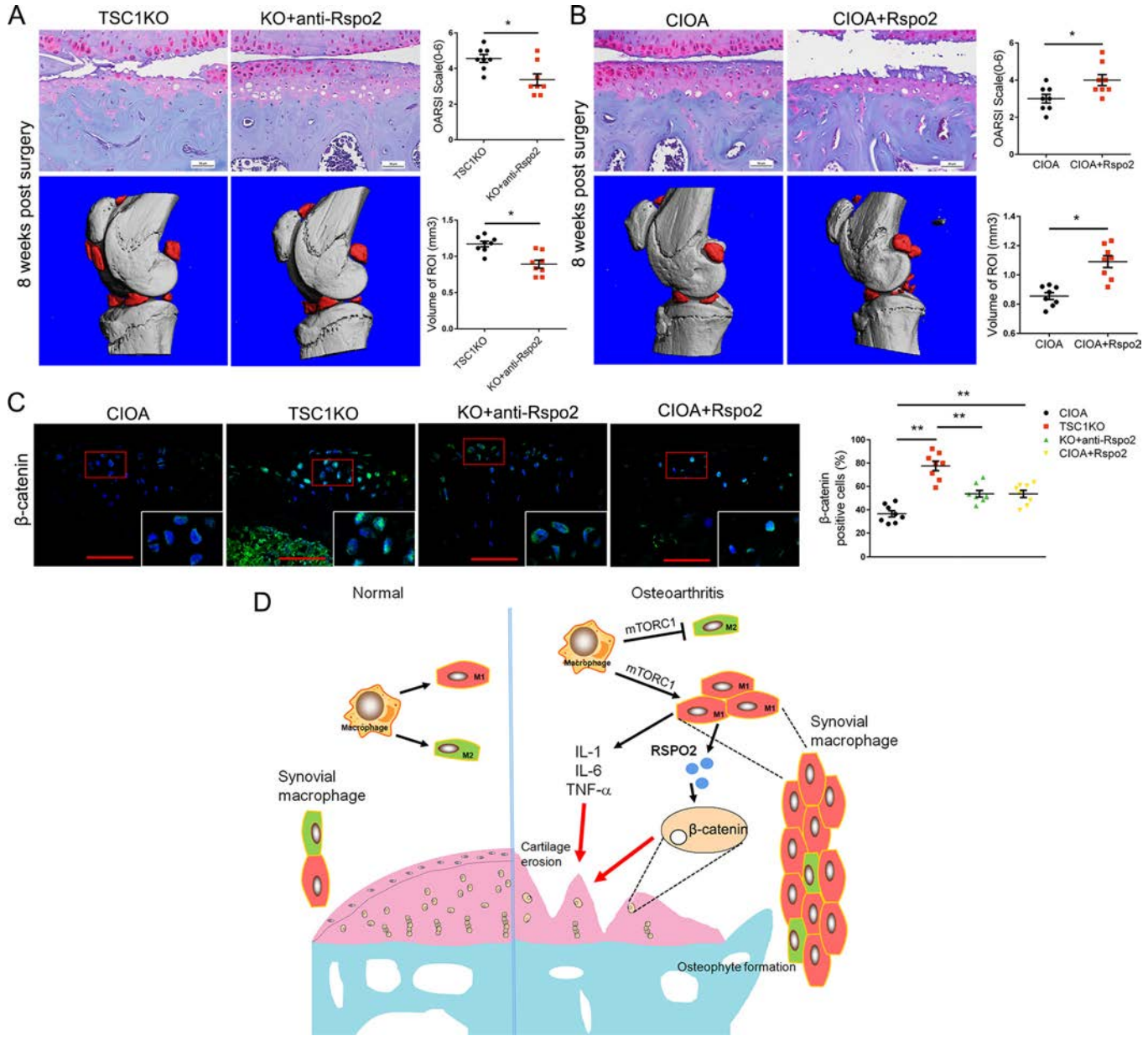

Figure 6 M1-polarised macrophages exacerbate experimental osteoarthritis (OA) partially through Rspo2. (A) Safranin O/fast green staining, micro-CT scan and three-dimensional reconstruction of joints from TSC1KO mice at 8 weeks after intra-articular injection of collagenase with or without anti-Rspo2 antibody, and quantitative analysis of OARSI scale and volume of the ROI. The ROI is marked in red for periarticular osteophytes; n=8 per group. Student's t-test. *P<0.05. (B) Safranin O/fast green staining, micro-CT scan and three-dimensional reconstruction of joints from mice at 8 weeks after intra-articular injection of collagenase with or without rhRspo2, and quantitative analysis of OARSI scale and volume of ROI. The ROI is marked in red for periarticular osteophytes; n=8 per group. Student's t-test. *P<0.05. (C) Representative images of immunofluorescence of β-catenin in articular chondrocytes of mice described in (A) and (B), with quantification analysis of β-catenin-positive chondrocytes as a proportion of total articular chondrocytes. Scale bar: 50 µm; n=8 per group. One-way analysis of variance (ANOVA) and Tukey's multiple comparison test. **P<0.01. (D) Model of Rspo2 secreted by M1-polarised macrophages in regulating hypertrophic chondrocyte terminal differentiation and osteophyte formation during OA. β-catenin: green; DNA: blue. Data are shown as mean±SEM. CIOA, collagenase-induced osteoarthritis; IL, interleukin; OARSI, Osteoarthritis Research Society International; ROI, region of interest; TNF, tumour necrosis factor.

role in OA. We propose a model in which mTORC1-induced macrophage M1 polarisation stimulates production of inflammatory cytokines and Rspo2 secretion to promote expression of matrix-degrading enzymes, hypertrophic differentiation of articular chondrocytes and cartilage degeneration during OA development (figure 6D).

In OA, disease affects all joint tissues and therapy that is able to target the multiple pathological changes in joints is desirable. Accumulating evidence highlights the vital role of mTORC1 in the pathogenesis and progression of OA. Previous studies have established that activation of mTORC1 inhibits autophagy to promote articular chondrocyte apoptosis during OA development.[41 42] Our previous study demonstrated the key role of the chondrocyte mTORC1 pathway in regulating cell proliferation and differentiation in OA.[29] We also found that chondrocyte mTORC1 promoted OA partially through formation of vascular endothelial growth factor-A-stimulated subchondral H-type vessels.[43] The current study demonstrates

an important role of macrophage mTORC1 in OA synovitis. We have clearly demonstrated the important role of mTORC1 signalling in regulating synovial macrophage M1 polarisation during OA development. Our results showed that synovial macrophage mTORC1 activation occurred throughout OA development, combined with enhanced M1 macrophage polarisation. Hyperactive mTORC1 in synovial macrophages enforced cartilage degeneration in TSC1KO mice. In contrast, macrophage mTORC1 inactivation enhanced M2 polarisation and attenuated OA. Together, these studies suggest that multiple OA pathological joint compartments may benefit from targeting of mTORC1.

Although Rspo2 has been widely studied as an activator of Wnt/β-catenin signalling,[44] its role in OA has not been reported. It has been shown that Rspo2 activates Wnt/β-catenin signalling to facilitate cultured chondrocyte hypertrophic differentiation during endochondral ossification.[31] Another in vitro experiment in MC3T3-E1 cells (a preosteoblastic cell line) demonstrated that Rspo2 might be a positive regulator of bone metabolism.[45] An interesting finding of this study was that high levels of Rspo2 were detected in synovial tissues and in the serum of TSC1KO mice. Importantly, abolishing Rspo2 with a neutralising antibody effectively reduced β-catenin in articular chondrocytes and attenuated the development of OA in mice. These data indicate that M1 macrophages secrete Rspo2 to induce terminal differentiation of articular chondrocytes, degradation of matrix proteoglycan and cartilage during OA development.

To conclude, our study found an association between M1 macrophages and Rspo2 on cartilage degeneration. Future studies are warranted to explore the underlying mechanisms and further management of the behaviour of polarised synovial macrophages seems a suitable approach to prevent OA.

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Contributors HZ and XB conceived the ideas for experimental designs, analysed data and wrote the manuscript. CL and CZ conducted the majority of the experiments and helped with manuscript preparation. ZW, JL and HW performed microcomputed tomography analyses and provided suggestions for the project. XL and YS performed immunohistochemistry and immunofluorescence and confocal imaging. SX and YZ conducted cell cultures and western blot experiments. CZ and DX collected human tissue samples. XB and DC developed the concept, supervised the project, conceived the experiments and critically reviewed the manuscript. HZ, CL and CZ contributed equally to this work.

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Competing interests None declared.

Patient consent Obtained.

Ethics approval Human study was approved by the Ethics Committee of the Third Affiliated Hospital of Southern Medical University. All animal experiments were approved by the Southern Medical University Committee Animal Care and Use Committee.

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Transmission of rheumatoid arthritis through blood transfusion: a retrospective cohort study

The long preclinical phase of rheumatoid arthritis (RA), where some factors involved in RA pathogenesis circulate peripherally, raises concern of RA transmissibility through blood transfusion.[1] Specifically, this possibility is suggested by murine RA models in which anticitrullinated peptide/protein antibodies may induce and enhance arthritis, and precursors of the RA-fibroblast-like synoviocyte cells may aggravate and spread the disease between joints.[2 3]

We used a large Danish–Swedish population-based research donations and transfusions database (SCANDAT2) with health register information on 1.5 million blood donors and 2.1 million recipients of their blood to investigate (1) RA occurrence in recipients of blood from donors who later developed RA and (2) clustering of RA among recipients of blood from individual donors, regardless of the donor's RA status.[4–6]

We used two different approaches to analyse RA transmission. First, we identified all donors who developed RA after blood donation. For each of these index donors, we identified up to 10 donors matched on age, sex, county, date of first donation, number of donations and ABO blood group, who were free of RA at the date of index donor diagnosis. We then identified all recipients of blood from the two donor populations and followed them from date of transfusion originating in said donors until date of RA diagnosis, death, emigration or end of 2012 (Sweden)/2013 (Denmark), whichever came first.

In the second approach, we investigated if RA clustered among recipients of blood from individual donors. Here, we used a modified time-dependent donor riskiness score by simply counting the RA occurrences among past recipients of each donor.

In both approaches, we only followed up recipients whose first registered transfusions occurred after 1996 and 1998 in Sweden and Denmark, respectively. We used Cox regression with age as underlying time scale, with an exposure lag of 1 year and strata defined by sex and hospital. All analyses were adjusted for calendar period and number of transfusions as restricted cubic splines with five equally spaced knots and ABO blood group. Persons with RA were identified using contemporary national International Classification of Diseases (ICD) 7, 8, 9 and 10 classifications. To reduce misclassification, we defined RA as having two registrations of RA within the course of a 2-year period. RA diagnoses were further subclassified as seropositive RA or seronegative RA.

Among a total of 938 942 blood donors, 2412 were diagnosed with RA during follow-up. We identified 13 369 patients (exposed) who received at least one unit from donors with later RA and 139 470 patients (unexposed) who received blood units from the matched donors who were free of RA at selection.

Recipient RA risk did not vary by donor RA occurrence, whether overall, by RA serotype, donor age at RA diagnosis or interval between donation and donor RA diagnosis (table 1).

Similarly, recipient RA risk did not also vary by RA occurrence in previous recipients of blood from the same donor, neither for all types of RA combined (HR per previous recipient with RA, 0.96; 95% CI 0.86 to 1.07) nor for specific RA subtypes.

The association between RA and blood transfusions has previously been explored only in two investigations, which based on self-reported transfusion history arrived at opposite conclusions.[7 8]

Table 1 Number of cases of rheumatoid arthritis (RA) overall and of seropositive RA and seronegative RA, respectively, observed among recipients whose blood donors were (exposed recipients) and were not (unexposed recipients) correspondingly diagnosed, with person-years of observation and HRs with 95% CIs for RA overall and in different strata

	Recipient outcome				
	Exposed		Unexposed		
Recipient exposed to:	Events	Person-years	Events	Person-years	HR (95% CI)
Overall RA					
Donor diagnosed with RA	63	82 551	610	756 275	1.04 (0.80 to 1.35)
Shortest latency of donor RA*					
<10 years	46	59 890	610	756 275	0.99 (0.56 to 1.72)
10+ years	17	22 661	610	756 275	1.05 (0.64 to 1.70)
Lowest age at donor RA diagnosis					
<65 years	51	69 827	610	756 275	0.80 (0.43 to 1.51)
65+ years	12	12 724	610	756 275	1.24 (0.70 to 2.20)
Seropositive RA					
Donor with seropositive RA	4	17 030	27	186 775	1.68 (0.58 to 4.84)
Seronegative RA					
Donor with seronegative RA	9	39 829	129	402 138	0.72 (0.37 to 1.43)

*Interval between donation and donor RA diagnosis.

In contrast, we exclusively analysed patients who underwent transfusion and focused on the link between donor-specific factors (eg, donor RA diagnosis) and recipient RA risk.

In conclusion, we found no evidence that RA or RA risk is transmitted through blood transfusion. In light of the study's strengths, including low likelihood of confounding and large study size ensuring meaningful statistical power, we believe the possibility of RA transmission is unlikely to be clinically relevant.

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Ultrasonography in psoriatic arthritis: which sites should we scan?

In psoriatic arthritis (PsA), ultrasonography (US) plays a growing role in the differential diagnosis and in monitoring treatment response.[1] PsA is a heterogeneous disease with different domains and peculiar sites involved.[2] Therefore, a dedicated US composite score is needed to monitor disease activity and to identify structural damage progression. A recently published Systematic Literature Review (SLR) identified only two US scores specifically developed for PsA (ie, 5TPD and PsA-Son) and, although these had a good sensitivity to detect inflammation and a good feasibility, they have not been validated in other series.[1 3 4] Recently, the Study Group for US of the Italian Society of Rheumatology promoted the Ultrasound in PSoriatic Arthritis TREAtMent (UPSTREAM) study (registered at ClinicalTrial.gov, NCT03330769). UPSTREAM is a multicentre observational prospective cohort study and it represents the first example of integration between clinical examination and US with the aim to identify predictors of achieving minimal disease activity in patients with PsA starting a new course of therapy.

Our first step, towards the development of a US composite score to be used as an outcome in UPSTREAM, was a qualitative research aimed to define those anatomic sites that are considered relevant by rheumatologists, expert in management of PsA and US. For this purpose, a web-based ranking exercise on the relative relevance of different anatomic structures was done.

Bilaterally, seven entheses, eight joints, 10 tendons with sheath, two tendons without sheath and seven anatomic sites for bursae and soft tissues were identified on the basis of the previous SLR and submitted to vote in the survey.[1] See table 1 for detailed localisation of each anatomic sites. Within every anatomic structure, a further ranking of sites (eg, for joints: metacarpophalangeal, proximal, distal interphalangeal and metatarsophalangeal joints, wrist, knee) was asked. The between and within weights were calculated as mean of reciprocal rank normalised to 0%–100%. In order to balance for the number of items in each category, the final weight was calculated as product of between and within weights multiplied for the number of items and normalised to 0%–100%. Final ranking identified those items candidate to be incorporated in the scanning US protocol of the UPSTREAM study. To satisfy content validity requirement, at least one item per anatomic structure was included. Twenty rheumatologists, with experience in musculoskeletal US and in the management of PsA, participated in the web exercise. The anatomic structures with a better ranking were: entheses, joints, tendons with sheath, tendons without sheath and ultimately soft tissues and bursae. Considering the overall weight for each site, enthesis of Achilles tendon achieved the best results. The full results are reported in table 1. Through this web-based exercise, we identified the anatomic sites considered useful in revealing typical US changes of PsA and they will be incorporated in the US protocol of the UPSTREAM study. This is a first essential step to assess the content of a simplified US score that will encompass both joint and extra-articular structures, most informative in the US evaluation of PsA.

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Table 1 Ranking of the anatomical sites

Structure	Between structure rank	Between structure weight*	Site	Within structure rank	Within structure weight*	Overall weight†	Overall rank
Enthesis	1	27%	Achilles tendon	1	26%	7%	1
			Patellar ligament (proximal insertion)	2	15%	4%	7
			Patellar ligament (distal insertion)	3	14%	4%	10
			Quadriceps tendon	4	13%	4%	11
			Common extensor tendon (lateral elbow)	5	11%	3%	13
			Plantar aponeurosis	6	11%	3%	14
			Distal insertion of extensor tendon of the finger	7	9%	2%	20
Joint	2	26%	Proximal interphalangeal joint (hand)	1	17%	5%	2
			Metacarpophalangeal joint	2	16%	5%	3
			Wrist joints	3	15%	4%	5
			Knee	4	13%	4%	8
			Distal interphalangeal joint (hand)	5	12%	3%	12
			Metatarsophalangeal joint	6	10%	3%	16
			Ankle joints	7	9%	2%	19
			Elbow joints	8	8%	2%	21
Tendon with sheath	3	18%	Flexor tendon of the digit	1	17%	4%	4
			Compartment of extensor tendons (wrist)	2	16%	4%	6
			Posterior tibialis tendon	3	11%	3%	15
			Anterior tibialis tendon	4	10%	3%	17
			Peroneal tendons	5	10%	3%	18
			Extensor hallucis longus tendon	6	8%	2%	22
			Extensor digitorum longus tendon	7	8%	2%	25
			Flexor digitorum longus tendon	8	7%	2%	27
			Flexor hallucis longus tendon	9	7%	2%	29
			Flexor tendons of the toes	10	6%	2%	30
Tendon without sheath	4	16%	Extensor tendons of the digits	1	63%	2%	24
			Extensor tendons of the toes	2	37%	1%	34
Bursa and subcutaneous tissue	5	14%	Achilles bursa	1	26%	4%	9
			Olecranic bursa	2	15%	2%	23
			Semimembranosus and gastrocnemius bursa	3	15%	2%	26
			Soft tissue oedema of the hands	4	14%	2%	28
			Medial malleolar bursa	5	11%	2%	31
			Lateral malleolar bursa	6	10%	1%	32
			Soft tissue oedema of the toes	7	10%	1%	33

The sites included in ultrasound scanning protocol of the Ultrasound in PSoriatic Arthritis TREAtMent study are marked in green in the 'rank' column.
*Calculated as inverse of mean rank normalised from 0% to 100%.
†Calculated as product of structure weights*site weight*number of sites within structure and normalised to 0%–100%.

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Ankylosing Spondylitis Disease Activity Score (ASDAS): 2018 update of the nomenclature for disease activity states

The Ankylosing Spondylitis Disease Activity Score (ASDAS) is a measure of axial spondyloarthritis (axSpA) disease activity with validated cut-offs endorsed by the Assessment of SpondyloArthritis international Society (ASAS) and Outcome Measures in Rheumatology (OMERACT).[1 2] In the 2016 update of the ASAS-European League Against Rheumatism (EULAR) management recommendations for axSpA, it is recommended that biological disease-modifying antirheumatic drugs should be considered in patients with persistently high disease activity despite conventional treatments, and that the preferred measure to define active disease should be the ASDAS (ASDAS of at least 2.1, ie, high disease activity).[3] The 2017 update of treat-to-target recommendations in axial and peripheral SpA recommends that the treatment target should be inactive disease/clinical remission and that low/minimal disease activity may be an alternative treatment target. The same recommendations state that the preferred measure to define the target in axSpA is the ASDAS.[4]

ASDAS cut-offs for disease activity states are 1.3, separating 'inactive disease' from 'moderate disease activity', 2.1, separating 'moderate disease activity' from 'high disease activity', and 3.5, separating 'high disease activity' from 'very high disease activity'. While inactive disease equates to a remission-like state, there is no low/minimal disease equivalent in the above nomenclature of ASDAS cut-offs. At the 2018 ASAS annual meeting in Lisbon, Portugal, ASAS members discussed the proposal of changing the nomenclature of ASDAS cut-offs in order to fill this gap.

Arguments to change the designation of 'moderate disease activity' to 'low disease activity' were presented. The compelling argument is the fact that the majority of patients in this ASDAS category have indeed mild disease activity, an observation that is in line with the external constructs that were used to derive the ASDAS cut-off of 2.1: patient and physician global assessments <3 using the 90% specificity criterion to determine the optimal cut-off.[1 5] Furthermore, recent publications have shown that the majority of patients with ASDAS values in the 'moderate disease activity' category consider themselves as being in a patient-acceptable symptom state (PASS), which can be defined as the maximum level of symptoms with which patients consider themselves to be well. Godfrin-Valnet *et al*[6] found that the agreement between ASDAS-C-reactive protein (CRP) and ASDAS-erythrocyte sedimentation rate (ESR) was good and that values of ≤2.3 for each were associated with the PASS. A previous study by Rodríguez-Lozano *et al*[7] suggested cut-off values between 2.5 and 3.0 for ASDAS-CRP and cut-off values between 2.8 and 3.5 for ASDAS-ESR (PASS as external construct), depending on the method used to determine the cut-off value. A more recent study by Sellas *et al*[8] suggested that the ASDAS cut-off of 2.04 was associated with patient-PASS while the ASDAS cut-off of 2.44 was associated with physician-PASS.

Following an open discussion among ASAS members about the topic, the proposed nomenclature change from ASDAS 'moderate disease activity' to ASDAS 'low disease activity' was voted and approved by ASAS members (figure 1). Other possibilities that were considered but rejected by ASAS members were the use of the wording 'low/moderate disease activity', 'mild disease activity' and 'moderate/high disease activity' (the latter proposed to replace 'high disease activity' state).

In conclusion, the nomenclature of ASDAS disease activity states was updated by ASAS. The 'moderate disease activity' state is replaced by 'low disease activity' state, better reflecting the opinion of patients and physicians about what ASDAS values ≥1.3 and <2.1 represent. This change will improve the interpretability of ASDAS scores and will facilitate the implementation of treat-to-target strategies in axSpA.

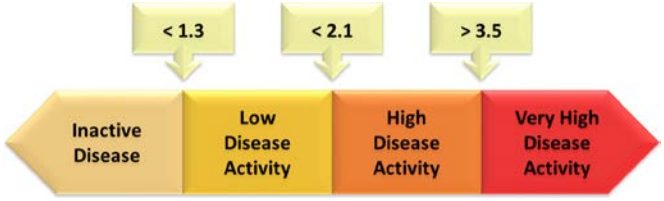

Figure 1 2018 update of the nomenclature for Ankylosing Spondylitis Disease Activity Score (ASDAS) disease activity states.

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Impaired long-term immune protection following pneumococcal 13-valent/23-valent polysaccharide vaccine in systemic lupus erythematosus (SLE)

Although patients with systemic lupus erythematosus (SLE) are at increased risk for *Streptococcus pneumoniae* infection,[1 2] vaccination coverage remains dramatically low in SLE,[3] and efficacy of the now recommended prime-and-boost strategy using 13-valent pneumococcal conjugate vaccine (PCV13) and 23-valent pneumococcal polysaccharide vaccine (PPSV23) is not known.

Consecutive patients with SLE admitted in our daycare hospital unit were prospectively enrolled to receive PCV13 followed by PPSV23 8 weeks later. Immune protection, defined by an antigen-specific IgG concentration ≥1.3 µg/mL for at least 70% of seven pneumococcal serotypes (4, 6B, 9V, 14, 18C, 19F and 23F), was assessed at baseline, 2 and 12 months. Patients were defined as 'long-term protected (LTP)' when seroconversion was observed 2 months and 12 months after PCV13 shot. Patients were considered 'short-term protected (STP)' when seroconversion was observed 2 months but no more at 12 months after PCV13 shot. Patients were 'not protected (NP)' when seroconversion occurred neither 2 months nor 12 months after PCV13 shot. Laboratory tests included the analysis of blood B lymphocyte subsets (online Supplementary figure S1) and immunoglobulin levels (online Supplementary data).

Thirty-seven patients with SLE were screened for pneumococcal vaccination. Among them, eight patients refused to be vaccinated, seven patients accepted the vaccination but refused to complete the 12 months immune response follow-up and one patient had already been vaccinated against pneumococcal infection. Eventually, 21 (40 (25–75) years; 85.7% female) patients were included in the study (table 1 and online Supplementary data).

No patient with SLE had a protective immune status against pneumococcal infection at baseline. Overall, only 12 patients (57.1%) showed a sustained immune protection against pneumococcal infection 12 month after PCV13 (LTP). Nine patients had no immune protection following pneumococcal vaccination with seroconversion that never (n=4, NP) or only transiently (n=5, STP) occurred (online Supplementary figure S2). No SLE flare was observed during the 12 months following pneumococcal vaccination.

As compared with patients with transient (STP) or sustained (LTP) immune protection, patients with SLE who totally failed to reach seroconversion (NP) had received immunosuppressive drugs for a longer period of time. Accordingly, deep B cell defects and hypogammaglobulinaemia were associated with the NP status (table 1). On the other hand, STP patients had a lower serum level of IgG2, a higher frequency of pregerminal centre B cells and a lower frequency of postgerminal centre B cells than LTP (table 1 and online Supplementary figure S3).

To help identifying patients with SLE actually protected against pneumococcal infection after vaccination, we next determined whether pneumococcal antigen-specific IgG measured 2 months after the PCV13 could be predictive of long-term protection. Accordingly, the receiver operating characteristic curves calculated for each of the seven tested serotypes showed that the IgG responses against serotype 19F had the best ability to discriminate between short-term and long-term protection (online Supplementary figure S2). Serotype 19F IgG titres of 4.35 ug/mL or more at M2 had a sensitivity of 100% (95% CI 47.8 to 100) and a specificity of 91.7% (95% CI 61.5 to 99.8) for long-term protection.

We show, in this 12-month prospective monocentric study, that more than 40% of patients with SLE fail to reach immune protection against pneumococcal infection despite a prime-and-boost PCV13/PPSV23 vaccine strategy. This finding is consistent with recent published data analysing the rate of SLE immune responders following a sequential administration of PCV7/PPSV23.[4] Importantly, we identify two distinct populations of patients with SLE who clearly do not benefit from pneumococcal vaccination. First, immune protection never occurs in patients with severe B cell defect. Because such a B cell defect was associated with the duration of immunosuppressive treatment, pneumococcal vaccination should be recommended early in the course of SLE disease. Second, immune protection occurs but is not maintained overtime in patients with SLE who shared a 'common variable immunodeficiency-like' immune phenotype.[5 6] In such cases, we further show that serotypes IgGs titres (more particularly serotype 19F IgGs titre) measured 2 months after PCV13 reliably discriminate between LTP and STP patients.

In conclusion, our data demonstrate the poor immune protection conferred by the recommended sequential PCV13/PPSV23 vaccines in SLE and identify several key factors associated with long-term protection that may help to design new pneumococcal vaccination strategy.

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Table 1 Clinical and immunological characteristic of patients with SLE

	LTP (n=12)	STP (n=5)	NP (n=4)	P
Age, years	37.5 (25–75)	50 (31–67)	42 (38–45)	ns
Female, n (%)	10 (83.3)	5 (100)	3 (75)	ns
History of PI, n (%)	1 (8.3)	0 (0)	2 (50)	ns
Duration of SLE disease, years	9.5 (1–32)	7 (1–23)	13.5 (2–19)	ns
Lupus nephritis, n (%)	8 (66.7)	2 (40)	2 (50)	ns
Steroid at study time, n (%)	6 (50)	2 (40)	4 (100)	ns
Steroid dose at study time, mg/d	10 (7–15)	10.5 (7–14)	9 (7–10)	ns
Cumulative years of steroid	6.5 (0.5–26)	2 (0–7)	7.5 (2–17)	ns
IS drugs at any time, n (%)	9 (75)	2 (40)	4 (100)	ns
IS drugs at study time, n (%)	4 (33.3)	1 (20)	4 (100)	<0.05*
Cumulative years of IS drugs	2.5 (0–14)	0 (0–3)	4 (2–6)	<0.05*
SELENA-SLEDAI score	2 (0–4)	0 (0–0)	1 (0–4)	ns
Anti-dsDNA antibodies titres, UI/mL	36 (0–258)	0 (0–108)	21 (0–148)	ns
High levels of anti-dsDNA titres, n (%)	6 (50)	1 (20)	1 (25)	ns
Lymphocytes <1000/mm^3	2 (16.7)	0 (0)	4 (100)	<0.01*
B cells/mm^3	166 (17–634)	243 (184–424)	16 (0–46)	<0.05†*
Naive B cells/mm^3	64 (2–500)	148 (90–317)	14 (1–25)	<0.01*
Transitional B cells/mm^3	3 (0–67)	13 (3–65)	0 (0–0)	<0.05*
Unswitched B cells/mm^3	7 (2-44)	11 (3–38)	6 (0–16)	ns
Germinal centre B cells/mm^3	0.1 (0–2.4)	0.2 (0–1.7)	0 (0–0.1)	ns
Postgerminal centre B cells/mm^3	27 (9–77)	18 (13–57)	5 (4–13)	<0.05†
Plasma cells/mm^3	0.5 (0–4.8)	0.3 (0–1.2)	0.1 (0–1.3)	ns
Gammaglobulin level <5 g/L, n (%)	3 (25)	0	4 (100)	<0.01† *
IgG, g/L	12.1 (1.3–23.8)	9.7 (9–15.7)	4 (1.8–6.2)	<0.05†
IgG1, g/L	6.4 (4.1–18.6)	7.9 (6.6–10.5)	3.7 (2.2–7.2)	ns
IgG2, g/L	3.1 (1.3–7.3)	1.3 (1.3–3.4)	1.7 (0.8–1.9)	<0.05‡
IgG3, g/L	0.5 (0.1–1)	0.7 (0.2–1.6)	0.2 (0.1–0.5)	ns
IgG4, g/L	0.2 (0.1–1.3)	0.2 (0.2–0.4)	0.1 (0–0.3)	ns
IgA, g/L	2.6 (1.4–6.4)	2.5 (1.5–3.5)	1.1 (0–1.9)	ns
IgM, g/L	0.9 (0.6–1.1)	0.8 (0.4–1.4)	0.8 (0–1.6)	ns
Low levels of complement, n (%)	1 (8.3)	1 (20)	1 (25)	ns

Naive B cells, CD19+CD27−IgD+CD10CD38−/low cells; transitional B cells, CD19+CD27−IgD+CD10+CD38high; unswitched B cells, CD19+CD27+IgD+ cells; germinal centre B cells, CD19+CD27+IgD−CD10+CD38+; postgerminal centre B cells, CD19+CD27+IgD−CD10−CD38low/+; plasma cells, CD19+CD27+IgD−CD10−CD38high.
High levels of anti-dsDNA titres referred to a twofold change threshold.
Low levels of complement referred to a low level of both C3 and C4.
Lupus nephritis was class III or class IV.
Significance determined using Kruskal-Wallis one-way analysis of variance on ranks test for continuous variables and X^2 test (or Fisher) for categorical variables.
*Significant difference: P<0.05 between STP and NP.
†Significant difference: P<0.05 between LTP and NP.
‡Significant difference: P<0.05 between LTP and STP.
dsDNA, double stranded DNA; IS, immunosuppressive drugs (including mycophenolate mofetil, cyclophosphamide, methotrexate and azathioprine); LTP, long-term protected; NP, not protected; ns, no significant difference; PI, pneumococcal infection; SELENA, Safety of Estrogens in Lupus Erythematosus National Assessment; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; SLE, systemic lupus erythematosus; STP, short-term protected.

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Reduced salivary secretion contributes more to changes in the oral microbiome of patients with primary Sjögren's syndrome than underlying disease

The oral microbiome of patients with primary Sjögren's syndrome (pSS) differs from that of healthy individuals.[1–3] Whether these changes are the consequence of reduced salivary secretion or specific for pSS is yet unknown. Therefore, the aim of our study was to assess whether patients with pSS have a specific oral microbiome compared with symptom controls (patients without SS, indicated as non-SS patients) and healthy controls (HCs).

To capture the overall bacterial composition in the mouth, we collected oral washings instead of buccal swabs from 121 consecutive patients referred for a diagnostic pSS workup and 14 volunteers without oral or ocular dryness (HCs). Patients fulfilling the 2016 American College of Rheumatology/European League Against Rheumatism classification criteria were classified as pSS (n=36) and patients not fulfilling the criteria as non-SS (n=85) (table 1).[4]

Bacterial composition was assessed with 16S rRNA gene sequencing of the V4 region. Reads were processed and diversity was analysed with QIIME V.1.9.1.[5] All samples were equated to 8000 reads/sample. Comparative statistics were performed in R V.3.4.2.[6] For details see online Supplementary text.

Alpha diversity, that is, the number of bacterial taxa and proportion in which each taxon is represented per sample was measured by the number of observed operational taxonomic units and Shannon index. Alpha diversity was similar between pSS, non-SS and HCs, indicating that oral washings of patients with pSS harbour a similar number of bacterial taxa and a similar distribution of relative abundances of taxa as non-SS patients and HCs (figure 1A, B).

Beta diversity, that is, the dissimilarity between samples based on the relative abundance of bacterial taxa, showed a similarly large variation in bacterial composition in samples from patients with pSS and without SS compared with HCs (figure 1C, D). This suggests that the oral microbiome in patients with pSS and

Table 1 Patient characteristics*

	Healthy controls	non-SS patients	Patients with pSS
n	14	85	36
Male gender, n (%)	1 (7.1)	18 (21.2)	5 (13.9)
Age, mean (SD)	41.7 (13.7)	54.9 (14.6)	59.1 (11.9)
Natural teeth present, n (%)	14 (100)	68 (88.3)	30 (88.2)
Smoking	0	16 (19.0)	0
Fulfilling 2016 ACR/EULAR criteria, n (%)	0 (0.0)	0 (0.0)	36 (100.0)
Xerostomia, n (%)	0 (0.0)	80 (94.1)	34 (94.4)
UWS mL/min, median (IQR)	0.51 (0.19)	0.13 (0.19)	0.07 (0.09)
SWS mL/min, median (IQR)	1.23 (0.6)	0.40 (0.47)	0.18 (0.34)
Hyposalivation (UWS≤0.1), n (%)	0 (0.0)	35 (41.2)	22 (61.1)
SSA positive, n (%)	NA	10 (11.8)	23 (63.9)
RA	0	4 (4.7)	3 (8.3)
SLE	0	0	0
Xerogenic medication	NA	47 (55.3)	17 (47.2)
PPI	NA	37 (43.5)	14 (38.9)
DMARD	NA	11 (12.9)	8 (22.2)
NSAID	NA	19 (22.4)	7 (19.4)
Methotrexate	NA	4 (4.7)	1 (2.8)
Antimalarial	NA	7 (8.2)	3 (8.3)
Corticosteroids	NA	2 (2.4)	4 (11.1)
Mean reads per sample (SD)	149 568 (134 936)	106 729 (76 116)	85 105 (44 158)

*Natural teeth present, any natural teeth present (ie, not edentulous); xerostomia, symptoms of oral dryness; hyposalivation, defined according to 2016 ACR/EULAR criteria; xerogenic medication, using a drug with a reported moderate to high level of evidence of inducing salivary gland dysfunction xerostomia or subjective sialorrhoea[10]. Age, UWS and SWS differed significantly between groups, while dental status (natural teeth present) was not significantly different.
ACR, American College of Rheumatology; DMARD, disease-modifying antirheumatic drug; EULAR, European League Against Rheumatism; NSAID, non-steriod anti-inflammatory drug; PPI, proton pump inhibitor; pSS, primary Sjögren's syndrome; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SSA, Sjögren's syndrome autoantibody A; UWS/SWS, unstimulated/stimulated whole salivary secretion.

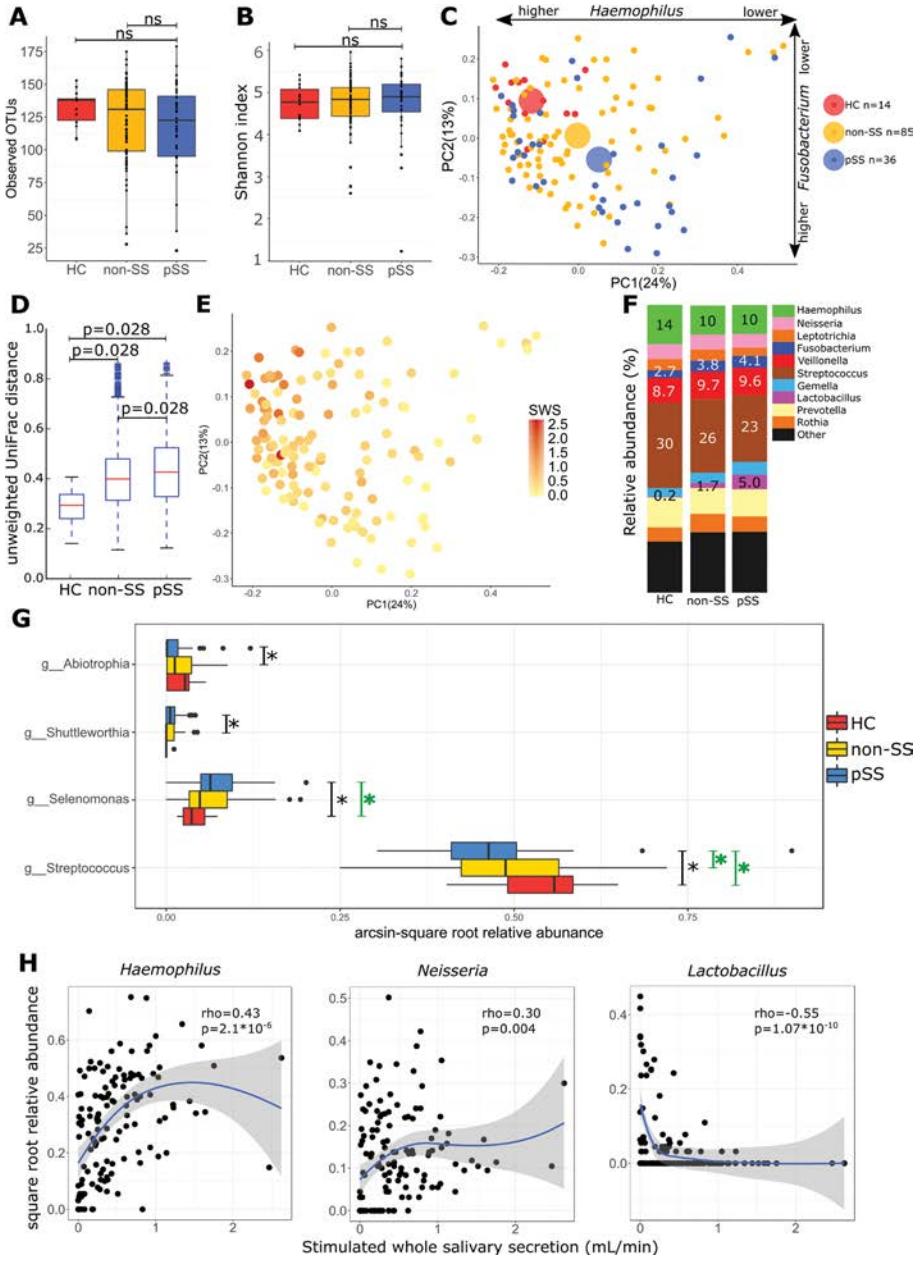

Figure 1 Characteristics of the oral microbiome in patients with primary Sjögren's syndrome (pSS), symptom-controls without SS (non-SS) and healthy controls (HC). (A, B) Alpha diversity measured by the number of observed operational taxonomic units (OTUs) and Shannon index (ns=not significant, Wilcoxon test with Benjamini Hochberg correction for multiple comparisons, alpha ≤0.05). (C) Beta diversity showing the dissimilarity between individual samples (small dots) and between group means (large dots) in a principal coordinate analysis (PCoA) based on unweighted Unifrac distance. *Haemophilus* had the strongest (negative) correlation with PC1 (coefficient –0.45, FDR p=1.6×10^{-9}) and *Fusobacterium* correlated strongly negatively with PC2 (coefficient –0.34, FDR p=2.3×10^{-13}, Multivariate Associations with Linear Models (MaAsLin)). The smaller the distance between dots, the more similar the bacterial composition. At group level, pSS and non-SS differed significantly from HCs in PC1 (p=0.005 and p=0.008, respectively) and PC2 (p=0.003 and p=0.008, respectively), while pSS was significantly different from non-SS in PC2 (p=0.02), but not in PC1 (p=0.17, Wilcoxon test with Benjamini Hochberg correction). (D) Distance box plot showing a significantly larger unweighted UniFrac distance between samples within patients with pSS, followed by non-SS patients in comparison with HCs (two-sided Student's two sample t-test, Bonferroni corrected non-parametric p values using 999 permutations). (E) PCoA as in (C), coloured according to stimulated whole salivary secretion (SWS, mL/min). (F) Mean relative abundance of the 10 most abundant genera per group. (G) Bacterial taxa significantly associated with pSS compared to HCs and non-SS patients, taking into account age, dental and smoking status (black bars) and additionally accounting for SWS (green bars) (MaAsLin, *FDR p<0.10) (H) Three of the 10 most abundant genera were significantly correlated with SWS (Spearman, Bonferroni corrected p value). Blue line shows the locally weighted scatterplot smoothing line (non-parametric regression method) and in grey the 95% CI.

symptom-controls without SS (non-SS) is more heterogeneous than that of HCs. This large heterogeneity in bacterial composition makes it impossible to identify individual patients with pSS based on overall oral bacterial composition. Disease status (pSS, non-SS or HCs) explained only 5% of the variation between samples (p<0.001, Adonis), while stimulated whole salivary secretion (SWS) explained 9% (figure 1E) (p<0.001). Despite the large variation between individual patients with pSS, average

overall bacterial composition in patients with pSS differed from non-SS patients and HCs, located at highest principal coordinate (PC) 1 and lowest PC2 (figure 1C, F). *Haemophilus* and *Fusobacterium* were negatively correlated with PC1 and PC2, respectively, indicating that patients with pSS have lower *Haemophilus* and higher *Fusobacterium* relative abundance on average.

Multivariate Association with Linear Models found lower *Streptococcus* and higher *Selenomonas* relative abundances significantly associated with pSS compared with HCs (false discovery rate (FDR) corrected $p<0.10$, taking into account age, dental and smoking status) (figure 1G).[7–9] Non-SS patients showed more similar relative abundances of these genera. *Abiotrophia* and *Shuttleworthia* were associated with pSS compared with non-SS (figure 1G, online Supplementary table S1). Surprisingly, previous studies reported higher oral *Streptococcus* relative abundance in patients with pSS compared with HCs.[1–3] These studies used different samples (saliva, buccal and tongue swabs) and sequenced different variable regions, which may explain the discrepancy with our study (online Supplementary table S2).[1–3]*Streptococcus* relative abundance in our study was not correlated with SWS (Spearman, $p=0.52$) and was significantly associated with pSS compared with HCs and non-SS independent of SWS (figure 1G, FDR $p<0.10$). This suggests that lower *Streptococcus* relative abundance is more disease specific than related to SWS. In contrast, *Haemophilus*, *Neisseria* and *Lactobacillus* relative abundance was significantly correlated with SWS, but not with disease status, indicating that lower SWS in patients with pSS can explain changes of these genera (figure 1F, H).

We conclude that salivary secretion has a stronger influence on the microbiome in oral washings than disease status. However, lower *Streptococcus* relative abundance in patients with pSS appears to be a disease-specific effect.

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Coffee consumption and gout: a Mendelian randomisation study

Observational studies have found that coffee consumption is inversely associated with risk of incident gout but inconsistently with serum uric acid concentrations.[1–3] The causality of the associations is, however, uncertain since observational studies are susceptible to confounding and reverse causation bias. Genetic variants with an explicit impact on a modifiable exposure, such as a biomarker or habitual behaviour like coffee consumption, can be used as instrumental variables (proxies) for the exposure to improve causal inference.[4] This method, known as Mendelian randomisation, builds on Mendel's second law and the fact that genetic variants are randomly assorted during meiosis. Therefore, results from Mendelian randomisation studies are less prone to bias due to confounding and reverse causality. We used the Mendelian randomisation approach to examine whether coffee consumption

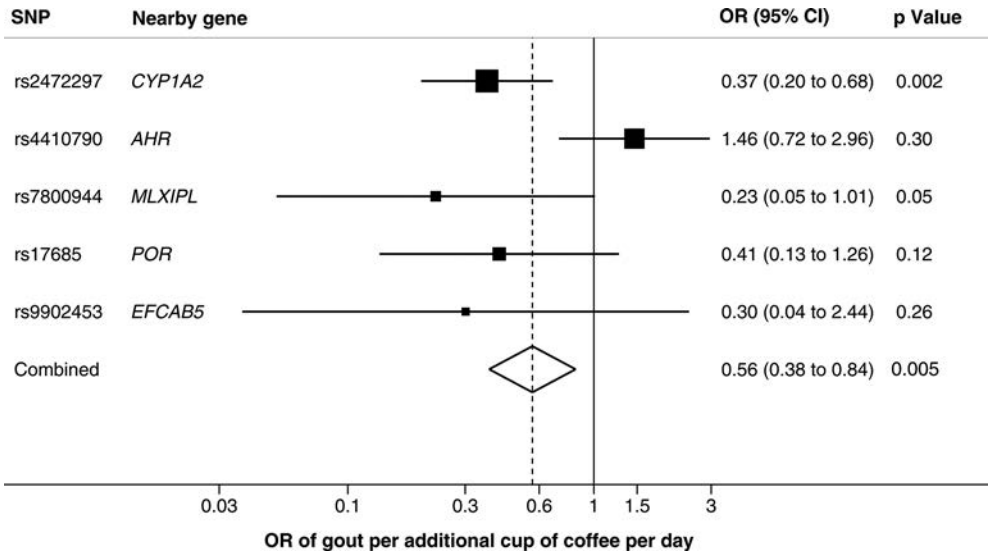

Figure 1 Association between genetically predicted coffee consumption and gout. Squares represent the OR of gout per genetically predicted one cup per day increase in coffee consumption; horizontal lines represent 95% CIs; diamond represent the combined OR, that is, the genetic association between coffee consumption and gout with its 95% CI. Test for heterogeneity between SNPs: I^2=63.4%; p=0.03. After exclusion of the outlying SNP near *AHR* (rs4410790), the OR per genetically predicted one cup per day increase of coffee consumption was 0.35 (95% CI 0.22 to 0.58) (test for heterogeneity: I^2=0%; p=0.93). SNP, single-nucleotide polymorphism.

is associated with gout and whether the association may be mediated by serum uric acid concentrations.

A genome-wide association study from the Coffee and Caffeine Genetics Consortium identified 10 single-nucleotide polymorphisms (SNPs), at eight loci, associated with coffee consumption (online supplementary table S1).[5] We excluded three SNPs that did not exceed the genome-wide significance threshold ($p < 5\times10^{-8}$) and two SNPs in linkage disequilibrium ($r^2 > 0.6$) (online supplementary table S1), leaving five independent SNPs for the main analyses. The Global Urate Genetics Consortium[6] provided summary statistics data for serum uric acid concentrations (110 347 individuals) and gout (2115 cases and 67 259 controls). Analyses were conducted using conventional (inverse-variance weighted) and complementary (weighted median and MR-Egger) Mendelian randomisation approaches.[4]

All but one of the coffee-increasing alleles of the five SNPs with a genome-wide significant association with coffee consumption were inversely associated with gout, and the association with rs2472297, near the gene encoding CYP1A2 (the major enzyme responsible for caffeine metabolism), was statistically significant (figure 1). In conventional Mendelian randomisation analysis, using data from all five SNPs, the OR of gout was 0.56 (95% CI (CI) 0.38 to 0.84; p=0.005) per genetically predicted one cup per day increase in coffee consumption (figure 1). Rs2472297 (*CYP1A2*) and rs7800944 (*MLXIPL*) but not the other three SNPs were inversely associated with serum uric acid concentrations (online supplementary table S1). Overall, serum uric acid concentrations decreased by 0.15 mg/dL (−0.15; 95% CI −0.22 to −0.09; $p=7.9\times10^{-6}$) per genetically predicted one cup per day increase in coffee consumption.

Results for gout and serum uric acid were consistent in sensitivity analyses including two additional SNPs (in *GCKR* and *ABCG2*) associated with coffee consumption at borderline genome-wide significance ($p<1\times10^{-7}$) (online supplementary table S2), and which were outliers (online supplementary figure S1). Further inclusion of rs6265 (in *BDNF*), which is weakly associated with coffee consumption and strongly with smoking and body mass index,[5] did not change the results markedly (online supplementary table S2). Results for gout but not serum uric acid were also consistent in complementary analyses using the weighted median method, and there was no evidence of pleiotropy (online supplementary table S2).

In summary, this study based on genetics provides evidence that coffee consumption may lower the risk of gout. Our finding corroborates and extends the results from two prospective cohorts of 89 000 US women[1] and 46 000 US men[2] which showed that self-reported high coffee consumption (≥4 cups/day) was associated with a significant reduced risk (40%–60%) of gout. Coffee is a major source of caffeine, a methylxanthine that has been demonstrated to competitively inhibit xanthine oxidase in rats.[1] Hence, like allopurinol, caffeine may exert a protective effect against gout by lowering serum uric acid concentrations. This study provided limited support that the association between coffee consumption and gout may be mediated by serum uric acid concentrations. Coffee consumption may reduce gout risk through other mechanisms, for example, by reducing inflammation, oxidative stress, glucose and insulin concentrations, and insulin resistance.[1–3]

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EXTENDED REPORT

Baseline ultrasound examination as possible predictor of relapse in patients affected by juvenile idiopathic arthritis (JIA)

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ABSTRACT

Objectives To define the correlation between joint ultrasonography and clinical examination in patients with juvenile idiopathic arthritis (JIA) and to assess whether synovitis detected by ultrasonography in clinically inactive patients predicts arthritis flares.

Methods 88 consecutive patients with JIA—46 (52%) with persistent oligoarthritis, 15 (17%) with extended oligoarthritis, 15 (17%) with rheumatoid factor-negative polyarthritis and 12 (14%) with other forms of JIA, all clinically inactive for a minimum of 3 months—underwent ultrasound (US) assessment of 44 joints. Joints were scanned at study entry for synovial hyperplasia, joint effusion and power Doppler (PD) signal. Patients were followed clinically for 4 years.

Results US was abnormal in 20/88 (22.7%) patients and in 38/3872 (0.98%) joints. Extended oligoarthritis and rheumatoid factor-negative polyarthritis were more frequent in US-positive than in US-negative patients (35.0% vs 11.8% and 30.0% vs 13.2%, respectively; P=0.005). During 4 years of follow-up, 41/88 (46.6%) patients displayed a flare; 26/68 (38.2%) were US-negative and 15/20 (75%) were US-positive at baseline. Abnormality on US examination, after correction for therapy modification, significantly increased the risk of flare (OR=3.8, 95% CI 1.2 to 11.5). The combination of grey scale and PD abnormalities displayed a much higher predictive value of relapse (65%, 13/20) than grey scale alone (33%, 6/18).

Conclusions US abnormalities are a strong predictor of relapse at individual patient level. Irrespective of treatment, the risk of flare in US-positive versus US-negative patients was almost four times higher. In case of US abnormalities, patients should be carefully followed regardless of both the International League of Associations for Rheumatology and Wallace categories.

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INTRODUCTION

Achieving remission is the target in treating children affected by juvenile idiopathic arthritis (JIA). Preliminary clinical and laboratory criteria to define remission and clinically inactive disease have been published.[1 2] Unfortunately, the risk of flare after discontinuation of methotrexate or biological therapy is high,[1–9] and tools predicting relapse are urgently needed. A recent paper by Guzman *et al*[10] showed that there are no clinical features reliably associated with the risk of flare except the JIA subtype.

In rheumatoid arthritis, the role of ultrasonography (US) to predict disease relapse and structural damage in rheumatoid arthritis is well established.[11–14] Studies on US of joints in healthy children and in JIA have already been published.[15–21] It has been suggested that US may be more sensitive in detecting joint inflammation in patients with JIA than the clinical evaluation.[22–27] However, the role of US-detected synovitis when clinical manifestations in JIA are absent is unknown. Furthermore, the only published study on the role of US in predicting JIA relapse showed no predictability.[28]

We, therefore, performed a prospective study with the aim of determining the value of US in clinical remission and its power to predict flare.

MATERIALS AND METHODS

Population and study design

We enrolled the following groups of subjects (see online supplementary table 1): (1) 88 consecutive patients with inactive JIA to study the association between US and synovitis flare; (2) 30 consecutive patients with active JIA and (3) 30 healthy subjects for US interpretation setting and calibration; and (4) 30 patients with active JIA recruited during an educational event with the aim to determine the intraobserver and interobserver reliability.

All patients in group 1 were classified according to the International League of Associations for Rheumatology (ILAR) criteria[29] with the disease being inactive for a minimum of 3 months. The recruitment occurred between January 2011 and December 2012. At study entry, all patients in groups 1 and 2 and the healthy subjects (group 3) underwent a clinical assessment. The following data were recorded: sex, birth date and, in case of patients with JIA, ILAR category, age and medications at baseline, and disease activity status (ie, clinical remission off medication—CR; clinical remission on medication—CRM; inactive disease—ID). Within the same session, each subject (groups 1, 2 and 3) underwent a US assessment, which was performed separately, blindly and immediately after the clinical evaluation, by one of two rheumatologists (ODL, VR) skilled in paediatric musculoskeletal US (MSUS). All inactive patients were followed for 4 years, every 3–6 months, without repeating US examination, according to our usual

clinical practice. We recorded the flares during the follow-up, the time to flare from baseline, the joints affected by flare, the therapy administered at baseline and its modification during the follow-up. A flare of synovitis was defined as a recurrence of clinically overt active arthritis, in one or more joints.

Group 4 was included only to assess the reliability between the machines and among the examiners and did not take part in the main study. Informed consent was obtained.

Clinical assessment

Clinical remission was defined according to Wallace criteria as CR, CRM and ID.[1] Joint assessment was performed by experienced paediatric rheumatologists blinded to US findings for groups 1, 2 and 3. Joints were assessed for swelling, tenderness/pain on motion and restricted motion. A joint with active arthritis was defined as a joint with swelling or, in the absence of swelling, with tenderness/pain on motion and restricted range of motion.

Ultrasonographic evaluation

The US examination technique and the standard scans were based on published Outcome Measures in Rheumatology (OMERACT) guidelines and on the studies on paediatric MSUS available at study entry.[15 30–33]

US setting and calibration

To standardise evaluation, the two examiners shared two consensus sessions: on active patients (group 4) and on stored images of groups 2 and 3, discussing all issues related to evaluation. Definite guidelines on paediatric US evaluation were not available at the study entry. However, the adopted criteria fit very well with the recent OMERACT preliminary definition of synovitis in children.[34] In each joint, three elements were assessed by MSUS: synovial hyperplasia, synovial effusion and power Doppler (PD) signal.

Synovial hyperplasia was defined as an abnormal hypoechoic joint space, distinct from the intra-articular fat pad and non-displaceable with the transducer. Synovial effusion was detected as the presence of an abnormal hypoechoic or anechoic space within the joint, which was displaceable. Synovial hyperplasia and synovial effusion were considered indifferently as a single abnormality in grey scale examination. PD signal was considered pathological in the presence of vessel dots only inside an area of synovial hyperplasia. In each joint, synovial hyperplasia, effusion and PD signal were graded semiquantitatively on a 0–3 scale.[32] For statistical calculations, however, we used the categories present (if ≥ 1) or absent (if <1) only. The possible presence of a physiological small amount of synovial fluid in the joints of healthy children was considered.

In the presence of synovial fluid, the joint recess was measured and the effusion scored ≥ 1, if greater than the average value plus 2 SD of the normal joint space, for each joint according to published data.[15 18] As for some joints published data are missing, we referred to data collected in our cohort of healthy children (online supplementary table 2).

A total of 44 joints for each patient (2 shoulders, 2 elbows, 2 wrists, 10 metacarpophalangeal, 10 proximal interphalangeal of hands, 2 hips, 2 knees, 2 subtalar, 2 tibiotalar and 10 metatarsophalangeal) were scanned for the presence of synovial hyperplasia/joint effusion and PD signal. To perform the examination, an Esaote MyLab 70 with a 6–18 MHz linear probe and a GE Logiq S8 with an 8–18 MHz linear probe were used. The grey scale frequency was adjusted according to the depth of the joint (18 MHz for small superficial joints, 1412 MHz for medium size and 10 or less for deeper joints); the highest frequencies were used for younger children. The PD frequency was set to 9–10 MHz for superficial joints and 8–6 MHz for medium and deep joints. The pulse repetition frequency was 750 Hz and the gain was set at a level just below the disappearance of the noise under the cortical bone.

Intraobserver and interobserver reproducibility

Given the problem of various Doppler sensitivities between different US machines,[35] a comparison of the two devices in their ability to detect blood flow in the volar aspect of the finger pulp and the feeding vessel of the capitate bone in five subjects of group 4 was made prior to the inter-rater assessment to ensure their comparability. A good agreement was found (Cohen's $k=0.80$).

The interobserver reproducibility was determined in group 4. On each patient 44 joints were assessed by the two operators (ODL and VR) independently and on the same day. The intraobserver reproducibility was calculated using saved images of the same group of patients 3 months after the day of data acquisition. Cohen's kappa coefficient of agreement was calculated to evaluate the intraobserver and interobserver reliability.

Statistical analysis

Sample size calculation of group 1 was based on the main hypothesis and on the feasibility of this study considering the number of patients usually attending our outpatient clinic (see online supplementary statistical analysis paragraph for details).

The dimension of groups 2, 3 and 4 was determined in 30 subjects for each category requiring an absolute precision of 0.2 in Cohen's k calculation with $\alpha=0.05$.

Descriptive statistics were used to calculate mean and SD for continuous variables and percentage for categorical variables. Difference in mean age between US-positive and US-negative patients was analysed by unpaired t-test. Association between categorical variables was assessed by χ^2 and Fisher's exact tests when appropriate.

Diagnostic test performance was defined by accuracy, specificity, sensitivity and positive/negative predictive values (PPV and NPV, respectively). Wilson Score[36] was used to calculate CI on binomial probability ('binom' R package). McNemar's test without continuity correction was applied to determine the significance of the disagreement in group 2.

Risk was measured in terms of OR, and Baptista-Pike mid-p[37] was used to obtain CI on OR (ORCI R package). The probability of flare-free condition was calculated with Kaplan-Meier method, and log-rank test was applied to compare stratified curves. To assess the role of treatment in the ability of US to predict flare, a stratified analysis was performed and the stratum-specific ORs were calculated. Breslow-Day test was applied to test the null hypothesis of homogeneity between stratified OR estimates, and Cochran-Mantel-Haenszel method was performed to compute a weighted average of the ORs across the strata. A P value <0.05 was considered as significant.

Statistical analysis was performed using R V.3.0.2 for Windows.

RESULTS

Harmonisation of US evaluation between examiners

Demographic and clinical characteristics of group 4 are included in online supplementary table 3. Cohen's k value was 0.89 for intraobserver reliability and 0.82 for interobserver reliability.

The site-by-site inter-reader agreement is displayed in online supplementary table 4.

Setting and calibration of US evaluation

Demographic and clinical characteristics of groups 2 and 3 are summarised in online supplementary table 3.

All 30 healthy children (17 female and 13 male, mean age 8.6±2.9 years) were attending our outpatient clinics but without any clinical and laboratory evidence of inflammatory musculoskeletal disorders. They were negative both at physical and US examination (none of the elementary lesion reached a score grading ≥1, in particular no sign of synovial hyperplasia was found). As expected, all the healthy children showed physiological Doppler signal, typical of the immature joints (the feeding vessels of physis, epiphyses, fat pads, carpal and tarsal bones). Sixty-three per cent (19/30) of healthy subjects had a minimal amount of synovial fluid detectable in at least one joint.

Group 2 with clinically active disease (21 female and 9 male, mean age 7.9±4.2 years) included 16 patients with persistent oligoarthritis, 12 with extended polyarthritis, 1 with rheumatoid factor-positive polyarthritis and 1 with enthesitis-related arthritis. There were 173 joints positive at US examination and 144 positive at clinical examination; the clinical and US examinations were comparable (McNemar's test P value >0.05).

Association between baseline US and subsequent clinical flares in the follow-up of patients with inactive disease at study entry

Characteristics of patients with inactive JIA and risk of flare

The main features of the 88 clinically inactive patients are summarised in table 1. We scanned by US 44 joints per patient for a total of 3872 joints. In 20 (22.7%) out of 88 patients and in 38 out of 3872 joints (0.98%), the US examination was abnormal for at least one of grey scale elementary lesions (synovial hyperplasia/joint effusion), with or without abnormal PD signal.

Comparing clinical examination and US, the most discordant joints were 15 knees, 8 wrists, 7 tibiotalar, 4 subtalar, 3 metatarsophalangeal and 1 elbow.

We compared the demographic and clinical characteristics of the patients with (20/88) and without (68/88) US abnormalities (defined as US-positive and US-negative patients, respectively).

As shown in table 1, extended oligoarthritis and rheumatoid factor-negative polyarthritis were almost three times more represented in US-positive than in US-negative patients (35.0% vs 11.8% and 30.0% vs 13.2%, respectively). Regarding Wallace criteria, the ID category was three times more represented in US-positive (30.0%) than in US-negative patients (10.3%), whereas CRM was only slightly more frequent in US-positive than in US-negative ones (60.0% vs 51.5%). In other words, US-positive patients compared with US-negative ones showed a statistically different distribution of both ILAR categories and Wallace criteria (P=0.005 and P=0.015, respectively). Noteworthy, US-positive patients showed a remission duration significantly (P<0.001) shorter than US-negative ones (0.99 vs 1.96 years). This difference was confirmed in relapsed patients (1.11 vs 1.81 years; P=0.019).

Out of a total of 88 patients, 41 (46.6%) had a flare of disease during follow-up: 38.2% (26/68) of US-negative patients and 75% (15/20) of US-positive patients (table 2).

This means that the presence of an abnormal US examination increases the risk of flare by almost five times (OR=4.9, 95% CI 1.6 to 14.9; P=0.005). The PPV of US is equal to 75% (95% CI 56.0 to 94.0). Performance measures of US are shown in table 3.

Table 1 Main features of the 88 inactive patients stratified into US-positive and US-negative groups

	US-positive (n=20)	US-negative (n=68)	P value
Sex (female, %)	16 (80.0)	52 (76.5)	1.000
Age (mean±SD)	10±4.3	9.9±4.4	0.949
ILAR category			
Persistent oligoarticular JIA, n (%)	7 (35.0)	39 (57.4)	0.005
Extended oligoarticular JIA, n (%)	7 (35.0)	8 (11.8)	
Rheumatoid factor-negative JIA, n (%)	6 (30.0)	9 (13.2)	
Other	0 (0.0)	12 (17.6)	
Wallace criteria			0.015
CR, n (%)	2 (10.0)	26 (38.2)	
CRM, n (%)	12 (60.0)	35 (51.5)	
ID, n (%)	6 (30.0)	7 (10.3)	
Disease duration at baseline (years) (mean±SD)	4.52 (3.17)	7.70 (4.51)	0.095
Remission duration at baseline (years) (mean±SD)	0.99 (0.68)	1.96 (0.87)	<0.001
Therapy discontinuation			
Yes, n (%)	6 (30.0)	22 (32.4)	0.047
No, n (%)	11 (55.0)	19 (27.9)	
NA, n (%)	3 (15.0)	27 (39.7)	
Treatment*			
Intra-articular steroids, n (%)	10 (50.0)	47 (69.1)	0.528
Oral steroids, n (%)	2 (10.0)	4 (5.9)	
Methotrexate, n (%)	13 (65.0)	40 (58.8)	
Biological drugs, n (%)	2 (10.0)	3 (4.4)	

*The sum is not equal to the number of US-positive or US-negative patients because each patient can have more than one treatment at a time.
CR, complete remission off medication; CRM, complete remission on medication; ID, inactive disease; ILAR, International League of Associations for Rheumatology; JIA, juvenile idiopathic arthritis; NA, not applicable since they were not on therapy at study onset; US, ultrasound.

With regard to medical treatment, 30/88 patients (34.1%) were not on Disease modifying anti rheumatic drugs (DMARDs)/biological therapy at study entry, while 58 out of 88 were on such therapy (65.9%). Twenty-eight of fifty-eight (48.3%) discontinued, while 30 (51.7%) continued the therapy during the follow-up.

More than 50% of subjects on DMARDs/biological therapy (17/30 and 15/28 of those who continued and discontinued therapy, respectively) and 30% (9/30) of untreated patients had a flare. Although the association between treatment discontinuation and flare was not significant (χ^2=5.09, P=0.08), a stratified analysis was performed to better evaluate whether and how the therapy could affect the ability of US examinations to predict a flare (Breslow-Day test). Medical treatment did not result to have a modifier effect (χ^2=2.98, P=0.224), and an adjusted OR was calculated across strata. Irrespective of treatment status, the risk of flare in US-positive patients versus US-negative ones was 3.8 (95% CI 1.2 to 11.5). As the proportional difference between the unadjusted (4.9) and adjusted (3.8) ORs is more than 10%, the contribution of therapy as confounder effect cannot be ruled out.

Time to flare

On average, the US-negative patient group showed a significantly greater probability of remission than the US-positive group (log-rank test: χ^2=15.1, P<0.001). At 1 year, the

Table 2 Main clinical features of the 88 inactive patients stratified by the outcome at the end of follow-up and by the US abnormalities

	Overall patients (n=88)	Patients with inactive disease (n=47)		Patients with synovitis flare (n=41)	
		US-negative (n=42)	US-positive (n=5)	US-negative (n=26)	US-positive (n=15)
ILAR category					
PO JIA, n (%)	46 (52.3)	24 (57.1)	1 (20.0)	15 (57.7)	6 (40.0)
EO JIA, n (%)	15 (17.0)	4 (9.5)	2 (40.0)	4 (15.4)	5 (33.3)
RF-negative JIA, n (%)	15 (17.0)	7 (16.7)	2 (40.0)	2 (7.7)	4 (26.7)
Other	12 (13.6)	7 (16.7)	0 (0.0)	5 (19.2)	0 (0.0)
Wallace criteria					
CR, n (%)	28 (31.8)	20 (47.6)	0 (0.0)	6 (23.1)	2 (13.3)
CRM, n (%)	47 (53.4)	17 (40.5)	2 (40.0)	18 (69.2)	10 (66.7)
ID, n (%)	13 (14.8)	5 (11.9)	3 (60.0)	2 (7.7)	3 (20.0)

CR, clinical remission off medication; CRM, clinical remission on medication; EO, extended oligoarthritis; ID, inactive disease; ILAR, International League of Associations for Rheumatology; PO, persistent oligoarthritis; RF, rheumatoid factor-negative polyarthritis; US, ultrasound.

remission probability was 94.1% (95% CI 88.7 to 99.9) and 55.0% (95% CI 37.0 to 81.8) in US-negative and US-positive patients, respectively (figure 1). The difference remained statistically significant even after stratification by therapy (log-rank test: χ^2=8.9, P=0.0029) (online supplementary table 5).

Analysis of specific joints in US-positive patients at baseline

US examination at study entry was positive in 38 joints (from one up to six joints per patient): 20/38 were positive in both grey scale and PD signal; 18 only in grey scale (tables 4 and 5). Flares occurred in 34 joints during the follow-up. Nineteen of thirty-four (55.9%) were predicted by US examination (positive at baseline), while 15/34 (44.1%) were previously US-negative. Among joints that were initially US-positive and flared in the follow-up, 13/19 (68%) were positive in both grey scale and PD signal, while 6/19 (31.6%) only in grey scale. The combination of grey scale and PD abnormalities displays a much higher predictive value of relapse (65%) than grey scale alone (33%) (tables 4 and 5).

DISCUSSION

Our study shows that US signs of inflammation are detectable in joints of children with JIA in full clinical remission. Furthermore, they are predictive of relapses, notably in the largest series of patients reported in the literature regarding this issue.

We show that up to 22.6% of children with inactive disease display US signs of inflammation in one or more joints. The incidence of US-detected abnormalities in clinically inactive patients is lower than reported in another study (about 70% in grey scale)[28] but close to that of the Spanish cohort,[38] where 38% of children showed US abnormalities.

A reason for the lower percentage of subclinical synovitis could be the more stringent criteria for defining the synovitis by US, both in grey scale (small amount of physiological fluid measured and judged according to standard reference values[15]) and in Doppler mode (which was scored only inside synovial hyperplasia[18 38]).

In addition, the preliminary consensus sessions and the examination of healthy children and patients with active disease, respectively, could have improved the reliability of the US in clinically inactive children.

Stringent criteria to distinguish normal from pathology are mandatory; the definition of minimal disease activity in children is still unclear. In the report by Magni-Manzoni *et al*,[28] this might have precluded the ability of US to predict flares given the high number of grade 1 abnormalities both in grey scale and PD mode.

US in paediatrics has some additional pitfalls.[39] For instance, the epiphysial cartilage is anechoic as the synovial fluid. A grey scale alteration (particularly a small amount of synovial fluid) may not be associated with an inflammatory state. Moreover, PD may be easily misinterpreted.[39] We tried to minimise these limitations by enrolling healthy subjects and active patients and by harmonising the US interpretation.

An important point is the prospective nature of our study, which assessed 88 inactive patients, the largest series reported in literature.

Forty-one out of 88 inactive patients had a flare (46.5%), similar to a recent study.[10] The patients with US abnormality showed a higher percentage of flares compared with those without abnormalities. US abnormalities increased the risk of flare by almost four times after considering the therapy as a confounder. As expected, the vascularisation of the synovial membrane, detected by the combination of the PD signal in an area of synovial hyperplasia, is the most reliable US finding for synovial inflammation. This finding is consistent with the preliminary definition of synovitis in children.[34]

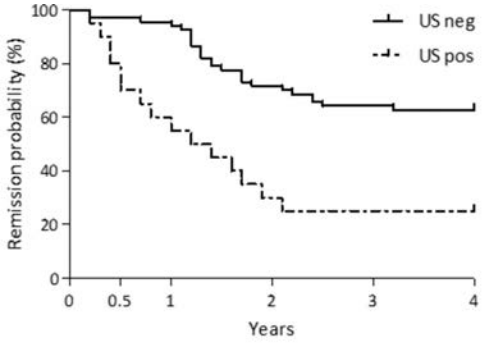
In a previous large study, physician global assessment (PGA) >30, active joint count >4, positive rheumatoid factor polyarthritis, antinuclear antibody (ANA) positivity and DMARDs or biological therapy before attaining inactive disease were associated with increased risk of flare, but US examination was not available.[10] Considering only significant flares and the results of a multivariate analysis, active joint count >4 and joint injections were still associated with the risk of relapse, with the maximum value of HR equal to 1.29. Our study focuses on US and if confirmed could support US examination as a useful tool for clinical management.

There are no differences in the probability of developing a relapse with regard to ILAR categories (persistent oligoarthritis=21/46, extended oligoarthritis=9/15, rheumatoid factor-negative=6/15; P=0.560). However, the association between US-positive and relapse is significant only for patients with persistent oligoarthritis (P=0.037). In particular, the probability of having a synovitis flare

Table 3 Estimates and relative 95% CI of accuracy, sensitivity, specificity and predictive values of ultrasound examination in juvenile idiopathic arthritis in clinical remission

	Estimate	Lower 95% CI	Upper 95% CI
Accuracy	64.8 (57/88)	54.4	73.9
Sensitivity	36.6 (15/41)	23.6	51.9
Specificity	89.4 (42/47)	77.4	95.4
Positive predictive value	75.0 (15/20)	53.1	88.8
Negative predictive value	61.8 (42/68)	49.9	72.4

CI was calculated using the Wilson Score method.



	Number of patients at risk (N events) Remission probability [95%CI]					
Time (years)	**0.5**	**1.0**	**1.5**	**2.0**	**3.0**	**4.0**
US negative	66 (2) 0.971 [0.931 to 1.000]	65 (2) 0.941 [0.887 to 0.999]	54 (11) 0.779 [0.687 to 0.884]	49 (5) 0.706 [0.605 to 0.823]	43 (5) 0.632 [0.528 to 0.758]	42 (1) 0.618 [0.512 to 0.745]
US positive	16 (6) 0.700 [0.525 to 0.933]	12 (3) 0.550 [0.370 to 0.818]	9 (2) 0.450 [0.277 to 0.731]	6 (3) 0.300 [0.154 to 0.586]	5 (1) 0.250 [0.117 to 0.534]	5 (0) 0.250 [0.117 to 0.534]

Figure 1 Kaplan-Meier curves showing 4-year remission probability by ultrasound (US) result. A US-positive result is significantly (log-rank test: χ^2 =15.1, P<0.001) related to a greater probability of flare than a US-negative result.

is about six times greater than the probability of not having the event (6/7 vs 1/7) in US-positive persistent oligoarthritis, while the probability of having a synovitis flare is about half of the probability of not having the event (15/39 vs 24/39) in US-negative persistent oligoarthritis (table 2).

Considering the Wallace criteria, although no statistical significance was achieved, the likelihood of developing a relapse in the presence of US positivity appears to be more associated with CR (PPV=2/2; P=0.074) and CRM (PPV=10/12; P=0.087). On the contrary, the number of false-positives in ID is far greater (37.5%, 3/8) than in the other two groups (0% (0/20) and 10.5% (2/19) for CR and CRM, respectively). This would support the hypothesis that the false-positives might be persistent US findings in the early stages of clinical remission that will diminish over time in patients with stable remission, a finding that has been documented in rheumatoid arthritis as well.[40]

Considering US as a potential predictor tool, false-positives are relatively few (5/47; specificity of 89.4%) and false-negatives are relatively many (26/41; sensitivity of 36.6%). In conclusion, in case of positive US, a follow-up should be carried out regardless of both the ILAR and Wallace categories because of the high probability of relapse (75%), while a negative result does not rule out the risk. Furthermore, patients with a clinical remission period around or less than a year should be followed more carefully since a US-positive result can confirm the flare. This finding is in line with the study showing that a deeper clinical remission was associated with a milder subclinical synovitis and a lower risk to relapse in adult patients with RA.[41]

A weakness of this study is that the discontinuation of DMARDs therapy seems not to influence disease relapse, in contrast to what was previously published.[4–8 10] However, a P value of 0.08 between those who stopped and those who maintained the therapy is very close to significance; the lower OR after adjusting for therapy confirms this statement. We may speculate that with a larger cohort

Table 4 Frequency of US abnormalities at study entry in specific joints of the 88 patients with clinically defined inactive disease

	n	Grey scale abnormalities, n (%)	Power Doppler signal, n (%)	Any US abnormalities, n (%)
All patients	88	20 (22.7)	13 (14.8)	20 (22.7)
Scanned joints	3872	38 (0.98)	20 (5.2)	38 (0.98)
Shoulder	176	0 (0.0)	0 (0.0)	0 (0.0)
Elbow	176	1 (5.7)	1 (5.7)	1 (5.7)
Wrist	176	8 (4.5)	4 (2.3)	8 (4.5)
MCP	880	0 (0.0)	0 (0.0)	0 (0.0)
PIP	880	0 (0.0)	0 (0.0)	0 (0.0)
Hip	176	0 (0.0)	0 (0.0)	0 (0.0)
Knee	176	15 (8.5)	9 (5.1)	15 (8.5)
Ankle	352	11 (3.1)	6 (1.7)	11 (3.1)
MTP	880	3 (0.34)	3 (0.34)	3 (0.34)

MCP, metacarpophalangeal; MTP, metatarsophalangeal; PIP, proximal interphalangeal of hands; US, ultrasound.

Table 5 Frequency of US abnormalities at study entry in the 44 joints which had a relapse of synovitis

		Joints with US abnormalities at study entry (n)		
Joint	**Joints with synovitis relapse (n=75)**	**Total* (n=19)**	**Grey scale abnormalities (n=19)**	**Power Doppler signal (n=13)**
Elbow	5	1 (20.0)	1	1
Wrist	11	4 (36.4)	4	2
MCP	6	0 (0.0)	0	0
PIP	2	0 (0.0)	0	0
Knee	28	8 (28.6)	8	6
Ankle	20	6 (30.0)	6	4
MTP	3	0 (0.0)	0	0

The total number of joints with synovitis relapse includes joints which were US-positive (19), US-negative (41) or belonging to US-positive patients, but with synovitis flare found in a different joint with respect to the US examination at study entry (15).
*Data are expressed as numbers (percentage).
MCP, metacarpophalangeal; MTP, metatarsophalangeal; PIP, proximal interphalangeal of hands; US, ultrasound.

of patients, we could have shown an effect of therapy discontinuation on relapses.

Another point to consider is that joints where JIA relapsed were different from those detected by US at study entry. We can hypothesize that US likely shows synovitis in patients with a higher systemic inflammation that can emerge over time in joints different from the ones detected at study entry.

In conclusion, US could be a predictor of relapse at the patient level since a subclinical synovitis increased by about four times the risk of flare. If confirmed by further studies, US may offer a useful tool in stratifying the risk of flare in patients with JIA.

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Cardiovascular effects of hydroxychloroquine: a systematic review and meta-analysis

In an article published in the *Annals of the Rheumatic Diseases*, Rempenault *et al*[1] suggested that hydroxychloroquine (HCQ) had a beneficial effect on the metabolic profile in patients with rheumatoid arthritis (RA). In this letter, we wish to add further data to support the conclusions of this article. We performed a meta-analysis (up to June 2017) to investigate cardiovascular risk in patients treated with HCQ for RA, lupus and other diseases.

We searched MEDLINE to identify all reports of interest published prior to June 2017 using the following search terms: 'hydroxychloroquine AND (cardiovascular OR myocardial OR stroke OR atherosclerosis OR lipid OR diabetes OR glycemia)'. We retrieved a total of 443 articles. In addition, we searched for abstracts from the European League Against Rheumatism and the American College of Rheumatology meetings between 2009 and 2016. Incidences of cardiovascular events or diabetes were calculated by a meta-analysis of proportions. The Mantel-Haenszel procedure was used to determine the risk ratio (RR) of diabetes and risk difference of cardiovascular events in HCQ users versus non-users. For cardiovascular risk factors, differences between HCQ users and non-users were expressed by standardised mean differences (SMD) using the inverse of variance method. We assessed the effects of 12 weeks of HCQ treatment on the glycaemic and lipid profiles with an estimation of paired SMD using fixed or randomised models.

We included 33 references, along with three abstracts, involving a total of 24 923 HCQ users and 36 327 non-users. In five studies, there were 431 diabetes reported in HCQ users (n=22 228) over a mean follow-up of 3 years: incidence=1.7% (1.0%–2.6%), while in HCQ non-users (n=32 367) 1323 diabetes were reported over the same period: incidence=5.3% (2.0%–10.0%). The meta-analysis showed a significant decrease in the occurrence of diabetes in the HCQ group (RR=0.36 (0.23–0.56)). This lower risk was found again in the four studies concerning only patients with RA (RR=0.33 (0.18–0.59)). In three studies, 78 cardiovascular events were reported in HCQ users (n=1211) and 238 events were found in the non-users (n=1074). The meta-analysis found a nearly significant decrease in the occurrence of cardiovascular events in the HCQ group (RR=−0.25 (−0.52 to 0.02)). The cardiovascular risk factors are compared in table 1, and there was a significant decrease in the lipid or glycaemic parameters in HCQ users. The same beneficial effect of HCQ was found after 12 weeks of treatment (table 2).

Our study results provide information that reinforces the conclusions of Rempenault *et al* that HCQ leads to an improvement in the cardiovascular risk profile in RA (15 studies[2]). This beneficial effect of HCQ on lipid or glycaemic profiles seems to also exist in patients with lupus (14 studies[3]) or other conditions (diabetes: three studies[4]; obese non-diabetic patients: two studies[5]; and Sjögren syndrome: one study[6]). These interesting effects suggest there are other indications for HCQ use, including the prevention of recurrent cardiovascular events in patients with myocardial infarction, coronary artery disease or pre-eclampsia.[7–9] Whatever the disease, HCQ treatment is associated with an improved cardiovascular risk profile. As in lupus,[10] patients with RA should consider systematic treatment with HCQ.

Table 1 Comparison of cardiovascular risk factors in case/control studies

Characteristics	Studies (n)	HCQ users	HCQ non-users	Standardised mean difference (95% CI) Fixed or random effects	P value	I^2 (%)
Glycaemia, g/L	6	0.89±0.19	0.99±0.31	−0.28 [(−0.41 to −0.15)	<0.001	0
Total cholesterol, g/L	12	1.87±0.37	1.92±0.38	−0.57 (−0.97 to −0.18)	0.004	95
LDL cholesterol, g/L	12	1.06±0.36	1.11±0.35	−0.23 (−0.40 to −0.06)	0.009	70
HDL cholesterol, g/L	11	0.53±0.14	0.50±0.15	0.20 [(0.05 to 0.34)	0.007	55
Triglycerides, g/L	12	1.18±0.58	1.37±0.78	−0.32 (−0.50 to −0.14)	<0.001	70
Atherogenic index (TC/HDL)	6	3.46±1.25	3.99±1.40	−0.28 (−0.37 to −0.18)	<0.001	35

Values are the weighted mean±SD.
HCQ, hydroxychloroquine; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TC, total cholesterol.

Table 2 Effects of 12 weeks of HCQ treatment on lipid and glycaemic parameters

Parameters	Studies (n)/ patients (n)	Standardised mean difference (95% CI) Fixed or random effect	P value	I^2 (%)
Total cholesterol	7/214	−0.32 (−0.52 to −0.13)	0.001	27
LDL cholesterol	6/193	−0.24 (−0.44 to −0.04)	0.019	0
HDL cholesterol	6/193	0.03 (−0.17 to 0.23)	0.750	0
Triglycerides	6/193	−0.20 (−0.40 to 0.002)	0.053	0
Glycaemia	4/160	−0.33 (−0.55 to −0.11)	0.004	43
Glycated haemoglobin	3/152	−0.34 (−0.57 to −0.12)	0.003	14

HCQ, hydroxychloroquine; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

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Response to: 'Cardiovascular effects of hydroxychloroquine: a systematic review and meta-analysis' by Mathieu *et al*

In their letter to the editor, Mathieu[1] and colleagues provide information that reinforce the conclusions of our recent paper.[2 3] We appreciate their interest in our study and complement the authors for their systematic review and meta-analysis of cardiovascular (CV) effects of hydroxychloroquine (HCQ) in rheumatoid arthritis (RA), lupus and other diseases.

In our study, we have chosen to focus only on RA for homogeneity reasons and because we think that demonstration of a positive influence of HCQ on CV risk has a stronger practical impact for RA than for other inflammatory rheumatisms. Indeed, while in lupus HCQ is anyway clearly recommended and prescribed for the disease itself, it is not the case in RA due to the rather low efficacy of HCQ on arthritis.[4] Consequently, showing the beneficial effect of HCQ on the metabolic profile of patients with RA could change practices and customs and could justify HCQ prescription in RA, in association to other disease-modifying antirheumatic drugs.

Interestingly, Mathieu *et al*'s study has retrieved the same beneficial effect of HCQ on CV risk factors and CV events in RA and in lupus, Sjögren syndrome, diabetes and in obese non-diabetic patients. In addition, evaluating several diseases may be responsible for the heterogeneity found in their meta-analysis that was superior than in our study. Anyway, their results are concordant with ours, highlighting notably a decrease of over 50% of diabetes occurrence in the HCQ group. Moreover, including more studies has allowed them to perform a meta-analysis for the occurrence of CV events, which we were not able to do, confirming the lower frequency of CV events in the HCQ group.

In our paper, we discussed the possibility of confounding bias due to the prescription of HCQ to patients with RA with benign disease rather than to patients with severe disease. Consequently, we were aware that the lower CV risk noted for the patients belonging to the 'HCQ users' was perhaps linked to the characteristics of these patients' disease and not to the HCQ treatment in itself. However, the fact that Mathieu *et al* have noted results similar to ours, regardless of the initial inflammatory rheumatism and even in patients without rheumatic disease, is an additional argument for a proper protective effect of HCQ on CV risk factors and CV events.

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Comment on l'Ami *et al* titled 'Successful reduction of overexposure in patients with rheumatoid arthritis with high serum adalimumab concentrations: an open-label, non-inferiority, randomised clinical trial'

We have read the recent paper of l'Ami *et al* titled 'Successful reduction of overexposure in patients with rheumatoid arthritis with high serum adalimumab concentrations: an open-label, non-inferiority, randomised clinical trial' with interest.[1] The topic of dose tapering is an important one and we welcome all research efforts in this field, especially controlled trials. However, the design choices and interpretation of current study have some important limitations that in our opinion merit more attention.

First, l'Ami *et al* set out to assess whether interval prolongation with 50% of adalimumab injections in patients with rheumatoid arthritis doing well is feasible when patients have a trough level of >8 mg/mL. These are some rather peculiar research questions and design choices. First, this design does not provide data on whether patients with trough level below <8 mg/mL can also extend their interval with equal success. This is especially relevant, since 63% of patients had trough levels below 8 mg/mL. In light of recent data from DRESS and STRASS, it seems that tapering is feasible in patients with lower trough levels.[2] Also, the reason to choose tapering only by 33%, when clinical trials have already demonstrated that further tapering up to cessation is successful in the majority of patients, is unclear.[3] Finally, there is no comparison with state-of-the-art usual care, being treat-to-target tapering using only a disease activity measure like DAS28.

The classical—and more informative—design in a strategy study using a test (a 'diagnostic study') would be to randomise all patients (regardless of adalimumab serum trough level) to a usual care tapering strategy or a test guided strategy. Such a design would—with similar efforts—have enabled us to answer several more important questions. First, whether adalimumab trough level guided treatment indeed results in similar or better clinical outcomes with less adalimumab use compared with disease activity guided tapering. And also, whether this is cost-effective, and what the predictive value of adalimumab trough levels is for successful tapering, as this can be assessed in the usual care arm. Such a study design is considered as the golden standard also in other diagnostic fields, although quality of design, execution and reporting of diagnostic trials have recently been shown to be very poor indeed, resulting in research waste.[4]

Of note, compared with the study registration in the trial register (NTR3509), the choice for non-inferiority design and the non-inferiority margin seems post hoc. While we appreciate current analyses, this should have been clearly stated in the method section.

So, although the results of the study certainly have some scientific value, important issues in trial design and execution unfortunately limit the recommendations that can be made based on this study. While we fully appreciate that increasing insights occur over time and that only hindsight has 20/20 vision, we feel that this study could and should have benefitted from a more appropriate design, and that this could have been addressed more in the discussion.

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Contributors AAB and BJFB have both made substantial contributions to the conception, drafting and revising of the work, and both approved the final version and are accountable for all aspects of the work.

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Response to: Comment on l'Ami *et al* titled 'Successful reduction of overexposure in patients with rheumatoid arthritis with high serum adalimumab concentrations: an open-label, non-inferiority, randomised clinical trial' by den Broeder *et al*

We thank den Broeder *et al* for their interest in our study and for their suggestions concerning further research.[1] Our study did not target the broad issue of dose tapering, but focused on the population of patients with rheumatoid arthritis with high adalimumab concentrations (>8 µg/mL).[2] We hypothesised that given the wide variation in serum concentrations on standard dosing and the established plateau in the concentration–response relationship—even in active disease[3]—there would be a role for individualised dosing to optimise adalimumab therapy. Unlike den Broeder *et al*, we find nothing peculiar about our research question or design to investigate whether overexposure could be reduced without an increase in disease activity. This study gives a clear positive answer.

We share with den Broeder *et al* a belief in the importance of research concerning optimal use of biological treatments. den Broeder *et al* highlight a stepwise dose reduction based on disease activity, where the dose is downtitrated to zero or until the occurrence of a flare[4]; we believe this may also be a valuable strategy in optimising adalimumab therapy. We note in passing that our study was designed in 2012, when discontinuation of biologics was still very contentious.

den Broeder *et al* claim that our non-inferiority analysis strategy was defined post hoc based on the record in the trial register, but we stated there as primary outcome 'Similar deltaDAS28 in patients with high serum adalimumab concentrations who are randomly assigned to continuation of the regular dose or to dose interval prolongation'. We think we adequately explained our problems with recruitment and our ways to handle this in the analysis. In addition, given the actual results, statistically favouring the dose reduction group, we think this point is moot.

In sum, we showed that patients with high adalimumab concentrations can safely reduce the dose. We think that this study highlights an important topic in biological treatment. Overexposure should be avoided regardless of treatment targets, in light of high drug costs and a possibly increased risk of adverse events.

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Acquiring new N-glycosylation sites in variable regions of immunoglobulin genes by somatic hypermutation is a common feature of autoimmune diseases

With great interest, we read the contribution of Vergroesen *et al* that was published recently in *Annals of the Rheumatic Diseases*.[1] In this manuscript, the authors describe the observation that immunoglobulin variable (V) region heavy and light chain transcripts from anti-citrullinated protein antibody (ACPA) IgG-expressing B cells in patients with rheumatic arthritis (RA) contain N-glycosylation sites (Nglycs) acquired by somatic hypermutation, whereas these acquired Nglycs (ac-Nglycs) were absent in tetanus toxoid (TT) specific B cells of healthy individuals. The authors postulate that the introduction of ac-Nglycs generates selective advantages that allow ACPA-expressing B cells to escape from classical selection mechanisms in germinal centres. We agree with the authors that this is an important finding which may have important implications for understanding citrulline-specific immunity in RA.

Here we would like to stress that ac-Nglycs, as a consequence of somatic hypermutation, might be important for RA and for many other rheumatoid and non-rheumatoid autoimmune diseases. We have shown previously[2] increased numbers of IgG encoding immunoglobulin variable heavy region gene (IGHV) transcripts with ac-Nglycs derived from B cells and plasma cells residing in the inflamed parotid salivary gland of patients with primary Sjögren's syndrome (pSS) compared with non-pSS sicca controls (24% vs 6%). Importantly, in pSS the IgG encoding transcripts exhibited no evidence for signs of antigen selection in the complementarity determining regions. Furthermore, most (60%) of the ac-Nglycs were seen in the framework (FR) 3 region, which is similar to the findings in ACPA-expressing B cells.[1] Together, these findings indicate that alternative selection mechanism may result in survival of aberrantly selected B cells in autoimmune diseases, like pSS and RA.

To test if ac-Nglycs created by somatic hypermutation could be a common phenomenon for B cells in rheumatic and non-rheumatic autoimmune diseases, we performed a meta-analysis of the presence of ac-Nglycs in IGHV sequences from a number of publicly available data sets from patients with pSS (n=576), RA (n=1331), systemic lupus erythematosus (SLE; n=361), multiple sclerosis (n=200), chronic Chagas' heart disease (cChD; n=70), ankylosing spondylitis (n=29) and granulomatosis with polyangiitis (GPA; n=242). As controls, we collected published data sets of IGHV sequences derived from various healthy, non-autoimmune individuals (n=2131) and from vaccination or infection studies on antigen-specific B cells (n=817) (see online supplementary table 1). Sequences identical to germline IGHV sequences (≤2 nucleotide mutations in the V and J genes), naturally occurring germline Nglycs and sequences lacking fully designated VDJ rearrangements were excluded from analysis. In each study, the number of ac-Nglycs was predicted by the NetNglyc V.1.0 program (http://www.cbs.dtu.dk/services/NetNGlyc/) based on the consensus sequence in the protein motif N-X-S/T (asparagine-X-serine/threonine). As shown in figure 1A, the combined frequency of ac-Nglycs in IGHV sequences is significantly higher (P<0.0001; Pearson's χ^2 test) in autoimmune disease data sets (9.0%; 95% CI 8.0 to 10.1) than that in control data sets (2.3%; 95% CI 1.7 to 3.0) as well as in antigen-specific data sets (2.7%; 95% CI 1.7 to 4.0).

Interestingly, the number of ac-Nglycs is elevated in nearly all autoimmune diseases with well-established B cell involvement except for GPA (figure 1B); we observed a higher amount of ac-Nglycs in the IGHV sequences from pSS (15%), RA (10%), SLE (6%), multiple sclerosis (9%) and for cChD (19%) compared with the normal control data set (3%). Although GPA is an autoimmune disease, well known for its B cell involvement, ac-Nglycs were completely absent in the IGHV sequences. Possible explanations could be the over-representation of IgM encoding sequences in these data sets (online supplementary table 1). In patients with ankylosing spondylitis, the number of ac-Nglycs (3%) is similar to normal controls.

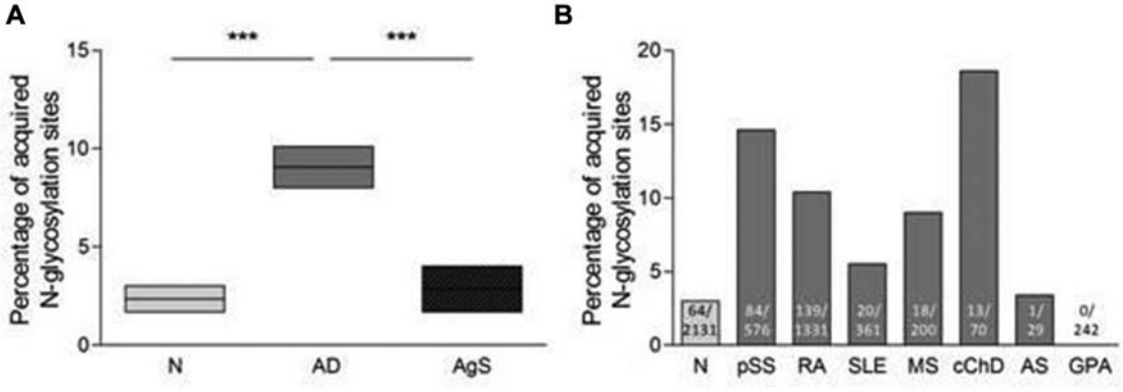

Figure 1 Prevalence of acquired N-glycosylation sites in IGHV sequences. (A) This figure depicts a simplified version of the meta-analysis shown in online supplementary figure 1. The percentages of ac-Nglycs were compared between autoimmune diseases and non-autoimmune or normal controls (N) and vaccination and infection controls (AgS) regardless of isotype. All the studies combined revealed 275 ac-Nglycs out of 2809 IGHV sequences from patients with autoimmune disease (9.0%; 95% CI 8.0 to 10.1) which was significantly higher than the 64 ac-Nglycs out of 2131 IGHV sequences in the healthy controls (2.3%; 95% CI 1.7 to 3.0) and the 23 ac-Nglycs out of 817 IGHV sequences in the vaccination and infection controls (2.7%; 95% CI 1.7 to 4.0). Because of the different sizes of all the data sets, analysis of the data was performed by using the Freeman-Tukey arcsine transformation method for meta-analysis of proportions to estimate the pooled proportion (read combined frequency) with the 95% CIs for each data set (see also online supplementary figure 1). The P values were calculated with the Pearson's χ^2 test. (B) Distribution of the frequency of ac-Nglycs in the IGHV sequences in the different autoimmune diseases. The percentage of ac-Nglycs found in the IGHV sequences from the data sets was calculated for each autoimmune disease. The number of observed ac-Nglycs per total number of analysed IGHV sequences is indicated in each bar. AS, ankylosing spondylitis; cChD, chronic Chagas' heart disease; GPA, granulomatosis with polyangiitis; MS, multiple sclerosis; pSS, primary Sjögren's syndrome; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

In conclusion, in addition to Vergroesen *et al* in RA, and our previous work in pSS,[2] the meta-analysis described here clearly indicates that there is an increase in ac-Nglycs by somatic hypermutation of immunoglobulin genes during humoral immune responses in various autoimmune diseases. This phenomenon is thus clearly not restricted to ACPA-expressing B cells in RA as shown by Vergroesen *et al*. It is not known yet whether the absence of ac-Nglycs in TT-specific cells is a property of TT specificity or due to the fact that these cells are from healthy, vaccinated individuals. It would therefore be of great interest to see whether also in patients with RA ac-Nglycs are absent in TT-specific B cells and other non-ACPA-expressing B cells. The explicit tendency for ac-Nglycs to occur also within the FRs strongly suggests that the increased frequency of ac-Nglycs in autoimmune diseases may offer Ig-producing cells alternative forms of selection to classical antigen selection. This could point to a very fundamental basis to understand the origin of autoreactive B cells in autoimmune diseases and possible targets for early intervention.

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Contributors AV wrote the concept of manuscript and performed the analysis of the data sets. NH performed the initial analysis of the data set. NAB and FGMK were involved in writing and discussion of the data.

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Response to: 'Acquiring new *N*-glycosylation sites in variable regions of immunoglobulin genes by somatic hypermutation is a common feature of autoimmune diseases' by Visser *et al*

We thank Visser *et al*[1] for their interesting correspondence to our recently published letter entitled 'B-cell receptor sequencing of anticitrullinated protein antibody (ACPA) IgG-expressing B cells indicates a selective advantage for the introduction of *N*-glycosylation sites during somatic hypermutation'.[2] Visser *et al* performed a meta-analysis on publicly available datasets to analyse acquired *N*-glycosylation sites in the variable region of B cell receptors (BCRs) derived from patients with different autoimmune diseases. BCR sequences of antigen-specific B cells isolated after vaccination or infection and BCR sequences of healthy donors (HD) served as comparison. The meta-analysis showed acquired *N*-glycosylation sites in 9% of BCR sequences derived from patients with autoimmune diseases and in 2.3% and 2.7% of sequences derived from HD and vaccine/infection-induced B cells, respectively. This enhanced frequency of acquired *N*-glycosylation sites (compared with controls) was observed for all autoimmune diseases, with the exception of ankylosing spondylitis (AS, 3%) and granulomatosis with polyangiitis (GPA, 0%).

The meta-analysis presented is a valuable addition to our work as it further highlights the relevance of variable domain *N*-glycosylation in general and its possible role in autoimmunity in particular. With regard to these data, it is important to note that our analysis referred selectively to BCR sequences of the IgG-isotype derived from B cells specific for citrullinated antigens. We reported that up to 88% of these ACPA-IgG BCRs carry at least one acquired *N*-glycosylation site. So far, this extensive presence of *N*-glycosylation sites is unique and has not been reported for another (auto)antigen-specific B cell response. Our control dataset consisted of BCR sequences derived from 12 HD and was matched for V-region and isotype (IgG) usage. We observed a frequency of 10% of acquired *N*-glycosylation sites in HD, which is higher than the 3% in the HD datasets presented by Visser *et al*. This discrepancy in frequency might be due to the inclusion of IgM and IgA sequences in the meta-analysis, while our analysis included only IgG isotypes. Indeed, the authors acknowledge a possible overrepresentation of IgM-encoding sequences in particular in the AS and GPA datasets. IgM-encoding sequences likely contain less *N*-glycosylation sites than IgA-encoding and IgG-encoding sequences due to a lower rate of somatic hypermutation. The latter notion is also supported by our recent observation, which suggests that ACPA-IgM molecules do not harbour additional *N*-linked glycans in the variable region.[3] Thus, matching for isotype usage might be critical for a direct comparison of *N*-glycosylation site frequency between datasets.

Extending these considerations, we observed a significant difference between the introduction of the *N*-linked glycosylation consensus sequence N-X-S/T (where X≠P) and N-P-S/T in ACPA-IgG B cell clones. Although highly similar to the consensus *N*-linked glycosylation site, the N-P-S/T sequence cannot accommodate *N*-linked glycans. In contrast to the BCRs from HD, ACPA-IgG BCRs specifically acquired the N-X-S/T-site and had not acquired the N-P-S/T sequence (table 1, P<0.005). This provides further evidence for a selective advantage of the introduction of *N*-linked glycosylation sites in the variable region of ACPA-IgG BCRs. Of note, we selected N-X-S/T and N-P-S/T sites in ACPA-IgG and HD sequences manually because, to the best of our knowledge, the NetNGlyc server (http://www.cbs.dtu.dk/services/NetNGlyc/) used by Visser *et al* includes both N-X-S/T and N-P-S/T sites in the analysis. As we did not observe acquired N-P-S/T sites in ACPA-IgG clones, it would be of great interest to perform additional analyses regarding the presence of N-P-S/T sites in a multiple autoimmune disease meta-analysis to further investigate the notion that autoreactive B cells selectively introduce N-X-S/T (X≠P) sites in a non-random manner.

Table 1 Selective preference for the introduction of *N*-glycosylation sites in ACPA-IgG compared with healthy donor IgG-expressing B cells

	N-P-S/T sites	N-X-S/T* sites
ACPA (next-generation sequencing)	0	102
Healthy donors (next-generation sequencing)	55	660
ACPA (single cell sequencing)	0	87

*X is any amino acid except proline.
Healthy donors were significantly different from ACPA (next-generation sequencing) and ACPA (single cell sequencing) using Fisher's exact test (P<0.005). No significant differences were observed between ACPA (next-generation sequencing) and ACPA (single cell sequencing) using Fisher's exact test (P=1).
ACPA, anticitrullinated protein antibody.

Irrespective of these considerations, the possibility that acquired *N*-glycosylation sites are more frequently observed in autoimmune diseases is intriguing. This is also supported by previous work of the authors on primary Sjögren's syndrome IgG-BCR sequences.[4] The findings presented by Visser *et al* contribute to the further understanding of the introduction of *N*-glycosylation sites by autoreactive B cells and its possible impact on selective advantages in B cell selection and/or the breach of tolerance of autoreactive B cells. Therefore, in future studies, it will be important to analyse full-length BCR sequences of different antigen-specific autoreactive B cells in RA and other autoimmune diseases to elucidate whether enrichment of *N*-glycosylation sites in the variable region of BCRs is a feature of autoreactive B cell responses that share common mechanisms for tolerance escape.

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Increased stroke incidence in systemic lupus erythematosus patients: risk factors or disease itself?

We read with great interest the recent study by Arkema *et al* showing that patients with systemic lupus erythematosus (SLE) are at increased risk for ischaemic (but not haemorrhagic) stroke as compared with Swedish general population (HR 2.2, 95% CI 1.7 to 2.8).[1] The risk was further increased in women and young (<50 years) patients, particularly in the first year following diagnosis. This study would indirectly suggest that SLE plays an independent role in inducing an accelerated atherosclerosis through a chronic inflammatory status. Such a phenomenon was suggested also for other rheumatic diseases, including rheumatoid arthritis, although data are still controversial.[2] However, by using data provided in table 1 where statistical comparisons were surprisingly lacking, we calculated that SLE patients have a significantly ($P<0.0001$) increased prevalence of atrial fibrillation, congestive heart disease, hypertension and diabetes. Of note, all these are well-known risk factors for atherosclerotic process and stroke. Moreover, additional risk factors, such as smoking habit and obesity, were not considered as the authors correctly highlighted. Overall, these data would suggest that SLE patients are at increased risk for ischaemic stroke due to a high prevalence of classical cardiovascular risk factors rather than the rheumatic disease itself.

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Response to: 'Increased stroke incidence in systemic lupus erythematosus patients: risk factors or disease itself?' by Bruzzese and Zullo

We thank Dr Bruzzese and Dr Zullo for their interest in our study on stroke risk in systemic lupus erythematosus (SLE).[1] In their letter, they speculate that it might not be SLE, per se, but other factors that could explain the increased risk.[2] We would first like to emphasise that the purpose of our study was to determine whether individuals with SLE have a higher risk of stroke compared with the general population, not to determine *how much* of a role SLE plays in this risk or what the drivers of stroke are above and beyond SLE. Our study was designed so that the risk of stroke could be communicated to patients and clinicians.

Bruzzese and Zullo are interested in better understanding whether SLE plays an independent role in the aetiology of stroke, and suggest that the group of people with SLE with atrial fibrillation, congestive heart disease, hypertension and diabetes are driving the increased risk. However, when we adjust for these risk factors in our 'model 2', adjustment does not greatly affect results. To further address this issue, we have excluded everyone in the population with a history of these comorbidities at start of follow-up. The HRs for each type of stroke are increased compared with the original results (see table 1). For example, the HR for ischaemic stroke increased from 2.4 to 2.7. This demonstrates that even in the absence of cardiovascular risk factors, individuals with SLE have an increased risk of stroke.

We agree that other risk factors such as smoking and obesity are important in stroke and may be risk factors for SLE, making them potential confounders of the association between SLE and stroke. To quantitatively assess the impact of unmeasured confounding on our results, we conducted a probabilistic bias analysis. The methodology is described in detail elsewhere.[3] Briefly, we performed a number of simulations to test the robustness of the HR for ischaemic stroke from the main analysis in the presence of unmeasured confounding by current smoking and obesity, separately or jointly. To define the bias parameters, we used internal data, results from previous studies and national health survey data.[4–7] We chose the largest differences in prevalence between SLE and non-SLE to test the robustness under extreme confounding. We assumed a prevalence of current smoking ranging 22%–26% in SLE and 16%–20% in non-SLE. For obesity, we defined prevalence distributions ranging from 12% to 16% for SLE and from 8% to 12% for the general population. We assumed that current smoking and obesity are associated with stroke by a relative risk of 3.8 and 1.6, respectively.[8 9] As shown in table 2, the HR remained robust under extreme confounding by smoking and obesity. This sensitivity analysis indicates that even in the presence of a strong confounder, the relative risk of stroke associated with SLE would still be almost twofold.

Table 2 Summary of probabilistic bias analysis where the robustness of the HR for ischaemic stroke from the main analysis was tested in the presence of unmeasured confounding by smoking and obesity in simulations

Bias analysis	HR (95% simulation interval)*
Unmeasured confounding by current smoking	1.8 (1.4 to 2.4)
Unmeasured confounding by obesity	2.1 (1.6 to 2.7)
Unmeasured confounding by current smoking and obesity	1.8 (1.3 to 2.3)

The adjusted HR from the main analysis was 2.2 (model 2).
*The CIs are bootstrapped intervals accounting for systematic error and random error originating from the main analysis.

Lastly, the authors suggest that there was no association between SLE and haemorrhagic stroke because the CI for the HR included 1. Haemorrhagic stroke was a relatively rare outcome, with only 11 cases in the SLE population and 37 in the general population comparator, and our analysis was not powered to detect a statistical significance. We urge our readers to consider the point estimate and the context rather than only focusing on P values and null hypothesis significance testing.[10] To address this, we used the results from our meta-analysis on stroke risk associated with SLE[11] and included our estimate in the pooled result to update the analysis. Pooling our study with Mok *et al*[12] and Wang *et al*[13] (not including Zöller *et al*[14] because their data overlap with ours), the relative risk of stroke is 2.11 (1.22, 3.65), with I^2 of 49% and P for heterogeneity of 0.14. This indicates that SLE is associated with haemorrhagic stroke. Our results should be taken into context with previous studies before coming to the conclusion that the relative risk for haemorrhagic stroke is not increased.

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Table 1 Number of strokes, person-years, incidence rates and HRs for incident stroke (all strokes, ischaemic stroke, intracerebral haemorrhage, subarachnoid haemorrhage and unspecified) comparing SLE (n=2679) with the general population (n=15 172) from 2003 to 2013 in Sweden

	General population		SLE		
	Strokes (n)	Incidence rate (95% CI)	Strokes (n)	Incidence rate (95% CI)	HR (95% CI)
Any stroke	208	2.6 (2.3 to 3.0)	75	5.5 (4.4 to 6.8)	2.5 (1.9 to 3.3)
Ischaemic stroke	156	2.0 (1.7 to 2.3)	59	4.3 (3.3 to 5.5)	2.7 (2.0 to 3.6)
Intracerebral haemorrhage	24	0.3 (0.3 to 0.4)	7	0.5 (0.2 to 1.1)	1.9 (0.8 to 4.5)
Subarachnoid haemorrhage	12	0.2 (0.1 to 0.3)	NA	NA	NA
Unspecified	16	0.2 (0.1 to 0.3)	6	0.4 (0.2 to 1.0)	2.7 (1.1 to 7.0)

Individuals with a history at baseline of atrial fibrillation, congestive heart disease, hypertension or diabetes were excluded.
NA marks cell counts <5, corresponding incidence rates and HRs were therefore not estimated.
Incidence rate per 1000 years. CI estimated based on the Poisson distribution.
Number of person-years in general population: 79 594. Number of person-years in SLE population: 13 736.
HR from a Cox regression model adjusted for age and sex.
SLE, systemic lupus erythematosus.

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Use of urate-lowering therapies is not associated with an increased risk of incident dementia in older adults

We read with interest the article by Singh *et al*, in which they investigated whether the use of urate-lowering therapies (ULTs) in the elderly is associated with a higher risk of developing dementia.[1] This study, which had a mean 683 days of follow-up, found that ULT use was not associated with increased risk of dementia.[1] In this study, there are some issues to comment. First, the results reported by Singh *et al* differ from those of a previous prospective population-based cohort study, which demonstrated that patients with gout undergoing uric acid-lowering treatment have a lower risk of developing dementia.[2] In addition, another population-based study found that gout is inversely related to the risk of developing Alzheimer's disease (AD) regardless of whether ULTs are used, supporting the purported potential neuroprotective role of gout or uric acid on the development of AD.[3] The main difference between the study by Singh *et al* and the other studies mentioned is the follow-up duration (683 days vs 5.1 and 4.3 years, respectively). A follow-up period of less than 2 years is too short to examine the long-term effects of ULTs. Second, drug non-compliance and non-persistence are common among patients undergoing uric acid-lowering treatment.[4] Thus, some patients on ULTs might have high serum uric acid levels. As the authors mentioned, they were unable to examine the baseline serum uric acid level due to the unavailability of laboratory data. Therefore, we cannot exclude the possibility that the results by Singh *et al* were confounded by the lack of baseline and subsequent follow-up serum uric acid levels, which could potentially be a very important factor in dementia. It is important to note that this study evaluated the relationship between dementia and ULTs, not hyperuricaemia or gout. Thirdly, it is concerning to see that hyperlipidaemia was associated with a decreased risk of dementia as this finding contrasts that of other studies.[5] In this regard, concerns may also extrapolated to ULTs. Taken together, the findings of this study should be interpreted with the limitations mentioned above. To date, no conclusive causal relationship between ULTs and dementia has been established. We believe that further large-scale longitudinal epidemiological studies are needed to clarify a definitive role of ULTs, uric acid and gout in the development of dementia.

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Response to: 'Use of urate-lowering therapies is not associated with an increase in the risk of incident dementia in older adults' by Lee and Song

We thank Lee and Song[1] for their interest in our paper[2] that found that compared with non-use, urate-lowering therapy (ULT) use was not associated with increased risk of dementia in adults aged 65 years or older.

We agree with Lee and Song that all observational studies have potential residual confounding related to study design, including our study and the observational studies they listed. Similar to our study, the two studies that Lee and Song have mentioned also did not adjust for serum urate[3 4] putting them at the same risk for potential confounding bias as our study. Although serum urate is thought to be a potential confounder for the association that others noted previously in their studies of gout and dementia or the the lack of association we noted in our study of ULT use and dementia, we conceptualise it more as a mediator/moderator of association of gout with dementia or of ULT use with dementia. One must also be very careful and not mix studies that are examining gout as a risk factor for dementia[3 4] with those that are examining the association of ULT use with the risk of dementia.[1] We included several potential confounders to reduce the risk of confounding bias, including demographics, comorbidities and cardiac medications. Additionally, sensitivity analyses were done limiting the cohort to patients with gout and additionally adjusting for cardiovascular disease (CAD) and CAD risk factors to reduce potential confounding, confirming our main findings. Lee *et al* restated the study limitations we listed, including the lack of serum urate levels and a limited follow-up duration of 2 years.

Lee and Song mentioned that our finding that 'hyperlipidaemia was associated with a decreased risk of dementia' contrasts with that of a meta-analysis of hypercholesterolaemia'.[5] Hyperlipidemia is not the main independent variable in our study, but only a covariate.[2] This meta-analysis concluded that there was no association between late-life hypercholesterolaemia and any dementia or Alzheimer's dementia.[5] Differences in studies included in the systematic review and our study may be related to differences in patient population (elderly in our study vs general populations), the definition of hypercholesterolaemia (diagnostic code in our study vs cholesterol levels in other studies) and the covariates adjusted in various analyses (including adjustment for statin use in our study vs lack of adjustment for it in some earlier studies). Hypercholesterolaemia is well known to be under-coded and database studies may underestimate the actual prevalence,[6] one reason why we included the use of statin drugs in our analyses.

We wholeheartedly agree that prospective studies and randomised trials are needed to confirm our observations related to ULTs and the risk of dementia and better understand the underlying mechanisms of association of serum urate with neuro-degenerative processes the lead to dementia.

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