LUPUS NEPHRITIS

Mysteries of kidney-protecting parasitic infection revealed

In 1970, Greenwood & Voller discovered that infecting lupus-prone mice with *Plasmodium* prevented the development of severe kidney disease. However, the mechanisms behind this intriguing finding have never fully been investigated. The results of a new study shed light not only on how malaria parasites can protect the kidney, but also on how lupus nephritis can progress to end-stage kidney disease.

"Previous work has connected the presence of kidney-infiltrating dendritic cells (DCs) with glomerulonephritis; however, our report goes beyond this concept by describing a system that fully separates the process of immune complex-driven glomerular inflammation (local innate responses) from the final stage of kidney disease, which is dependent on inflammatory DCs and involves an adaptive response that recruits T cells targeting the kidney," explains corresponding author Silvia Bolland. "We now fully explain how malaria parasites can reduce kidney pathology by averting the tissue infiltration of pro-inflammatory type 2 DCs with long-lasting effects."

After first confirming Greenwood & Voller's results, albeit in a different strain of lupus-prone mice, Bolland and colleagues discovered that *Plasmodium* infection specifically reduced expression of the chemokine CCL17 in the kidneys. *CCL17* is highly expressed by DCs, which were notably also absent from the kidneys of *Plasmodium*-infected mice. Further experiments confirmed that kidney-infiltrating DCs are pro-inflammatory type 2 DCs that produce CCL17, and that their absence correlates with a reduction in severe nephritis.

"We also show that high levels of type I interferon, TNF, IL-1 and hypoxia-induced gene expression, all of them factors previously thought to be triggers of nephritic damage, are by themselves not sufficient to cause immune-cell infiltration and tissue destruction in the kidney," states Bolland.

> "*Plasmodium* infection specifically reduced expression of the chemokine CCL17 in the kidneys"

The results of bone marrow reconstitution studies suggest that *Plasmodium* infection directly alters bone marrow cells in such a way as to prevent DCs from migrating to the kidneys; something Bolland and colleagues are keen to investigate further. Importantly from a translational perspective, CCL17-blocking antibodies could reduce glomerulonephritis in lupus-prone mice without the need for parasitic infection, suggesting that CCL17 could be a future therapeutic target for lupus nephritis.

Joanna Clarke

ORIGINAL ARTICLE Amo, L. et al. CCL17-producing cDC2s are essential in end-stage lupus nephritis and averted by a parasitic infection. *J. Clin. Invest.* https://doi.org/10.1172/JCI148000 (2021)
RELATED ARTICLE Greenwood, B. M. & Voller, A. Suppression of autoimmune disease in New Zealand mice associated with infection with malaria. I. (NZB × NZW) F1 hybrid mice. *Clin. Exp. Immunol.* **7**, 793–803 (1970)

PSORIATIC ARTHRITIS

Targeting the CCR6–CCL20 axis improves experimental PsA

Results of a new study highlight the importance of the CCR6–CCL20 axis in psoriatic arthritis (PsA) and suggest that a novel engineered protein that targets this pathway could offer a new approach to treating the disease. The protein, known as CCL20 locked dimer (CCL20LD), attenuated inflammation in the skin, synovium and enthesis in the IL-23 minicircle DNA mouse model of PsA.

The chemokine CCL20 and its receptor CCR6 have previously been implicated in the development of psoriasis, with CCR6 being critical for the migration of IL-17- and IL-22-producing T cells in skin. CCL20LD, which differs from the natural structure of CCL20 by only one amino acid, binds CCR6 and blocks its function. "Our current data suggest that the CCR6–CCL20 axis might be highly relevant in human PsA, which historically has been more challenging to treat than skin psoriasis," explains corresponding author Sam Hwang.

> "[CCL20LD] attenuated inflammation in the skin, synovium and enthesis"

In the study, systemic delivery of IL-23 minicircle DNA to autoimmune-prone B10.RIII mice led to the development of typical features of psoriasis and PsA, including erythema, swollen paws and enthesitis. In these mice, increased expression of CCL20 and CCR6 was seen in the skin and joints but was most striking in the enthesis, which also had increased numbers of $CCR6^+$ γδ T cells and expression of several pro-inflammatory markers.

Administration of CCL20LD was able to prevent IL-23-mediated skin and joint inflammation and also ameliorate established disease in a dose-dependent manner, with a therapeutic effect similar to that of an anti-IL-17A antibody. CCL20LD also markedly attenuated IL-23-mediated entheseal inflammation.

In human tissue, the researchers observed that CCL20 was present at high concentrations in the synovial tissue of patients with PsA, and $CCR6^+$ cells were detected in both healthy synovial tissue and tissue from patients with PsA. In vitro, IL-1β-stimulated healthy human tendon stromal cells were able to produce CCL20 and promote migration of $CCR6^+$ T cells.

The researchers plan to explore the development of CCL20LD as a new therapy for psoriasis and possibly PsA. Given that CCL20LD is minimally changed from the natural structure of CCL20, Hwang notes that one question to address is whether or not this engineered protein might be less prone to elicit anti-drug antibodies, which often neutralize the therapeutic effect of monoclonal antibody-based therapeutics.

Sarah Onuora

ORIGINAL ARTICLE Shi, Z. et al. Targeting the CCR6/CCL20 axis in entheseal and cutaneous inflammation. *Arthritis Rheumatol.* https://doi.org/10.1002/art.41882 (2021)

IN BRIEF

VASCULITIS

AAV remission possible with reduced steroid dose

Treatment with a reduced dose of glucocorticoids (0.5 mg/kg per day) in addition to rituximab was non-inferior to treatment with high-dose glucocorticoids (1 mg/kg per day) plus rituximab for remission induction in patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) in a randomized, open-label study from Japan. Of the 140 patients with newly diagnosed AAV included in the study, 71.0% of those treated with reduced-dose steroids and 69.2% of those treated with high-dose steroids achieved remission at 6 months, with fewer adverse effects reported in those treated with the reduced dose.

ORIGINAL ARTICLE Furuta, S. et al. Effect of reduced-dose vs high-dose glucocorticoids added to rituximab on remission induction in ANCA-associated vasculitis: a randomized clinical trial. *JAMA* **325**, 2178–2187 (2021)

SYSTEMIC SCLEROSIS

Rituximab shows promise for skin disease in SSc

A randomized controlled trial of rituximab in patients with systemic sclerosis (SSc) has shown good safety and efficacy results using skin sclerosis as the primary end point. In the Japanese study, 56 patients with SSc were randomly allocated to receive either rituximab or placebo once per week for 4 weeks, and the modified Rodnan Skin Score (mRSS) was calculated at baseline and after 24 weeks. The difference in mRSS from baseline to week 24 was –6.30 for those who received rituximab and 2.14 for those who received placebo, and the rate of adverse effects was similar for all participants.

ORIGINAL ARTICLE Ebata, S. et al. Safety and efficacy of rituximab in systemic sclerosis (DESIRES): a double-blind, investigator-initiated, randomised, placebo-controlled trial. *Lancet Rheumatol.* **3**, E489–E497 (2021)

SYSTEMIC LUPUS ERYTHEMATOSUS

B cell X-chromosome inactivation is faulty in SLE

Dysfunctional maintenance of X-chromosome inactivation (XCI) in B cells could help to explain the strong sex bias in systemic lupus erythematosus (SLE). RNA fluorescence in situ hybridization analysis and single-cell immunofluorescence profiling of B cells from patients with SLE has revealed how epigenetic changes to the inactive X chromosome in these cells can cause X-linked immunity-related genes to escape XCI and be aberrantly expressed. Notably, these changes were found in cells from both adults and children with SLE, suggesting a mechanism of sex bias that is not influenced by sex hormones.

ORIGINAL ARTICLE Pyfrom, S. et al. The dynamic epigenetic regulation of the inactive X chromosome in healthy human B cells is dysregulated in lupus patients. *Proc. Natl Acad. Sci. USA* **118**, e2024624118 (2021)

SJÖGREN SYNDROME

Molecular classification divides pSS into 4 groups

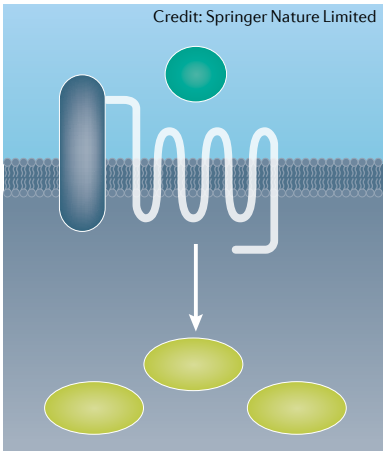
Molecular profiling of patients with primary Sjögren syndrome (pSS) and healthy individuals from the PRECISESADS project has revealed four clusters of patients with distinct patterns of disease, which could be useful for future precision medicine approaches. One cluster did not show an interferon signature, suggesting that interferons should not be therapeutically targeted in these patients. The other three clusters had strong interferon signatures, but differed as to which type of interferon predominated, the severity of disease and the types of immune cells and autoantibodies that were present.

ORIGINAL ARTICLE Soret, P. et al. A new molecular classification to drive precision treatment strategies in primary Sjögren's syndrome. *Nat. Commun.* **12**, 3523 (2021)

BONE DISEASES

Synthetic Wnt agonists rapidly rebuild bone in vivo

Wnt signalling is intricately linked to regulation of bone mass, making it an attractive target for the treatment of diseases and injuries that affect bone. A new publication in *Nature Communications* reports that novel, systemically delivered Wnt mimetics that can activate the Wnt signalling cascade are able to stimulate rapid and robust bone growth in vivo, suggesting their potential as bone anabolic therapies in a range of diseases.



The development of agents that directly activate Wnt signalling, with the aim of promoting bone accrual and repressing bone resorption, is hindered by the complexity of the Wnt signalling cascades and the properties of Wnt proteins themselves. To overcome these challenges, the researchers used an antibody-based platform to engineer Wnt mimetics that target combinations of LRP5 and LRP6 co-receptors and Frizzled family receptors with different specificities, and assessed their effects on bone repair, bone mass and bone strength in disease models including osteoporosis, ageing and bone fracture.

"In these disease models, the Wnt agonists induced rapid and robust bone-building effects and corrected bone-mass deficiency and bone defects," reports corresponding author Yang Li. "The results also suggested that combining Wnt mimetics with current clinical treatments for osteoporosis may enhance bone repair and help maintain bone mass," Li adds.

The Wnt mimetic antibodies induced bone accrual in young and older (16 weeks and 1 year old, respectively) naive C57BL/6 mice. They also reversed bone loss associated with ovariectomy-induced osteoporosis in C57BL/6 female mice, as shown by increases in whole-body bone mineral density (BMD) as well as cortical and cancellous bone growth and improvements in biomechanical bone strength.

Interestingly, BMD increases were greater in 12-week-old naive mice treated with a combination of a Wnt mimetic and bisphosphonate or anti-sclerostin antibody treatment than in mice treated with the individual therapies. In a model of fracture healing, Wnt mimetics increased bone tissue volume and BMD when administered 2 weeks post-fracture, with the gap allowing for callus formation.

"This work adds to the growing body of scientific evidence demonstrating the promise of targeted Wnt mimetics for the potential treatment of disease and injury in a broad spectrum of therapeutic areas where there is significant, unmet medical need," highlights co-corresponding author Wen-Chen Yeh.

"The proof of principle provided is impressive and the further path towards clinical application will generate great interest," notes Rik Lories, who was not involved in the study. "A major question will be on the safety of this approach," he adds, noting that the benefits for bone will need to overcome the potential negative consequences of excessive activation of Wnt signalling, which is deleterious in cartilage, for example.

Sarah Onuora

ORIGINAL ARTICLE Fowler, T. W. et al. Development of selective bispecific Wnt mimetics for bone loss and repair. *Nat. Commun.* **12**, 3247 (2021)

EXPERIMENTAL ARTHRITIS

Modifying exosomes to target macrophages in arthritis

Exosomes derived from mesenchymal stem cells (MSCs) contain immunomodulatory components and are a potential cell-free therapy for various diseases, including rheumatoid arthritis. However, the use of MSC-derived exosomes is limited by their low biodistribution in vivo. In a new study, researchers have modified the surface of MSC-derived exosomes to help target these vesicles to activated macrophages and improve their therapeutic efficacy in arthritis.


Credit: S.Harris/Springer Nature Limited

Using metabolic glycoengineering, the researchers first modified adipose-derived stem cells by adding dextran sulfate, a moiety that binds specifically to a receptor on activated macrophages, to the cell surface. Exosomes produced from these cells were then isolated at high yield using tangential flow filtration.

Analysis using confocal microscopy showed that the dextran sulfate-containing exosomes (DS-EXOs) were internalized by RAW264.7 macrophages and bone marrow-derived macrophages in vitro. Furthermore, following intravenous injection into mice with collagen-induced arthritis (CIA), the DS-EXOs accumulated effectively in the inflamed joints and at higher levels than bare exosomes.

> “engineered exosomes could be used to modulate the macrophage population in the synovium”

Further in vitro analysis found that the DS-EXOs could reprogramme pro-inflammatory (M1) macrophages towards an anti-inflammatory (M2) phenotype through a mechanism involving the microRNAs Let-7b-5p and miR-24-3p. In mice with CIA, systemic administration of DS-EXOs had therapeutic benefits compared with saline, including reducing cartilage and bone erosion, neutrophil infiltration and synovial inflammation. Notably, the DS-EXOs showed a similar or better therapeutic efficacy than bare exosomes, despite being administered at ten times lower the dose.

Overall, the data suggest that these engineered exosomes could be used to modulate the macrophage population in the synovium and, in turn, the surrounding cells, to promote resolution of arthritis. Such an approach shows promise as a next-generation therapy for rheumatoid arthritis.

Jessica McHugh

ORIGINAL ARTICLE You, D. G. et al. Metabolically engineered stem cell–derived exosomes to regulate macrophage heterogeneity in rheumatoid arthritis. *Sci. Adv.* **7**, eabe0083 (2021)

RHEUMATOID ARTHRITIS

Tumour protein linked to FLS phenotype in RA

In rheumatoid arthritis (RA), fibroblast-like synoviocytes (FLS) develop an aggressive phenotype that promotes invasion of the synovium into the surrounding joint structures, which is often described as ‘tumour-like’. The results of a new study lend support to this description by revealing that LIM and SH3 domain protein 1 (LASP1), an adapter molecule more commonly associated with metastatic breast cancer, is associated with aggressive RA FLS.

“LASP1 was initially identified in the context of cancer, and its overexpression is associated with an increased rate of metastasis,” explains corresponding author Adelheid Korb-Pap. “Therefore, it is very interesting for our research to identify LASP1 as a modulator of FLS function and disease in patients with RA and in mouse models of arthritis.”

The researchers found that expression of *LASP1* was increased in both synovial tissues and FLS from patients with RA compared with those from patients with osteoarthritis, and in FLS taken from mice with experimental arthritis compared with wild-type mice. Epigenetic changes in *Lasp1* and increases in *Lasp1* mRNA in two mouse models of arthritis led Korb-Pap and colleagues to hypothesize that alterations in LASP1 could be associated with destructive arthritis.

> “TNF transgenic mice lacking *Lasp 1* had reduced arthritis severity”

The transformation of FLS in RA involves changes in cell migration, cytoskeletal rearrangement and cell-to-cell contact pathways. Notably, Korb-Pap and colleagues found LASP1 to be associated with all of these processes. Compared with FLS from wild-type mice, FLS from arthritic mice had increased migration rates that returned towards normal upon *Lasp1* deletion. The ability of FLS to form cell-to-cell contacts through the cadherin 11 complex was also impaired upon *Lasp1* deletion. TNF transgenic mice lacking *Lasp1* had reduced arthritis severity, less cartilage damage and less bone destruction than wild-type mice.


Credit: Springer Nature Limited

“Our next aim is the investigation of how LASP1 influences the inflammatory microenvironment in RA and at what stage of RA LASP1 mediates FLS function,” notes Korb-Pap. “In this context, we want to identify whether these alterations exist before the onset of disease symptoms, and whether these can be specifically inhibited to prevent the progression of destruction of articular structures.”

The researchers also plan to investigate the epigenetic changes in RA FLS to potentially identify new therapeutic approaches.

Gabriella Szylar

ORIGINAL ARTICLE Beckmann, D. et al. Lasp1 regulates adherens junction dynamics and fibroblast transformation in destructive arthritis. *Nat. Commun.* **12**, 3624 (2021)

OSTEOARTHRITIS

Targeting calcium-related mechanotransduction in early OA

Michelle L. Delco and Lawrence J. Bonassar

Sensation of mechanical stimuli by chondrocytes is critical to cartilage homeostasis and osteoarthritis development. The earliest responses in chondrocyte mechanotransduction pathways involve calcium influx and changes in mitochondrial function, which occur in seconds to minutes. Deeper understanding of these events can elucidate new therapeutic targets for early intervention to prevent osteoarthritis.

Refers to Lee, W. et al. Inflammatory signaling sensitizes Piezo1 mechanotransduction in articular chondrocytes as a pathogenic feed-forward mechanism in osteoarthritis. *Proc. Natl Acad. Sci. USA* **118**, e2001611118 (2021) | Agarwal, P. et al. A dysfunctional TRPV4–GSK3β pathway prevents osteoarthritic chondrocytes from sensing changes in extracellular matrix viscoelasticity. *Nat. Biomed. Eng.* https://doi.org/10.1038/s41551-021-00691-3 (2021).

The process by which chondrocytes sense and respond to mechanical stimuli has been a subject of great interest to the osteoarthritis (OA) community for more than 30 years. Several mechanically mediated factors are known to contribute to the development of OA, including chronic joint overloading from obesity, acute overload from joint trauma, and mechanical stress concentrations arising from anatomic anomalies such as hip dysplasia. Despite these well-established mechanical promoters of the disease, the connection between mechanics and the well-known catabolic and inflammatory pathways that cause OA are still not fully understood. Two newly published studies from Lee et al.[1] and Agarwal et al.[2] shed light on the ways chondrocytes sense their mechanical environment and the importance of calcium signalling in understanding the earliest pathologic events, with an eye towards developing new treatments for OA.

Mechanical loading of cartilage induces a wide range of physical stimuli to which chondrocytes are sensitive, including changes in extracellular and pericellular matrix strain, pH, fluid velocity, hydrostatic pressure and streaming potentials[3]. The downstream effects of mechanical loading include changes in gene expression and protein synthesis, which occur in minutes to hours; apoptosis and extracellular matrix alterations, which occur in hours to days; and changes in tissue-scale mechanical properties, which occur in days to weeks. In the past few years, researchers have also identified even faster responses to mechanical loading that occur on the peracute time scale, in the seconds or minutes after injury, including mitochondrial dysfunction[4,5]. Both Lee et al.[1] and Agarwal et al.[2] have investigated a pathway that is likely upstream of these effects: cellular calcium influx.

Many mechanosensation and mechanotransduction pathways in cartilage involve ion channels[6], with transient receptor potential vanilloid 4 (TRPV4) and Piezo 1 and 2 being arguably the most important. TRPV4 was first characterized as an osmotically sensitive Ca^{2+} ion channel in articular chondrocytes and subsequently shown to be sensitive to physiologic dynamic compression loading. Further, the mechanically gated Ca^{2+} channels Piezo 1 and Piezo 2 were identified in articular chondrocytes and found to be responsive to high (supraphysiologic) strain and responsible for mechanically induced chondrocyte death[7]. In addition to ion channels, integrins are an important physical link between the extracellular and pericellular matrix and cytoskeletal elements such as actin filaments, and therefore constitute a critical transducer of physical cues to physiologic responses in chondrocytes.

In their latest work[1], Lee et al. have fit another important piece into the cartilage mechanotransduction puzzle. While investigating whether inflammatory signalling sensitizes articular chondrocytes to mechanical trauma, they found that IL-1α treatment upregulates Piezo 1 (mRNA and protein) in primary porcine chondrocytes and human OA cartilage. By contrast, IL-1α did not have similar effects on Piezo 2 and TRPV4. Increased activity of Piezo 1, indicated by an accelerated rise in intracellular Ca^{2+} concentration, was observed in chondrocytes pretreated with IL-1α. This increased activity resulted in higher resting-state Ca^{2+} and increased calcium signalling in response to mechanical deformation. The researchers concluded that IL-1α sensitizes chondrocytes to injurious loading through Piezo 1 mechanotransduction.

Lee et al.[1] went on interrogate this IL-1α-amplified influx of Ca^{2+} in response to single-cell mechanical stimulation via atomic force microscopy. The researchers found that treating the chondrocytes with Piezo 1 inhibitors or knocking down Piezo 1 prevented the IL-1α-induced increase in resting Ca^{2+}, whereas inhibitors of TRPV4 and voltage-gated Ca^{2+} channels had no effect. These findings suggest that Piezo 1 is responsible for increased resting Ca^{2+} and mechanically induced Ca^{2+} influx in response to inflammatory priming. The lack of involvement of TRPV4 in this pathway indicates that this inflammatory sensitization to mechanical stimulation is selective (that is, specific to supraphysiologic loading via the Piezo pathway) and suggests an opportunity for targeted therapy.

In a second recently published paper, Agarwal et al.[1] shed light on a distinct form of mechanosensation. In addition to sensing externally applied loads, chondrocytes are known to respond to the stiffness of their surrounding matrix through integrins[8]. The researchers investigated the effect of changing the effective stiffness of the environment by suspending chondrocytes in 3D alginate gels[2]. An increase in stiffness of the hydrogels was associated with a decrease in the expression of important matrix molecules such as aggrecan and type II collagen, and an increase in the expression of pro-inflammatory cytokines, such as IL-1β. This response suggests

a homeostatic feedback loop, whereby chondrocytes attempt to bolster a compliant environment by synthesizing more extracellular matrix, and conversely respond to a stiff environment by decreasing matrix synthesis and activating catabolic pathways. This ability to sense matrix mechanical properties was notably deficient in human osteoarthritic chondrocytes. Such a deficiency suggests that in OA, chondrocytes are missing an important component of mechanically driven homeostasis, leading to insufficient remodelling of damaged tissue.

Notably, this sensing of matrix stiffness by the chondrocytes was dependent on calcium signalling[2]. The addition of glycogen synthase kinase 3β (GSK3β), an enzyme that inhibits TRPV4-mediated calcium signalling, to the 3D cell cultures inhibited the ability of healthy chondrocytes to respond to matrix stiffness. This inhibition points both to the importance of calcium signalling in the sensation of matrix stiffness and to the role of TRPV4 in cartilage extracellular matrix homeostasis. Disruption of this homeostasis might have a role in the development of OA, and, as such, the restoration of this pathway is an interesting new avenue for OA therapy.

The perception of and response to mechanical loading is a complex process that occurs over multiple length scales and time scales (FIG. 1). Historically, the parts of these pathways that have been targeted for OA therapy have been downstream events, which occur at later time scales. Extracellular targets such as degradative proteinases and pro-inflammatory cytokines have shown promise in preclinical studies, but have not provided benefit in clinical trials. Cellular targets such as apoptosis inhibitors have also not proven to be effective clinically[9].

These exciting studies on the roles of Piezo 1 and TRPV4 in mechanical sensation by chondrocytes point to a new frontier in OA research: therapeutic targeting of calcium-dependent pathways and other peracute events related to mechanical signalling. The importance of calcium signalling in this process presents interesting opportunities because of the variety of approaches that have been used to image calcium concentration in real time. Modern microscopy enables interrogation of these mechanically driven signalling events on the scale of a single cell and with a potential time resolution of milliseconds. As such, a single cartilage sample might effectively enable hundreds of individual 'experiments', with individual cells tracked spatially and temporally to reveal the contributions of the local mechanical and biochemical environment[10]. This spatial and temporal monitoring of calcium pathways can also be coupled with measurements of downstream events such as mitochondrial dysfunction[5] and apoptosis to understand the consequences of aberrant mechanical signalling. A better understanding of the very early mechanisms linking mechanical inputs and calcium-mediated catabolic responses in cartilage will enable the development of novel therapeutics to prevent end-stage OA.

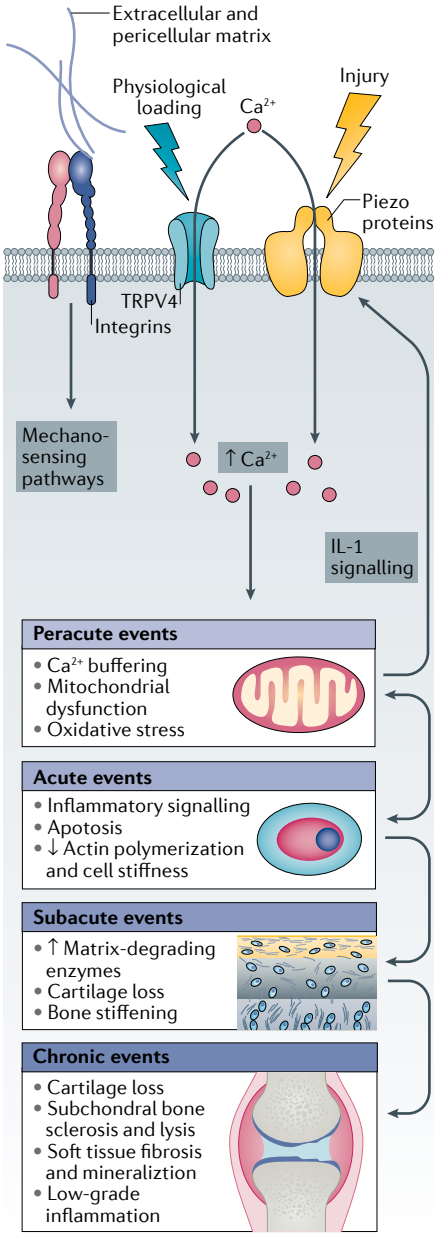


Fig. 1 | **Chondrocyte mechanotransduction pathways in posttraumatic osteoarthritis pathogenesis.** Extracellular and pericellular matrix components transmit mechanical forces to the chondrocyte cytoskeleton via integrins. Physiologic loading is transduced via transient receptor potential vanilloid 4 (TRPV4), whereas injurious loading is transduced via Piezo 1 and Piezo 2. These mechanosensitive ion channels increase intracellular Ca^{2+} concentrations, which is buffered by mitochondria. Excessive intra-mitochondrial Ca^{2+} can lead to mitochondrial dysfunction, which promotes oxidative stress, inflammatory signalling (including IL-1 signalling) and cell apoptosis. Increased IL-1 signalling increases the expression of Piezo 1, exacerbating this process, leading to hypermechanotransduction. Increased Ca^{2+} signalling can also inhibit actin polymerization, resulting in decreased cell stiffness and likely exposing the chondrocyte to 'microtrauma'. Ongoing mechanical inputs and inflammatory signalling lead to an increase in matrix-degrading enzymes, resulting in further biological and mechanical dysfunction of the tissue. These catabolic pathways ultimately lead to chronic pathological outcomes that escalate joint dysfunction and pain in osteoarthritis.

Michelle L. Delco[1] *and Lawrence J. Bonassar*[2,3]✉

[1]*Department of Clinical Science, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA.*

[2]*Meinig School of Biomedical Engineering, Cornell University, Ithaca, NY, USA.*

[3]*Sibley School of Mechanical and Aerospace Engineering, Cornell University, Ithaca, NY, USA.*

✉*e-mail: lb244@cornell.edu*

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Competing interests
L.J.B. is a co-founder and holds equity in 3DBio Therapeutics, which develops engineered cartilage for therapeutic applications. M.L.D has no competing interests.

HEALTH DISPARITIES RESEARCH

Rheumatology resources need reform to represent all patients

Lisa Zickuhr and Heather A. Jones

Underrepresentation of patients with non-white skin in rheumatology educational resources impairs practitioners' competence in caring for patients with skin of colour and reduces patients' confidence in the medical system. Medical publishers and educators should reconsider how to promote equal representation and care of patients with all skin types.

Refers to Strait, A. et al. Race, ethnicity and disparities in rheumatology educational materials. *Arthritis Care Res.* https://doi.org/10.1002/acr.24602 (2021).

Non-white patients experience worse health outcomes than their white counterparts, a disparity most notable in the field of rheumatology among patients with systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA)[1]. Medical education materials inadequately inform practitioners about the nuances of disease manifestations among patients with skin of colour (SOC), thereby contributing to poor health outcomes in these patients[1–3]. New findings published by Strait et al.[1] reveal that rheumatology educational resources underrepresent patients with SOC. These results complement prior research reporting that information relating to SOC is largely missing from medical resources and highlights that this facet of education needs remediation to promote equitable care for all patients[2,3].

Strait et al. collected more than 1,000 images from rheumatology educational materials and coded the images as representing 'light,' 'dark' or 'indeterminate' skin colour using Fitzpatrick's skin phototypes (FSTs)[1]. Overall, 13.4% of the clinical images in the collection portrayed dark skin (that is, FSTs V and VI), whereas 84.0% depicted light skin (FSTs I–IV). Using FSTs as a proxy for race and ethnicity, the researchers compared the representation of dark skin colours in the published images with the estimated representations of Asian (FST V), Native American (FST V) and Black (FST VI) individuals in the general US population as well as in subpopulations with SLE or RA. The researchers found that the bias to publish images of light skin over those of dark skin persisted in all three of these groups and was most pronounced among the SLE and RA subpopulations. These findings highlight a mechanism through which medical education materials could marginalize vulnerable patients, especially those with SLE in whom cutaneous findings often help form a diagnosis.

Educational resources and training experiences are intended to help practitioners develop their skills in assessing, diagnosing and treating patients. If they exclude representation SOC, then they fail to teach practitioners to equitably care for non-white patients[4,5]. This deficiency negatively affects patients with SOC — delaying their diagnosis, increasing the risk of misdiagnosis, reducing their confidence in the health-care system and perpetuating the system in which non-white patients receive poorer care[1]. Such factors could explain why patients with SOC perceive poorer quality of care when receiving treatment from a practitioner of a different race and experience worse health outcomes overall[6].

Notably, when using FSTs to code skin colour in their analysis, Strait and colleagues employed an imperfect scale[1]. The FST classification was developed to assess the response of skin to ultraviolet light, ranging from 'always burns' to 'never burns,' which makes it insufficient for discerning the skin type portrayed in images[2]. Also, as it is a six-point scale, the FST classification underrepresents diversity in skin tones. Research similar to that of Strait et al. has employed FSTs to measure the frequency of SOC in published images and reduced their findings to the binary categories of 'light' and 'dark'[1,2]. An alternative tool, the New Immigrant Survey (NIS) skin colour scale, portrays 11 shades of skin tone without relying on response to ultraviolet light[7]. The NIS skin colour scale produced more granular results when applied to evaluations of medical education materials as researchers could group the 11 shades of skin tone into three categories of 'light', 'medium' and 'dark'[3].

> "…information relating to [skin of colour] is largely missing from medical resources…"

The use of skin tone as a surrogate for race and ethnicity limits the generalizability of research investigating educational materials. A trend exists among studies, including the report by Strait et al., to compare the frequencies of depicted skin colours with models estimating the racial and ethnic make-up of populations[1,3]. However, race and ethnicity are social constructs, and patients with the same skin colour can identify with different racial or ethnic groups[7]. Post hoc estimation of race and ethnicity reduces the validity of results and overlooks the importance of skin colour as a unique outcome. Skin colour can carry more societal implications than race and ethnicity, which prompted sociologists to develop the NIS scale to investigate its independent effects[7]. Researchers could report more valid as well as more clinically and socially relevant results if they were to focus on skin colour without equating it to race and ethnicity.

Strait and colleagues' argument that medical resources underrepresent SOC because the frequency of images with 'dark' skin is less than what exists in the US population can perpetuate disparities in medical education[1]. If resources were to include SOC at a rate relative to what exists in the general population, then non-white minority subgroups would continue to have less representation in medical resources, maintaining their vulnerability to marginalization and perpetuating disparities in health outcomes. To provide equitable care for patients, we propose that practitioners should be trained to diagnose and care for patients with all skin tones equally rather than with a competence commensurate to a colour's presence within the population. This training would require medical resources to include images and examples that represent skin tones with equal frequency, thereby creating materials that prepare practitioners to recognize the nuances of disease patterns among different skin types with equal skill.

Educational materials communicate the importance the medical profession places on the patients for whom it provides care; thus, the underrepresentation of SOC within educational resources reflects poorly on the medical profession. Strait et al. outline strategies to correct this inequality within rheumatology[1];

they ask editors to increase the publication of images depicting SOC across all rheumatic diseases and the ACR to enhance the representation of SOC in its Image Library, and they call on educators to teach about the manifestations of rheumatic diseases in patients of colour as well as incorporate images in their materials that reflect a variety of skin tones.

> “…practitioners should be trained to diagnose and care for patients with all skin tones equally…”

Several initiatives exemplify that these aims are achievable. Dermatologists have created specialty SOC clinics dedicated to cutaneous conditions occurring in non-white skin to enhance patient outcomes, clinical research and practitioner training[8]. Experts have shared suggestions for capturing high-quality images of SOC[9], and, encouragingly, Strait et al. report that almost half of the images featuring sarcoidosis and discoid lupus in their study depicted SOC[1]. VisualDx, a software system that supports general medical education and clinical practice, has grown the proportion of images showing SOC to almost one-third of its collection[2]. The ACR's 2021 Image Competition is 'dedicated exclusively to images of rheumatic disease in SOC', with the purpose of increasing representation in the ACR Image Library[10].

Studies thus far have focused on the representation of SOC in educational resources. Future research should describe the images available through internet search engines because such pictures are most readily accessible. Educators should also investigate which methods and clinical environments best teach the assessment of cutaneous findings in SOC. In order to expand the mission of SOC clinics, leaders should evaluate specialty clinics' effect on practitioner training in SOC, quantify their effect on patient outcomes and share barriers to caring for patients with non-white skin so that the medical community can address these challenges. These research initiatives would offer opportunities for educators and implementation scientists to collaborate while providing evidence to advance the medical profession's progress towards equity.

The work by Strait et al. highlights that patients with rheumatic diseases and SOC are underrepresented in educational materials[1]. This shortcoming reflects a profession-wide problem that reduces practitioners' skill in identifying cutaneous manifestations in SOC and adversely affects patients' health outcomes and confidence in the medical profession. In order to ensure practitioner competence with every skin type, researchers should explore equal representation of all skin tones in resources rather than reflecting the composition of the general population, and should recognize the importance of skin colour without equating it to the social constructs of race and ethnicity. As leaders commit to correcting the SOC disparity observed in published images, the profession of medicine will dismantle this inequity, thereby improving the training of practitioners and the care of patients.

Lisa Zickuhr ✉ *and Heather A. Jones*

Department of Medicine, Washington University School of Medicine, St. Louis, MO, USA.

✉*e-mail: zickuhrl@wustl.edu*

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Competing interests
The authors declare no competing interests.

Lyme arthritis: linking infection, inflammation and autoimmunity

Robert B. Lochhead[1], *Klemen Strle*[2], *Sheila L. Arvikar*[3], *Janis J. Weis*[4] *and Allen C. Steere*[3]✉

Abstract | Infectious agents can trigger autoimmune responses in a number of chronic inflammatory diseases. Lyme arthritis, which is caused by the tick-transmitted spirochaete *Borrelia burgdorferi*, is effectively treated in most patients with antibiotic therapy; however, in a subset of patients, arthritis can persist and worsen after the spirochaete has been killed (known as post-infectious Lyme arthritis). This Review details the current understanding of the pathogenetic events in Lyme arthritis, from initial infection in the skin, through infection of the joints, to post-infectious chronic inflammatory arthritis. The central feature of post-infectious Lyme arthritis is an excessive, dysregulated pro-inflammatory immune response during the infection phase that persists into the post-infectious period. This response is characterized by high amounts of IFNγ and inadequate amounts of the anti-inflammatory cytokine IL-10. The consequences of this dysregulated pro-inflammatory response in the synovium include impaired tissue repair, vascular damage, autoimmune and cytotoxic processes, and fibroblast proliferation and fibrosis. These synovial characteristics are similar to those in other chronic inflammatory arthritides, including rheumatoid arthritis. Thus, post-infectious Lyme arthritis provides a model for other chronic autoimmune or autoinflammatory arthritides in which complex immune responses can be triggered and shaped by an infectious agent in concert with host genetic factors.

[1]*Department of Microbiology and Immunology, Medical College of Wisconsin, Milwaukee, WI, USA.*

[2]*Laboratory of Microbial Pathogenesis and Immunology, Division of Infectious Diseases, Wadsworth Center, Albany, NY, USA.*

[3]*Center for Immunology and Inflammatory Diseases, Division of Rheumatology, Allergy and Immunology, Harvard Medical School, Massachusetts General Hospital, Boston, MA, USA.*

[4]*Department of Pathology, Division of Microbiology and Immunology, University of Utah School of Medicine, Salt Lake City, UT, USA.*

✉*e-mail: asteere@mgh.harvard.edu*

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Microbial infections have long been hypothesized to have a role in triggering autoimmunity in chronic inflammatory diseases[1]. However, the clinical onset of autoimmune disorders often develops over years or decades, making it difficult to establish a causal link between exposure to an infectious trigger and subsequent disease. Uniquely in Lyme arthritis, a late manifestation of Lyme disease, the triggering event, *Borrelia burgdorferi* infection, is known with certainty.

Lyme disease (also known as Lyme borreliosis) occurs in temperate regions of North America, Europe and Asia (FIG. 1), and causes ~300,000 cases annually in the USA[2]. Lyme disease is caused by the tick-transmitted spirochaete *B. burgdorferi* sensu lato (*B. burgdorferi* in the general sense), which consists of 20 different species[2]. However, the human infection is caused primarily by three species, *B. burgdorferi* sensu stricto (*B. burgdorferi* in the strict sense, hereafter called *B. burgdorferi*) in the USA, and *Borrelia afzelii* and *Borrelia garinii* in Europe and Asia. Less common species that can infect humans include *Borrelia mayonii* in upper midwestern USA, *Borrelia bavariensis* (which is closely related to *B. garinii*) in Europe and Asia, and *B. burgdorferi* in Europe. Each species or subtype is associated with distinct clinical features; for example, the common subtypes of *B. burgdorferi* that are found in north-eastern USA are particularly arthritogenic, whereas *B. garinii* and *B. afzelii* rarely cause Lyme arthritis[2].

Infection with *B. burgdorferi* usually begins with an expanding erythema migrans skin lesion, which develops at the site of the tick bite[2] (FIG. 2a). Within weeks, spirochaetal strains from north-eastern USA can disseminate to a number of sites[3], a process that is often accompanied by flu-like symptoms and can be shortly followed by organ-specific involvement, particularly neurological or cardiac abnormalities[4]. Months later, many patients develop Lyme arthritis, which is characterized by intermittent or persistent joint swelling and pain, primarily in large joints (especially the knees) for a period of several years[5,6]. In some patients, early infection is asymptomatic and Lyme arthritis is the presenting manifestation of Lyme disease.

Most patients with Lyme arthritis respond to appropriate oral and, if needed, intravenous antibiotic therapy, and the arthritis resolves (termed antibiotic-responsive Lyme arthritis)[7,8]. However, in a small percentage of patients, joint swelling lessens but synovitis persists or worsens after spirochaetal killing with antibiotic therapy[7,9].

Key points

- A combination of spirochaetal and host genetic factors shape the outcome of Lyme arthritis, which ranges from mild, antibiotic-responsive joint inflammation to persistent, antibiotic-refractory autoinflammatory or autoimmune synovitis.
- Certain highly inflammatory strains of *Borrelia burgdorferi* most commonly found in north-eastern USA are present at an increased frequency among patients who subsequently develop post-infectious Lyme arthritis.
- The histology of post-infectious Lyme arthritis synovia is similar to that in other chronic inflammatory arthritides, such as rheumatoid arthritis, but there is greater microvascular damage in Lyme arthritis.
- *B. burgdorferi* is no longer present in synovia after treatment with antibiotics, but *B. burgdorferi* peptidoglycan might persist and could be an important promoter of innate immune responses.
- Dysregulated, excessive IFNγ responses and inadequate amounts of the anti-inflammatory cytokine IL-10 are a central feature of post-infectious Lyme arthritis, and contribute to persistent inflammation and the development of autoimmunity.
- Synovial fibroblasts, the most common cell in the synovial lesion, become immune effector cells capable of altering the innate and adaptive immune microenvironment in Lyme arthritis.

These patients develop massive synovial hyperplasia[10], often accompanied by autoimmune T and B cell responses that can last for several years[11–16], called post-infectious (or post-antibiotic or antibiotic-refractory) Lyme arthritis. After appropriate oral and intravenous antibiotic therapy, such patients are treated with DMARDs[17], the standard of care for chronic autoimmune or autoinflammatory types of arthritis. As only one knee is usually affected in post-infectious Lyme arthritis, synovectomy is also an option[18]. The synovial lesion in post-infectious Lyme arthritis is similar to that seen in other forms of chronic inflammatory arthritis (FIG. 2b), including rheumatoid arthritis (RA)[9,10,19].

In addition to relevant human studies, several in-bred, congenic and knockout strains of mice have provided critical insights into Lyme arthritis pathogenesis (TABLE 1). *B. burgdorferi*-infected C3H/HeN (C3H) mice develop severe arthritis of the tibiotarsal joint with thickening of the tibiotarsal tendon sheath, which peaks several weeks following infection and then spontaneously resolves[20]. By contrast, *B. burgdorferi*-infected C57BL/6 (B6) mice have only mild arthritis and quickly repair damaged tissue, leading to a reduction in all parameters of joint disease[21]. Comparison of how these two strains respond to *B. burgdorferi* infection has led to the identification of genetic and immune factors that are important for arthritis development[22]. However, important differences exist between mice and humans. Mice primarily rely on innate immune responses to control *B. burgdorferi* infection, whereas humans employ both innate and adaptive immune responses throughout infection. In humans, arthritis usually only develops after months of infection within the context of expanded innate and adaptive responses, which can become excessive and maladaptive. Immune responses to *B. burgdorferi* in mice and humans are discussed in more detail elsewhere[23].

In this Review, we integrate human and mouse studies to detail the pathogenetic features of Lyme arthritis, from initial infection of the skin, to infection of joints, to post-infectious arthritis. We emphasize how, in genetically susceptible individuals, infection with certain *B. burgdorferi* strains can trigger an excessive, dysregulated immune response that results in post-infectious inflammatory synovitis similar to that seen in other forms of chronic autoimmune or inflammatory arthritis, including RA.

Skin infection and dissemination

After the injection of *Borrelia* spp. into the skin by an *Ixodes* tick, spirochaetes multiply in erythema migrans lesions[24] (FIG. 3a). The immune response in the skin includes T cells, macrophages, dendritic cells and a small number of B cells[25,26], and the main cytokines expressed are the pro-inflammatory cytokines IFNγ and IL-6, and the anti-inflammatory cytokine IL-10 (REFS[25,27]). In the USA, *B. burgdorferi* often disseminates in the blood during the first few weeks of infection in a process that requires the binding of *Borrelia* surface adhesins to host integrins on the vascular endothelium[28–30] (FIG. 3b). As shown in mice, the spread of *B. burgdorferi* through the vasculature or lymphatics is dependent on the interactions of spirochaetal surface molecules and endothelial cell membrane proteins. Bacterial–endothelial cell interactions result in the loosening of tight junctions and migration of spirochaetes into the synovial extracellular matrix via small vascular lesions[31,32] (FIG. 3c). In response, natural killer T (NKT) cells, tissue-resident macrophages, polymorphonuclear cells and stromal cells have an important role in maintaining endothelial cell barrier function, limiting spirochaetal invasion into extravascular tissues and suppressing tissue damage and arthritis development[33]. NKT cells secrete IFNγ in response to immunogenic *B. burgdorferi* glycolipids[34,35] that are presented by CD1-expressing antigen-presenting cells[36,37]. Macrophages, polymorphonuclear cells, fibroblasts and endothelial cells respond to spirochaetal invasion by producing large amounts of innate immune response and tissue repair proteins. Notably, variations in these responses greatly affect arthritis severity and outcome.

According to one subtyping system[38], out of 23 *B. burgdorferi* outer-surface protein C (OspC) subtypes, types A, B, I and K are the most likely to disseminate in humans[39]. In patients with disseminated early infection, many interferon-associated genes are upregulated in peripheral blood mononuclear cells[27,40]. Serum samples often show high concentrations of the macrophage-recruiting chemokine CCL2 and of the innate immune mediators IL-6 and TNF, although the anti-inflammatory cytokine IL-10 is also prominent[41]. Patients with disseminated infection also have high serum concentrations of T helper 1 (T_H1) cell-associated immune mediators, including IFNγ and the IFNγ-inducible T cell chemokines CXCL9 and CXCL10 (REFS[41,42]). Many patients' sera contain numerous T_H17 cell-associated mediators, particularly IL-23 (REF.[43]). Infection with the OspC type A (RST1) strain is particularly inflammatory, leading to more severe symptoms in patients with erythema migrans[41,42]. Similarly, strain-specific OspC also has an important role in spirochaetal joint invasion and colonization in mice[44].

Lyme disease spirochaetes are only transiently present in the blood[45] and rapidly migrate to extravascular tissues via transendothelial migration[46]. With their unique planar wave motion, these bacteria are highly adapted to move through dense connective tissue, which requires the binding of plasminogen or its activators to the surface of the organism[47]. The spirochaetal adhesins decorin binding protein A (DbpA) and DbpB bind to host decorin[48], a proteoglycan that is bound to collagen, and spirochaetes can also bind directly to, invade and colonize native type I collagen lattices[32]. The binding of DbpA and DbpB to host decorin probably explains the alignment of spirochaetes with collagen fibrils in connective tissue in joints, heart or nerves[49]. Genetic variability in *Borrelia* outer-surface adhesins at least partially explains differences in tissue tropism between strains[41,50,51]. For example, in a mouse study, *B. burgdorferi* OspC subtypes that bound dermatan sulfate were associated with joint invasion[44], which the authors suggest could explain the exceptional arthritogenicity of certain spirochaetal strains found in north-eastern USA.

The antibody response to *B. burgdorferi* develops slowly, and during the first few weeks of infection an IgM response is seen in only a minority of patients[52]. Total IgM concentrations can also be increased during early infection, suggestive of polyclonal activation of B cells[53]. As *B. burgdorferi* disseminates and infects host tissues, an increasingly higher percentage of patients develop IgM and IgG responses to the spirochaete[52,54]. To evade the host antibody response, spirochaetes seek protected niches and change the expression profile of their outer-surface proteins[55]. In particular, the lipoprotein VlsE undergoes extensive antigenic variation[56]. In addition, *B. burgdorferi* evade innate immune responses by binding host complement regulator proteins to their surface, which inactivate complement and induce innate immune tolerance[57].

Dysregulation of innate immune responses during early disseminated infection might promote subsequent arthritis development. In C3H mice, type I interferons (IFNα and IFNβ) have a particularly important role during the first week of infection and set the stage for the subsequent development of arthritis[58]. Importantly, the type I interferon response (typically associated with anti-viral immunity) is maladaptive and has no effect on host defence[59]. This type I interferon response is accompanied by downregulation of numerous genes involved in tissue repair and wound healing, such as extracellular matrix proteins and transforming growth factor-β-inducible genes[60]. By contrast, B6 mice, which develop only mild Lyme arthritis, lack the robust interferon signature seen in arthritogenic C3H mice and exhibit marked upregulation of tissue repair and wound healing genes in joints at 1 week post-infection[60].

As in C3H mice, early type I interferon responses are likely to be arthritogenic in humans with Lyme arthritis. Human peripheral blood mononuclear cells stimulated with the highly inflammatory OspC type A (RST1) strain of *B. burgdorferi* secrete type I interferons as well as type II interferon (IFNγ)[42]. In addition, type I and II interferons are predominant in erythema migrans skin lesions[27]. Moreover, type I interferons are known to be important in the development of a number of rheumatic and autoimmune diseases[61]. Given that patients with erythema migrans who are treated with antibiotics do not develop subsequent arthritis, it is difficult to directly test the importance of early type I interferon responses in the subsequent development of arthritis in humans. However, a role for type I interferons can be inferred from responses in C3H mice. On the basis of studies in this mouse model, we hypothesize that during early disease, dysregulated type I interferon responses to *B. burgdorferi* in the skin or joint set the stage for severe Lyme arthritis and autoimmunity later in the disease.

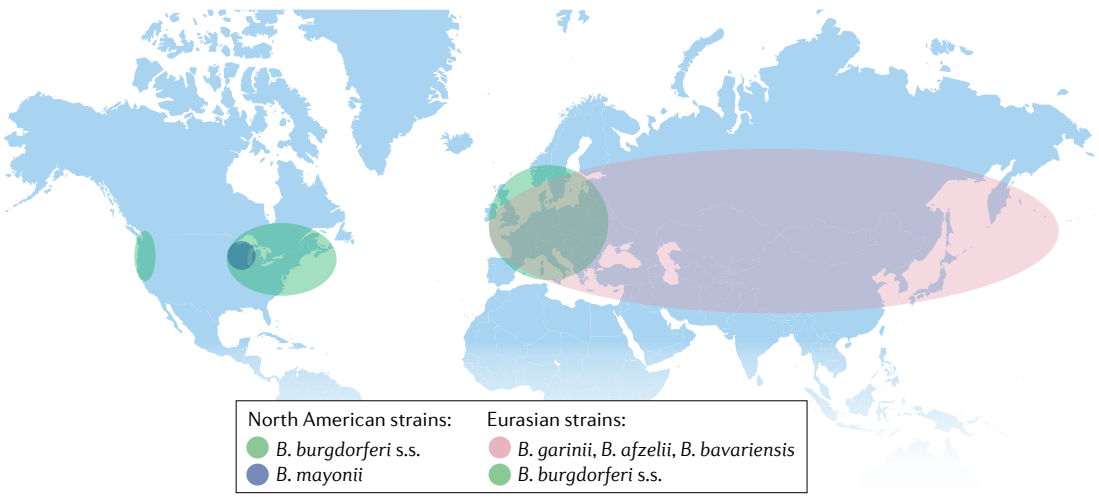


Fig. 1 | **Geographic distribution of *Borrelia burgdorferi* species relevant to human disease.** *Borrelia burgdorferi* sensu stricto (*B. burgdorferi* s.s.) is the major species in North America and is primarily found in the USA in the north-eastern and mid-Atlantic states, the upper Midwest, in northern California and, to a lesser degree, in Oregon and Washington. *B. burgdorferi* s.s. also extends into Canada at each of the bordering USA locations. *Borrelia mayonii* is much less common than *B. burgdorferi* s.s. and is restricted to the upper midwestern states in the USA. European strains include *Borrelia garinii* and *Borrelia afzelii* and, to a lesser extent, *B. burgdorferi* s.s. and *Borrelia bavariensis*, which is closely related to *B. garinii*. In Asia, *B. garinii* is the predominant species, but *B. bavariensis* and *B. afzelii* are also found there.

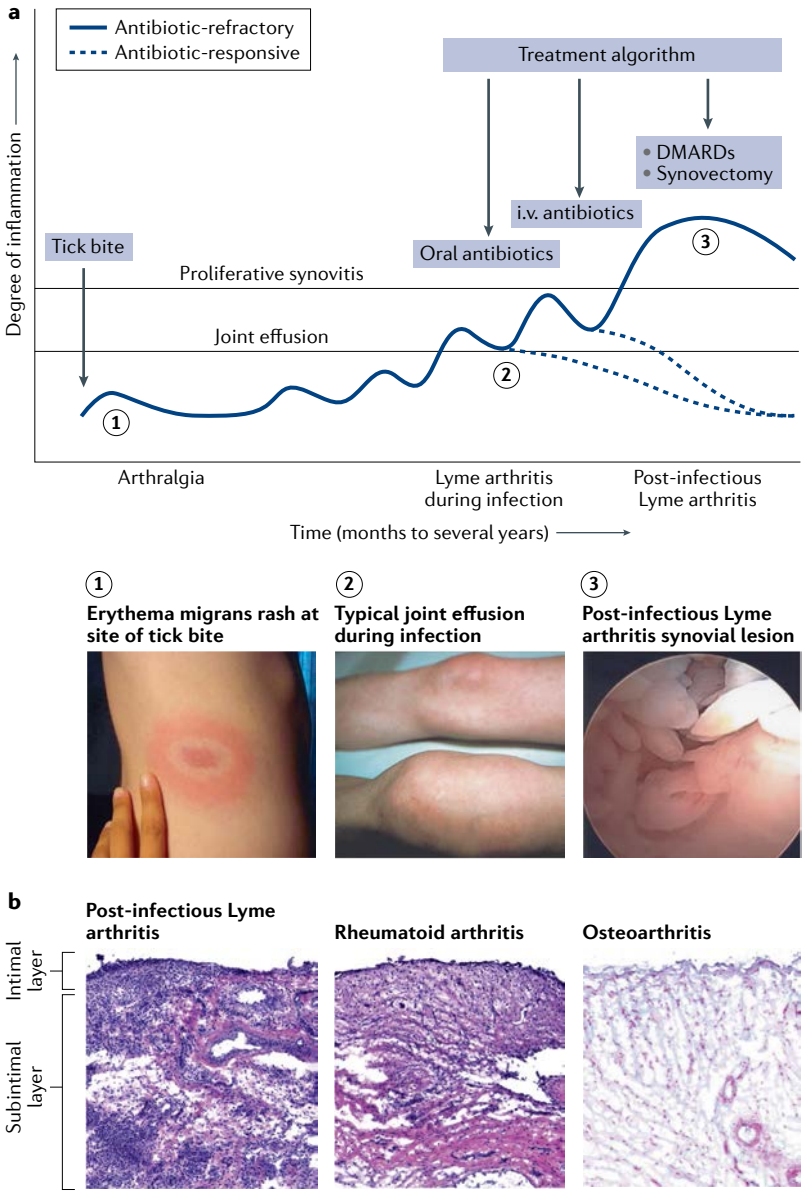


Fig. 2 | **Lyme arthritis stages and characteristics. a** | In untreated patients, Lyme disease occurs in stages, with different manifestations present at each stage. A slowly expanding erythema migrans rash commonly appears 3 to 32 days after a bite by a *Borrelia burgdorferi*-infected *Ixodes* tick (1), which can be accompanied by flu-like symptoms such as fever, headache, myalgias, arthralgias, malaise and fatigue. In the north-eastern states of the USA, Lyme arthritis typically causes large joint effusions, particularly affecting the knees (2), which develop a median of 6 months after the initial skin lesion. Arthritis usually resolves after 1–3 months of oral and, if necessary, intravenous (i.v.) antibiotic therapy (antibiotic-responsive Lyme arthritis). In a small subset of patients, arthritis persists or worsens despite 2–3 months of antibiotic therapy and apparent spirochaetal killing (post-infectious Lyme arthritis). These patients typically develop a highly proliferative synovial lesion (3) that does not respond to further courses of antibiotic therapy (antibiotic-refractory Lyme arthritis). Treatments such as DMARDs or arthroscopic synovectomy help to resolve their arthritis. **b** | The synovial lesion in post-infectious Lyme arthritis is similar to the lesion in rheumatoid arthritis and other inflammatory arthritides. By contrast, osteoarthritis synovium typically has minimal cellular infiltrate, the intimal layer is not inflamed or thickened, and the subintimal layer is composed of healthy, intact microvasculature and highly organized collagen fibres. In this figure, the synovial lesions from Lyme arthritis and rheumatoid arthritis are stained with haematoxylin and eosin (H&E), and osteoarthritis synovium is stained with H&E and Alcian blue to show acidic glycosaminoglycans on the outer surface of collagen fibre bundles and along the synovial lining. Image of the knee in part **a** reprinted with permission from REF.[137], Elsevier.

Lyme arthritis during active infection

Months after the initial infection, along with an expansion of the immune response to *B. burgdorferi*, untreated patients often develop marked joint swelling, frequently in one or both knees. *B. burgdorferi* has rarely been cultured from the synovial fluid of patients with Lyme arthritis, but prior to antibiotic treatment, *B. burgdorferi* DNA (but not mRNA) can be found in the synovial fluid of ~70% of these patients[24,62]. This finding suggests that live spirochaetes might survive only in protected tissue niches within joints and are killed if they escape into synovial fluid. During joint infection, immune responses are focused on spirochaetal killing, primarily through acute inflammatory responses to pathogen-associated molecular patterns (PAMPs), antibody production and the infiltration of polymorphonuclear cells into synovial fluid[9], which might be the principal barrier preventing spirochaetal escape. In addition, large amounts of NF-κB-induced acute pro-inflammatory cytokines and chemokines are found in synovial tissue and synovial fluid from patients with Lyme arthritis[41,42,63], typical of innate immune responses to bacterial infections.

Robust anti-*B. burgdorferi* antibody responses develop towards a large array of spirochaetal proteins[52,64]. Patients with Lyme arthritis can have antibody reactivity to as many as 89 spirochaetal proteins[65], primarily outer-surface proteins, many of which are lipidated and might serve as immune adjuvants[66]. Two spirochaetal glycolipids, acylated cholesteryl galactoside (*Bb*GL1) and monogalactosyl diacylglycerol (*Bb*GL2), are also highly immunogenic[34]. Moreover, patients with Lyme arthritis can have antibody responses to spirochaetal antigens that are ordinarily expressed only in the tick, such as OspA, OspD and *Borrelia* iron and copper-binding protein A (BicA), a phenomenon found almost exclusively in the highly inflammatory milieu of joints in North American patients with Lyme arthritis[67]. Similarly, *B. burgdorferi* can be induced to express tick-specific proteins in mice in a highly inflammatory environment[68].

Marked T_H1 cell responses to *B. burgdorferi* antigens also occur in patients with Lyme arthritis, particularly among synovial fluid mononuclear cells, which produce large amounts of IFNγ[69–71]. The role of these cells might be primarily to help B cells to produce neutralizing antibodies against the spirochaete. Anti-borrelial antibodies are predominantly T cell-dependent IgG1 and IgG3 isotypes, which are capable of inducing opsonization and activating complement[72]. Most synovial fluid mononuclear cells also express memory markers[71], which helps to explain why *B. burgdorferi* T cell and B cell responses typically persist for many years after the resolution of Lyme arthritis, and why reinfection occurs only rarely, if at all, after Lyme arthritis.

Animal model studies have provided insights into important innate immune effectors in Lyme arthritis (TABLE 1). Mice deficient in certain innate immune response pathways, particularly those involved in recognition of *B. burgdorferi* surface lipoproteins, including Toll-like receptor 2 (TLR2) and myeloid differentiation primary response protein MyD88, have impaired host defence and develop severe Lyme arthritis[73–76]. In addition, C3H mice have a hypomorphic allele (*Bbaa2* locus

on Chromosome 5) encoding the lysosomal enzyme β-glucuronidase, which allows the accumulation of arthritogenic glycosaminoglycans in infected joints[77,78]. Similarly, the *Bbaa1* locus on Chromosome 4 in C3H mice, which contains the type I interferon locus, is involved in dysregulated type I interferon responses and severe Lyme arthritis[79–81].

Untreated patients with Lyme arthritis often have intermittent flares of arthritis or persistent arthritis over a period of several years[5]. One theory is that spirochaetes might survive in relatively avascular sites, such as the tendons in and around joints, and then escape from these sites occasionally to repopulate the synovial tissue[82,83]. Consistent with this hypothesis, joint swelling might be more severe and prolonged in recurrent flares, and the very high antibody responses that occur in patients with Lyme arthritis are consistent with repeated waves of antigenic exposure to spirochaetes[5]. As affected joints are no longer swollen after treatment in antibiotic-responsive patients, post-infection immune responses cannot be assessed in these patients' joints. However, we predict that antibacterial responses are downregulated after spirochaetal killing and wound repair genes are upregulated, leading to tissue repair, a return to joint homeostasis and arthritis resolution (FIG. 4), similar to the strong tissue repair signature that occurs in the joints of infected B6 mice[60].

Post-infectious Lyme arthritis

Rather than resolution of arthritis after antibiotic therapy, a small percentage of patients with Lyme arthritis have persistent synovitis that can worsen in the post-antibiotic period[7]. In these patients, the synovial lesion — the target of the immune response — shows massive synovial fibroblast proliferation and fibrosis, infiltration of mononuclear cells, large amounts of antigen presentation, marked vascular proliferation and, in some patients, obliterative microvascular lesions and massive fibrin deposition suggestive of microscopic bleeding[84,85] (FIG. 5). These histological findings are similar to those that occur in other autoimmune or autoinflammatory forms of arthritis, including RA, albeit with a greater emphasis on microvascular damage in post-infectious Lyme arthritis[10,12,84–86]. Damage to the microvasculature, including obliterative microvascular lesions, seems to be a common feature of Lyme disease and can be found in other affected tissues, including the heart[49,87,88], skeletal muscle[49,89] and dura mater[90]. The inflammatory process in the joints can be accompanied by tendon sheath thickening (tenosynovitis) and tendon calcification (tendonitis) and, occasionally, by mild-to-moderate cartilage damage[91]. Although synovial fluid contains a very high percentage of neutrophils during active infection, in the post-infectious stage it contains relatively fewer neutrophils and proportionally more monocytes, macrophages and lymphocytes, suggestive of an expanded inflammatory response in the post-infectious phase[9]. In contrast with RA, post-infectious Lyme arthritis eventually resolves in all patients — often with the aid of DMARD therapy — usually within 1–2 years, but within a maximum of 4–5 years[7,17]. Presumably, without the immune stimuli provided by live spirochaetes, the immune response eventually regains homeostasis and the arthritis resolves.

These changes in the cellular infiltrate in synovial fluid result from an inadequately restrained, excessive pro-inflammatory immune response that begins during infection and continues into the post-infectious period[92]. Infection with the highly inflammatory OspC Type A (RST1) strain of *B. burgdorferi* more commonly results in this outcome[42,51]. In studies of *B. burgdorferi* isolates from erythema migrans skin lesions, OspC type A strains were found in the USA in 21 of 58 isolates (36%) from New England states[93], 46 of 291 isolates (16%)

Table 1 | **Characteristics of mouse models of Lyme arthritis**

Mouse model	Immune defect	Effect on arthritis	Effect on host defence	Relevance to human disease	Refs
C57BL/6 (B6)	NA	Mild, self-resolving Lyme arthritis	NA	Probably mimics patients who develop only mild Lyme arthritis	20
C3H/HeN (C3H)	NA	Severe, acute, self-resolving Lyme arthritis	NA	Most similar to severe Lyme arthritis during active infection	20
Il10$^{-/-}$ (B6)	Dysregulated NF-κB and T_H1 cell responses; impaired regulatory T cells	More severe (increased innate and adaptive inflammation)	Very few *Borrelia burgdorferi* in joints compared with B6 or C3H mice	Mimics dysregulated T_H1 cell responses seen in patients who develop post-infectious Lyme arthritis[9,10,43,70,71]	101,109, 110
Tlr1$^{-/-}$ or *Tlr2*$^{-/-}$ (B6 or C3H)	Impaired response to *Borrelia* lipoproteins (such as OspA and OspC)	More severe (probably owing to impaired host defence)	~100-fold more *B. burgdorferi* in joints compared with wild-type mice; OspA vaccine non-protective	Low TLR1 in vaccine low responders[75]; TLR1 hypomorph associated with severe Lyme arthritis[41]	74,75
Mir146a$^{-/-}$ (B6)	Hyperactive NF-κB signalling	More severe (increased acute inflammation)	Slightly fewer *B. burgdorferi* in joints compared with wild-type mice	Probably reflects the central importance of NF-κB regulation in host defence and arthritis during infection[9]	100
Ifnar$^{-/-}$ (C3H)	Defect in type I interferon signalling	Less severe (type I interferon is arthritogenic)	No effect	Unclear, might be important in early infection of skin	59
C3H *Gusb* allele (B6)	B6 mice with C3H *Gusb* allele are unable to clear ECM debris	More severe (accumulated glycosaminoglycans in joints)	No effect	Unclear, might be important in clearing *B. burgdorferi* peptidoglycan and host ECM debris	77

ECM, extracellular matrix; NA, not applicable; Osp, outer-surface protein; T_H1 cell, T helper 1 cell; TLR1, Toll-like receptor 1.

from New York state[39] and only 2 of 65 isolates (3%) from Wisconsin, an upper midwestern state[94], compared with 0 of 29 isolates from Slovenia[95]. These results might explain why post-infectious Lyme arthritis is most often found in New England. Nevertheless, a 2019 French study described patients with post-infectious Lyme arthritis that were similar to those found in the USA[96], suggesting that highly inflammatory strains of *B. burgdorferi* might occur in certain regions in Europe.

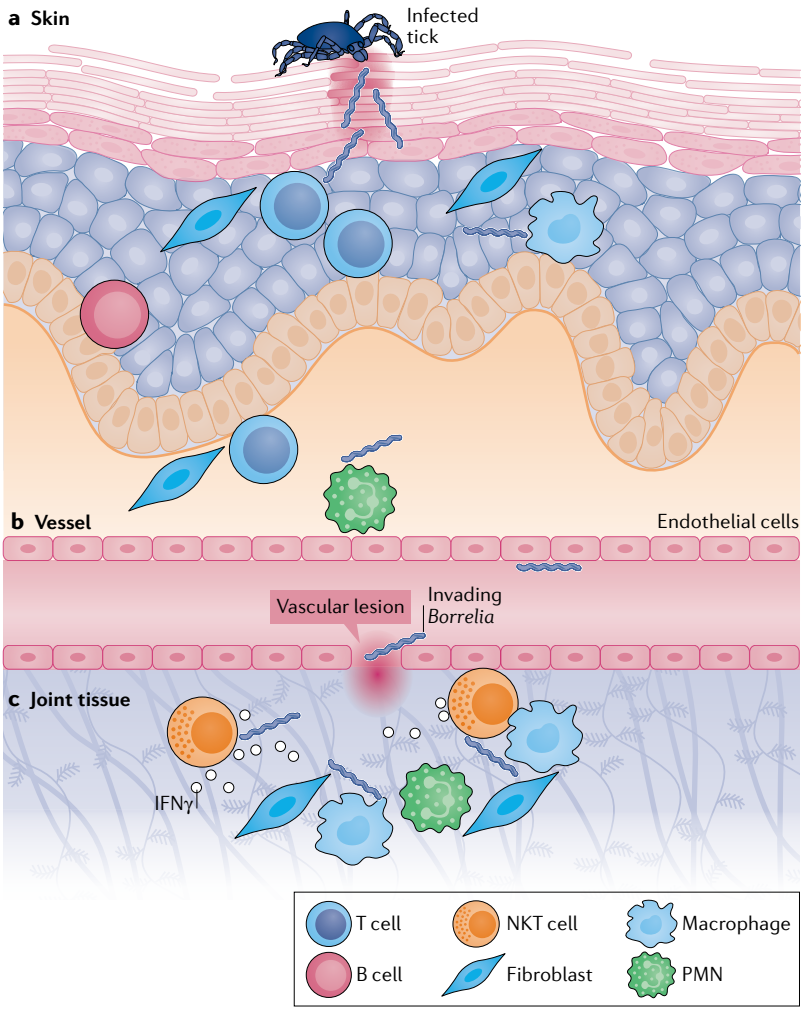


Fig. 3 | **Spirochaete invasion into joint tissue. a** | Spirochaetes invade the skin during the bloodmeal of an infected *Ixodes* tick. Upon entry, tissue-resident T cells, B cells, resident antigen-presenting cells (such as macrophages and dendritic cells), some polymorphonuclear cells (PMNs) and stromal cells (such as fibroblasts, keratinocytes, epithelial cells and endothelial cells) are responsible for early-acute immune responses to infection. **b** | A small number of spirochaetes escape the site of invasion and enter the vasculature, where *Borrelia* surface lipoproteins interact with vascular endothelial cells to induce the loosening of tight junctions. **c** | Spirochaetes enter the extracellular matrix of joint tissue through vascular lesions. Once in the joint tissue, they induce acute inflammatory responses by resident cells such as endothelial cells and synovial fibroblasts, which produce adhesion molecules, matrix metalloproteinases and innate immunity cytokines and chemokines. Natural killer T (NKT) cells produce IFNγ in response to CD1-presented immunogenic *Borrelia* glycolipids, thereby enhancing vascular barrier function and limiting spirochaetal invasion and chronic inflammation. The cytotoxic function of NKT cells might also directly contribute to spirochaetal killing. The nature and magnitude of these early immune responses in the skin and joint help to set the stage for subsequent arthritis development.

Although the strain of *B. burgdorferi* is an important factor in stimulating excessive immune responses during infection, culture and PCR results have been uniformly negative in synovial tissue obtained from patients with Lyme arthritis months to years after antibiotic therapy[24], hence the use of the term post-infectious Lyme arthritis[10]. Moreover, after oral and intravenous antibiotic therapy, re-emergence of infection has not been noted while patients are being treated with DMARDs[7]. However, spirochaetal remnants can persist during the post-infectious period[82]. A 2019 study found that *B. burgdorferi* peptidoglycan, a predominant cell wall component, is detectible in post-infectious Lyme arthritis synovial fluid up to several years after antibiotic treatment[97]. *B. burgdorferi* peptidoglycan is shed during cell replication and is uniquely difficult to clear[97]. Thus, uncleared peptidoglycan might be an important factor in promoting innate immune responses in the post-infectious period in genetically predisposed individuals.

Host factors associated with excessive immune responses. Transcriptomic analysis of synovia from patients with post-infectious Lyme arthritis shows prominent gene signatures associated with innate immune responses, antigen presentation and cell-mediated immune activation[10]. As in erythema migrans skin lesions, a large number of interferon-response genes are highly or moderately enriched in synovial tissue from all patients with post-infectious Lyme arthritis[10]. Importantly, this high interferon signature correlates inversely with tissue repair response gene signatures[10], indicating that high concentrations of interferons impair wound healing. Supporting the transcriptomic data, large percentages of T cells and natural killer (NK) cells isolated from synovial tissue or synovial fluid test positive for IFNγ by intracellular cytokine staining[10,70,71]. Thus, in patients with post-infectious Lyme arthritis, high numbers of IFNγ-producing lymphocytes present in synovial tissue might prevent appropriate repair of tissue damaged by *B. burgdorferi* infection, blocking the return to tissue homeostasis even after the bacteria themselves are cleared[9].

Both host and spirochaetal genetic factors can contribute to this exceptionally high IFNγ response. In individuals with a *TLR1* single nucleotide polymorphism (1805GG) that affects the recognition of PAMPs by innate immune cells, infection with OspC type A (RST1) strains of *B. burgdorferi* leads to exceptionally high levels of IFNγ and signal transducer and activator of transcription 1 (STAT1)-dependent cytokines in joints[41]. In an initial study, this *TLR1* polymorphism was present in 24 of 47 European Americans (51%), but in only 2 of 24 African Americans (8%) and 0 of 390 Vietnamese individuals[98].

Among patients with Lyme arthritis in New England, the 18055GG polymorphism was present in 35 of 76 patients with antibiotic-responsive arthritis (47%) compared with 62 of 101 patients with post-infectious arthritis (62%)[41]. This polymorphism is within the portion of the gene that encodes the transmembrane region of TLR1 and might impair cell surface localization and

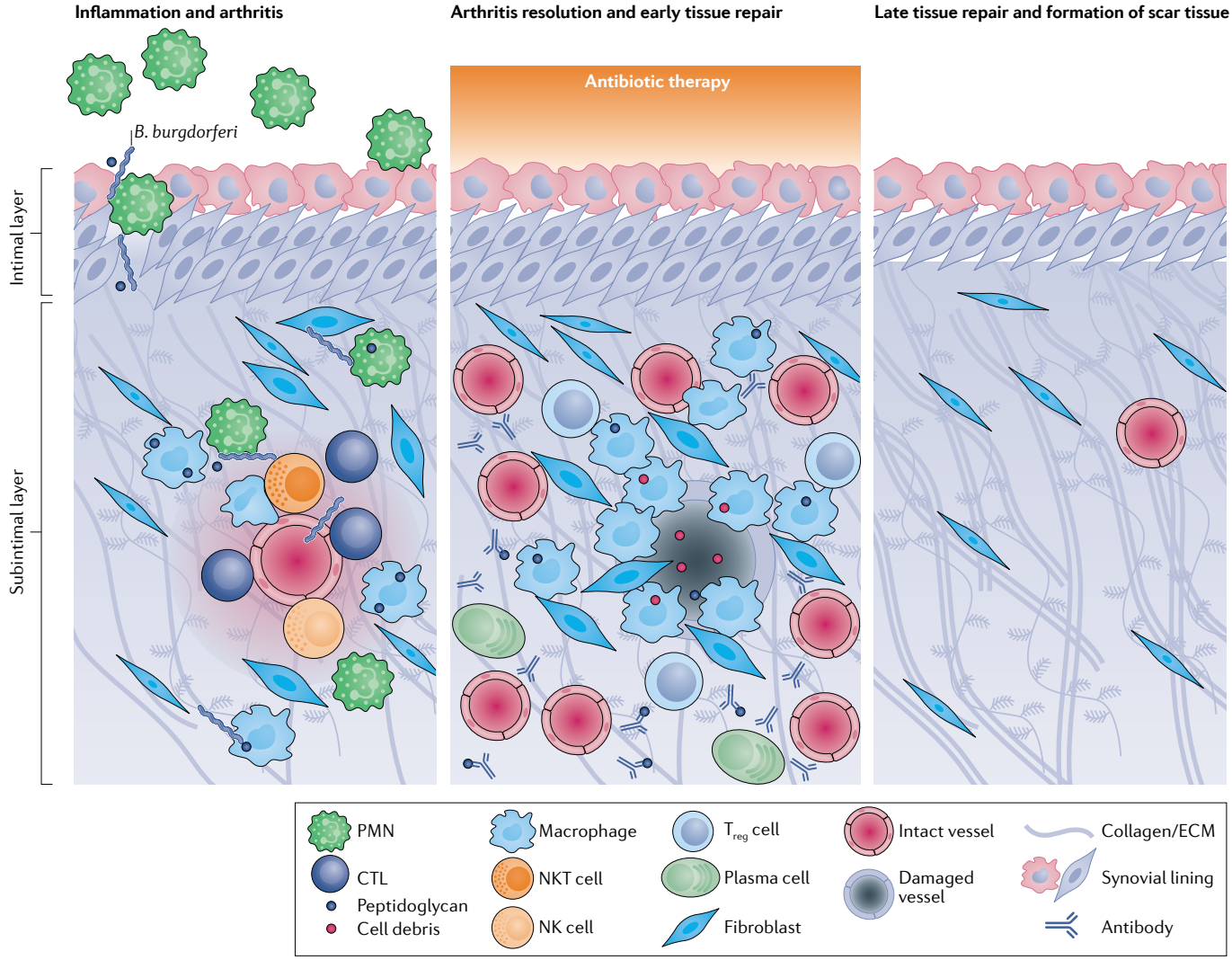


Fig. 4 | **Stages of arthritis and proposed tissue repair in antibiotic-responsive Lyme arthritis.** Immune responses to *Borrelia burgdorferi* and *B. burgdorferi* peptidoglycan by endothelial cells, fibroblasts, lymphocytes (such as natural killer (NK cells), natural killer T (NKT) cells and cytotoxic T lymphocytes (CTLs)) and myeloid cells (such as macrophages and polymorphonuclear cells (PMNs)) in the synovium trigger localized inflammation, tissue damage and arthritis. Antibiotic therapy is given during acute joint infection and inflammation, facilitating arthritis resolution and the initiation of early tissue repair responses. These responses are dominated by pro-angiogenic factors and the activation of tissue-repairing macrophages and fibroblasts, which remove bacterial debris and damaged cells from the damaged microvasculature, extracellular matrix (ECM) and fibrotic tissue. Other immune cells such as regulatory T (T_{reg}) cells and plasma cells might also be present during arthritis resolution. Over several months, synovial fibroblasts differentiate into myofibroblasts and lay down collagen and form scar tissue, leading to full recovery.

downstream NF-κB signalling in response to the TLR1 and TLR2 ligand Pam3CSK4 (REF.[99]). Paradoxically, patients with Lyme arthritis who have this polymorphism have exceptionally high amounts of IFNγ, as well as STAT1-regulated and NF-κB-regulated pro-inflammatory immune mediators in their joints, but unremarkable amounts of IL-10 (REF.[41]). This polymorphism is hypothesized to be associated with excessive inflammatory responses to *B. burgdorferi* because it results in a deficiency in Janus kinase–STAT, NF-κB and mitogen-activated protein kinase feedback loop inhibitors, such as the regulatory microRNA miR-146a[100] and the anti-inflammatory cytokine IL-10 (REF.[101]).

Antigen presentation of certain *B. burgdorferi* peptides by specific HLA-DR molecules might also lead to high IFNγ concentrations. In one study, 7 of 14 HLA-DRB molecules — HLA-DRB1*04:01 in particular — bound to a peptide from *B. burgdorferi* OspA ($OspA_{163–175}$), whereas the other seven HLA-DRB molecules (including HLA-DRB1*11:01) did not[102]. Among patients with post-infectious Lyme arthritis, 56 of 71 (79%) had at least one HLA-DRB molecule that bound *B. burgdorferi* $OspA_{163–175}$, compared with 23 of 50 patients with antibiotic-responsive Lyme arthritis (46%)[102]. As mentioned previously, immune responses to OspA are found primarily in the highly inflammatory joint milieu of patients with Lyme arthritis in North America[67]. In transgenic mice, those expressing the human HLA-DR4 allele had higher IFNγ responses and lower titres of anti-*Borrelia* antibodies than mice expressing the human HLA-DR11 allele, which had higher anti-*Borrelia* antibody titres but lower IFNγ

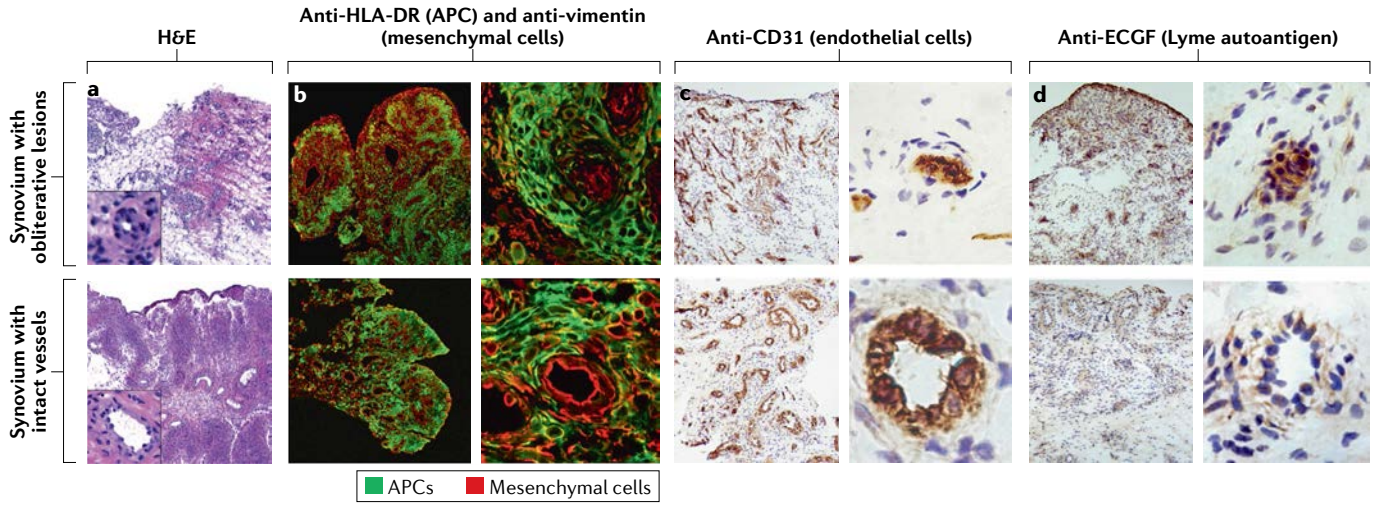


Fig. 5 | **Microvascular involvement in the synovial lesion of post-infectious Lyme arthritis. a** | Haematoxylin and eosin (H&E)-stained sections of synovial tissue from representative patients with post-infectious Lyme arthritis with varying degrees of inflammation, fibrosis and vascular damage (insets show enlarged images of vessels). **b** | Synovial tissue sections stained with fluorescently labelled anti-HLA-DR (green) and anti-vimentin (red) antibodies, showing localization of antigen-presenting cells (APCs) and mesenchymal cells (such as fibroblasts and endothelial cells), respectively. **c** | Around half of patients with post-infectious Lyme arthritis have evidence of vascular damage, including obliterative microvascular lesions, as shown by staining with the endothelial cell marker CD31. **d** | Obliterative microvascular lesions are also enriched with the Lyme disease autoantigen endothelial cell growth factor (ECGF). In panels **b–d**, the panels on the left show the general architecture of the synovial tissue at low magnification, and the panels on the right show single blood vessels from the same sections at high magnification. Parts **c** and **d** adapted with permission from REF.[12], Wiley. © 2014 by the American College of Rheumatology.

responses[103]. Thus, presentation of $OspA_{163–175}$ by certain HLA-DR molecules is associated with high IFNγ concentrations and with post-infectious Lyme arthritis.

Two NF-κB-regulated microRNAs, miR-146a and miR-155, which have been associated with a number of inflammatory joint diseases[104], including RA, are also prominent in Lyme arthritis[9,104]. Experiments in mice have shown that these two microRNAs fine-tune the amplitude of inflammatory responses to *B. burgdorferi* to balance host defence and tissue damage in Lyme arthritis[100,105]. miRNA-146a functions as a feedback inhibitor of NF-κB signalling, and mice lacking miR-146a develop more severe Lyme arthritis than wild-type mice, despite having fewer bacteria in their joints[100]. By contrast, miR-155 enhances acute inflammation by potentiating NF-κB and STAT1 signal transduction[105]. In humans with post-infectious Lyme arthritis, miR-155 is particularly enriched in synovial fluid and correlates positively with arthritis duration, but is below or near the limit of detection in patients with antibiotic-responsive Lyme arthritis[9]. Concentrations of both miR-146a and miR-155 remain persistently elevated in synovial tissue and fluid from patients with post-infectious Lyme arthritis, providing further evidence of chronic NF-κB activation in the inflamed synovium[9].

Several other types of immune regulation imbalance can result in high concentrations of IFNγ. In patients with post-infectious Lyme arthritis, a high percentage of $CD4^+CD25^+$ T cells, which are ordinarily regulatory T (T_{reg}) cells, become effector cells that secrete large amounts of IFNγ, thereby skewing the T_H1 cell–T_{reg} cell balance[71,106]. By contrast, in patients with antibiotic-responsive Lyme arthritis, T_{reg} cells secrete large amounts of anti-inflammatory IL-10 and negligible amounts of IFNγ[71]. IL-10 produced by T_{reg} cells and other immune cells is critical to limiting NF-κB and STAT1 signalling, which is necessary for the development of innate and adaptive immune responses to *B. burgdorferi*. In mice, T_{reg} cells are an important source of IL-10, and T_{reg} cell-depleted mice develop more severe Lyme arthritis than immunocompetent mice[107]. Similarly, HLA-DR4 transgenic mice that lack the co-stimulatory molecule CD28, which greatly reduces the number of T_{reg} cells, also develop persistent arthritis after spirochaetes have been killed[108].

The critical role of the balance between IFNγ and IL-10 in post-infectious Lyme arthritis is underscored by studies in IL-10 knockout (*Il10*$^{-/-}$) mice. Similar to patients with post-infectious Lyme arthritis, these mice have greatly increased innate and adaptive immune responses to infection with *B. burgdorferi*, resulting in severe arthritis despite having low to undetectable amounts of bacteria in inflamed joint tissues[105,109,110]. Longitudinal transcriptomic analysis of joints from infected B6 *Il10*$^{-/-}$ mice show marked upregulation in the transcription of IFNγ-stimulated genes and the pro-inflammatory microRNA miR-155, with a corresponding downregulation of mRNA transcripts and microRNAs involved in tissue repair and response to wounding, similar to human post-infectious Lyme arthritis[60,105]. This transcriptomic profile is probably the result of the impaired ability of IL-10 to regulate STAT1 activation in these mice[105]. The dysregulated IFNγ response in these mice is caused by TLR2-mediated bystander activation of both $CD4^+$ and $CD8^+$ T cells[110]. Importantly, spirochaetes are no longer detectable in synovial tissue at 16 weeks post-infection, and depletion of $CD4^+$ and $CD8^+$ T cells in B6 *Il10*$^{-/-}$ mice results

in less severe Lyme arthritis[110], demonstrating that a dysregulated T_H1 cell response is arthritogenic. In the human disease, bystander activation of T cells could also cause a break in immune tolerance, providing a critical intermediate step on the path towards autoimmunity.

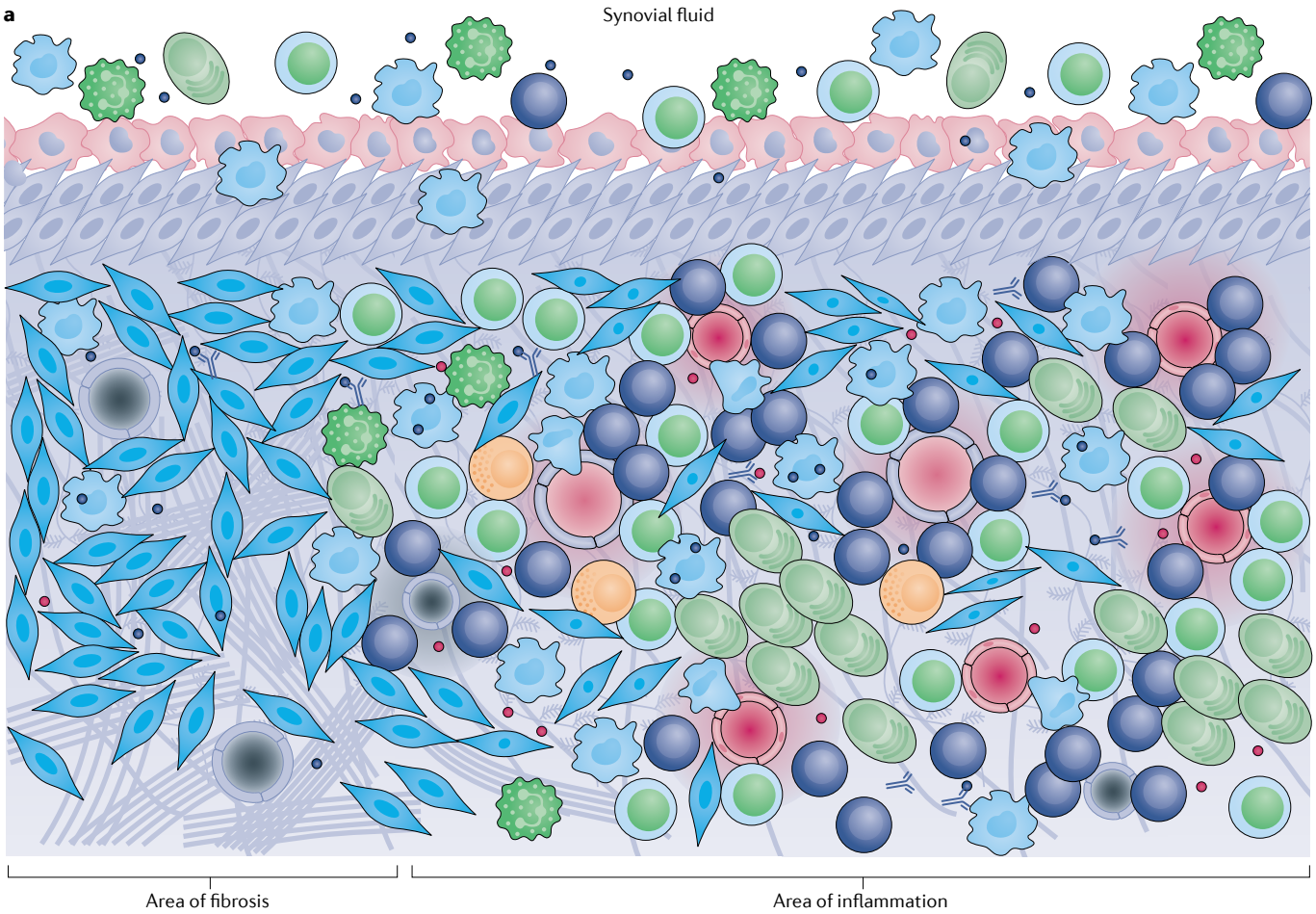
Consequences of excessive, dysregulated pro-inflammatory responses. A model of the cellular architecture in the synovial lesion of post-infectious Lyme arthritis and the proposed roles of the main immune cells, peptidoglycan, autoantigens and IFNγ responses are summarized in FIG. 6. Immune dysregulation during microbial infection, particularly pathogenic T_H17 cell responses, can trigger autoimmunity[1,111]. In a study in which HLA-DR-presented peptides were eluted from synovia of patients with post-infectious Lyme arthritis, four immunogenic peptides were identified that were derived from self-proteins[112], including endothelial cell growth factor[11], annexin A2 (REF.[13]), apolipoprotein B100 (REF.[14]) and matrix metalloproteinase 10 (REF.[15]). Similarly, HLA-DR molecules expressed on *B. burgdorferi*-stimulated dendritic cells obtained from healthy individuals presented peptides derived from all of these self-proteins, with the exception of matrix metalloproteinase 10 (REF.[113]). HLA-DR presentation of these self-proteins might reflect previous damage to endothelial cells and/or to the extracellular matrix by spirochaete invasion[12]. Moreover, autoantibodies to these self-proteins can sometimes be found in patients with early-stage *B. burgdorferi* infection, Lyme carditis, neuroborreliosis or antibiotic-responsive Lyme arthritis[11–16], albeit usually without T cell responses. Thus, initial autoimmune responses might be triggered by increased T_H17 cell responses during early infection, but T cell responses to autoantigens are not usually apparent at that time. By contrast, both T cell and B cell responses to these autoantigens are often found in patients with post-infectious Lyme arthritis, suggestive of further maturation of the immune response[11,13–15]. Amounts of T_H17 cell-associated cytokines, particularly IL-23, that correlate with anti-*B. burgdorferi* antibody titres in early disease, correlate strongly with autoantibody titres in post-infectious Lyme arthritis[43], suggesting a shift from protective anti-*Borrelia* responses to autoreactive immunity.

Although the pathogenic nature of Lyme autoantibodies has not yet been delineated, IgG4 Lyme disease autoantibody titres correlate with the magnitude of obliterative microvascular lesions and fibrosis in synovial tissue[72]. Curiously, a 2020 study using humanized mice indicated that loss of the inhibitory Fc receptor Fcγ-receptor IIb, which binds to IgG4 immune complexes, might contribute to infection-induced autoantibody responses in Lyme arthritis[114]. Although IgG4 responses are typically considered to be anti-inflammatory, clinical data imply a pathogenic role for these autoantibodies.

The timeline for the development of putative pathogenic autoantibodies in Lyme arthritis could have parallels with RA. In RA, anti-citrullinated protein antibodies (ACPAs) typically develop years before inflammatory arthritis manifests[115]. Prior to arthritis development, ACPAs can undergo epitope spreading, which, together with the appearance of innate immune mediators including IL-1, IL-6 and TNF, can lead to the development of clinical arthritis[116]. Moreover, in a study from the Netherlands, IgG4 ACPAs were noted in 104 of 373 patients with RA (28%), and anti-carbamylated protein antibodies were found in 209 of the 373 patients (56%)[117]. Similarly, in a study from France, 35 of 141 patients with RA (25%) had IgG4 antibodies that recognized citrullinated fibrinogen, a common target of ACPAs[118]. In a Chinese study, patients with IgG4 antibodies were more difficult to treat successfully with DMARDs[119], suggesting that serum IgG4 autoantibodies might define a specific clinical phenotype associated with more severe disease.

Cytotoxic immune responses had not previously been thought to be involved in synovial pathology, yet a 2019 transcriptomic analysis of synovial tissue from patients with post-infectious Lyme arthritis or RA showed marked upregulation of genes associated with cell-mediated cytotoxicity[10]. Cells associated with cytotoxic potential include $CD8^+$ T cells, NK cells and, less commonly, γδ T cells[120]. However, each of these cell types can also secrete cytokines, which has been thought to be their primary function in chronic inflammatory arthritides. During spirochaete dissemination and in the infectious phase of Lyme arthritis, these cells probably function as part of a complex web of inflammatory responses that are important for spirochaetal killing. For example, innate-like cytotoxic lymphocytes, such as NK cells and NKT cells, could initially have a role in trapping spirochaetes in obliterative microvascular lesions[35,84,121]. However, the role of cells with cytotoxic potential and the cellular targets of such responses are yet to be clarified in post-infectious Lyme arthritis and in other chronic inflammatory arthritides.

Synovial fibroblasts, the most common cells in the synovial lesion, function as important immune effector cells in inflamed synovial tissue[70]. When stimulated with IFNγ and *B. burgdorferi* in vitro, primary synovial fibroblasts derived from patients with post-infectious Lyme arthritis secrete large amounts of NF-κB-regulated and STAT1-regulated cytokines, chemokines and pattern recognition receptors[70]. These cells also secrete T_H1 cell-promoting immune mediators and proteins involved in antigen presentation to T cells, including HLA-DR molecules and co-stimulatory molecules. These data[70] suggest that synovial fibroblasts function as non-professional antigen-presenting cells and might contribute to T cell reactivity to HLA-DR-presented Lyme autoantigens. Furthermore, when synovial fibroblasts obtained from patients with post-infectious Lyme arthritis were grown in culture, the gene signature of IFNγ-stimulated cells in vitro was quite similar to that found in vivo in the synovia from such patients[10], confirming a central role for IFNγ and synovial fibroblasts in post-infectious Lyme arthritis. Thus, immune dysregulation in post-infectious Lyme arthritis leads to pro-inflammatory and tumour-like proliferative responses by synovial fibroblasts, rather than the wound healing and appropriate tissue repair responses that are probably orchestrated by these cells in antibiotic-responsive Lyme arthritis following antibiotic



therapy. In RA, genetic, epigenetic and phenotypic changes in synovial fibroblasts are likely to contribute to inflammatory synovitis and the development of autoimmunity[122–124]. Taken together, these studies indicate a central role for synovial fibroblasts in the pathogenesis of a number of forms of chronic inflammatory arthritis, including Lyme arthritis and RA[125].

Linking infection and autoimmunity

The main message from post-infectious Lyme arthritis for other forms of chronic autoimmune or autoinflammatory arthritis is that this complex immune response can begin with an antimicrobial immune response, and is shaped by complex interactions between pathogen and host. Such an immune response could be triggered by an invading pathogen, as is the case in Lyme arthritis, or by commensals in the host microbiome. In RA, evidence is emerging that bacteria-induced inflammation at mucosal sites in the periodontium, lung or bowel might trigger or enhance autoimmunity and joint disease in predisposed individuals[126]. For example, the periodontal pathogen *Porphyromonas gingivalis* is associated with RA[127,128], as is the gut commensal *Prevotella copri*[129,130]. In ankylosing spondylitis and Crohn's disease-associated spondyloarthritis, strains of *Escherichia coli* or *Prevotella* spp. that adhere to the

Fig. 6 | **Cellular architecture of the post-infectious Lyme arthritis synovial lesion.** **a** | The post-infectious Lyme arthritis synovial lesion is characterized by widespread fibrosis and areas of marked inflammation. Fibrotic areas contain large numbers of synovial fibroblasts, obliterative microvascular lesions, disordered collagen and other extracellular matrix (ECM) proteins. Areas of inflammation are found primarily in highly vascularized synovial intimal and subintimal layers that can contain obliterative microvascular lesions and/or intact vessels. Immune cells, such as macrophages, $CD4^+$ T helper (T_H) cells, cytotoxic T lymphocytes (CTLs; mostly $CD8^+$ T cells with a few γδ T cells), natural killer (NK) cells, and large numbers of antibody-producing plasma cells, are found primarily in vascularized areas, but can be found throughout the tissue. Vascularized areas also contain many HLA-DR-expressing synovial fibroblasts; however, they tend to have less fibrotic tissue. Bacterial peptidoglycan is present in synovial fluid and might additionally be present in inflamed tissue, along with degraded cellular and ECM debris. Only a few polymorphonuclear cells (PMNs) are present in post-infectious Lyme arthritis synovial tissue, but more are present in synovial fluid. **b** | In this panel, a hypothesis is developed regarding the roles of important cells and immune mediators in autoimmune-mediated damage to the endothelium in inflamed synovia. Large amounts of IFNγ produced by T_H cells, CTLs and NK cells induce potent responses by HLA-DR-expressing synovial fibroblasts and macrophages, which upregulate proteins associated with antigen presentation, T cell activation and inflammation. *Borrelia burgdorferi* peptidoglycan and cell debris might amplify these responses. Synovial fibroblasts and macrophages present MHC class II-restricted peptides derived from Lyme autoantigens, which are abundant in synovial tissue, to autoreactive T_H cells, perpetuating IFNγ responses in the tissue. Endothelial cells, which were damaged during infection with *B. burgdorferi*, can be targeted for killing by CTLs, either through direct CTL-mediated killing or through autoantibody-dependent cell-mediated cytotoxicity (ADCC), or both. Further damage to the microvasculature releases more autoantigens and debris, leading to a feedback loop of chronic inflammation and tissue damage.

bowel mucosa have been implicated in the pathogenesis of joint disease[131,132]. Similarly, in psoriatic arthritis, skin flora might have a role in pathogenesis[133,134]. As with other arthritides, changes in host microflora could also affect the pathogenesis of Lyme arthritis. *B. burgdorferi* modulate the host microbiomes of their tick vectors to facilitate colonization[135] and this process could also occur during tick-to-mammal transmission, disrupting the normal skin flora and altering the local immune environment.

Interestingly, patients have been reported to develop systemic autoimmune diseases, including RA and spondyloarthritis, within months of having Lyme disease[136]. Although these occurrences could be coincidental, we speculate that latent autoimmunity might be induced non-specifically by the adjuvant effects of infection, or alternatively, that autoimmune-promoting conditions that develop during Lyme disease might trigger other systemic autoimmune diseases. The Lyme arthritis story underscores the importance of research into the potential role of specific infectious agents in various forms of chronic inflammatory arthritis, research that is hoped to provide breakthroughs in approaches to diagnosis and treatment.

Conclusions

After *B. burgdorferi* infection of the skin, early dissemination of spirochaetes to joints accompanied by dysregulation of innate immune responses (particularly type I interferon responses), might promote subsequent arthritis development. Months later, clinical arthritis develops within the context of an expanded adaptive immune response to the spirochaete. Rather than the arthritis resolving following antibiotic therapy, a small percentage of patients have persistent synovitis that can worsen in the post-antibiotic period. In these patients, the central pathogenetic feature is an excessive, dysregulated pro-inflammatory immune response characterized by exceptionally high amounts of IFNγ coupled with inadequate amounts of the anti-inflammatory cytokine IL-10. The consequences of this dysregulated response in synovia include chronic vascular damage and impaired tissue repair, autoimmune T cell and B cell responses, and tumour-like fibroblast proliferation and fibrosis. These histological characteristics are similar to those seen in other chronic inflammatory arthritides, including RA. Thus, post-infectious Lyme arthritis might serve as a model to aid our understanding of other forms of arthritis in which an infectious agent triggers or shapes the complex interactions between pathogen and host immune responses, leading to joint inflammation. However, important gaps remain in understanding the link between infection and autoimmunity in Lyme arthritis. Future research should focus on determining the initial steps in the break in immune tolerance during infection, on elucidating the role of *B. burgdorferi* peptidoglycan or other spirochaetal remnants in the pathogenesis of Lyme arthritis, on studying antibody specificities and function, and on identifying how autoimmune responses seem to evolve over time to become increasingly T cell dependent and more pathogenic.

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Genome editing to define the function of risk loci and variants in rheumatic disease

Yuriy Baglaenko[1,2,3], *Dana Macfarlane*[1,2], *Alexander Marson*[4,5,6,7,8,9,10], *Peter A. Nigrovic*[2,11] *and Soumya Raychaudhuri*[1,2,3,12,13]✉

Abstract | Discoveries in human genetic studies have revolutionized our understanding of complex rheumatic and autoimmune diseases, including the identification of hundreds of genetic loci and single nucleotide polymorphisms that potentially predispose individuals to disease. However, in most cases, the exact disease-causing variants and their mechanisms of action remain unresolved. Functional follow-up of these findings is most challenging for genomic variants that are in non-coding genomic regions, where the large majority of common disease-associated variants are located, and/or that probably affect disease progression via cell type-specific gene regulation. To deliver on the therapeutic promise of human genetic studies, defining the mechanisms of action of these alleles is essential. Genome editing technology, such as CRISPR–Cas, has created a vast toolbox for targeted genetic and epigenetic modifications that presents unprecedented opportunities to decipher disease-causing loci, genes and variants in autoimmunity. In this Review, we discuss the past 5–10 years of progress in resolving the mechanisms underlying rheumatic disease-associated alleles, with an emphasis on how genomic editing techniques can enable targeted dissection and mechanistic studies of causal autoimmune risk variants.

Rheumatic diseases are highly complex and affect up to 7–10% of the general global population[1]. Characterized by chronic inflammation, intermittent flares and progressive tissue damage, these diseases can result in morbidity and mortality if left untreated. Some of the most common rheumatic diseases are caused by issues within the immune system, leading to autoimmunity and deterioration of the body. Although the aetiology differs between disorders, the risk of developing a rheumatic disorder is increased among those individuals with affected relatives; indeed, studies of monozygotic and dizygotic twins show that rheumatic diseases share a strong genetic component[2].

Defining the genetic variants that increase the risk of rheumatic disease and the genes that the disease-associated variants influence will improve our ability to both diagnose and treat these complex conditions. For example, characterizing drug targets on the basis of human genetic data can increase the likelihood of developing a successful therapeutic agent[3–6]. In this regard, large-scale collaborative efforts over the past decade have conducted genome-wide association studies (GWAS) to identify hundreds of loci associated with autoimmune disorders[7]. These studies use genotyping information from millions of single nucleotide polymorphisms (SNPs) in thousands of affected individuals and thousands of unaffected individuals to pinpoint regions associated with disease. The findings of a particular SNP allele that is more (or less) frequent in individuals with a particular disease than in healthy individuals indicates that the SNP, or a nearby variant in tight linkage with that genomic location, contributes to disease risk (FIG. 1).

Despite the plethora of information gained from GWAS, the overall goal of assigning genetic mechanisms remains unexpectedly elusive. To achieve this goal, we need to ascribe causality for SNPs by identifying the affected genes and the downstream effects of the gene modulation on immune cell function. Experimental efforts to define allelic function over the past decade have been restricted largely to molecular biology assays and mouse models of disease. Both strategies are useful but have various limitations[8–13]. In vitro cellular assays might not retain the relevant chromatin context (that is, the chromatin organization present in the local tissue environment) and therefore cannot fully recapitulate disease, leading to technical noise. Mouse models are unable to recapitulate the full pathophysiology of human disease, including species-specific gene regulatory mechanisms. Fortunately, advances in genome editing and CRISPR–Cas technologies — the discovery of which

✉e-mail: soumya@broadinstitute.org

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Key points

- Hundreds of autoimmune risk loci have been discovered in coding and non-coding regions of the genome; however, their function and the causal alleles functioning within these loci have been difficult to discern.
- Advances in genomic editing have made it possible to quickly and effectively investigate autoimmune disease-associated loci and variants using a number of approaches in both cell lines and primary cells.
- CRISPR–Cas genomic editing can be used to induce insertions and deletions, correct precise mutations and induce epigenetic changes to investigate loci and variants associated with rheumatic diseases.
- CRISPR–Cas screening approaches are effective tools for whole-genome investigation of autoimmune disease-related genes and detailed resolution of autoimmune risk regions.
- Resolving the heterogeneity of cell types in rheumatic disorders with unbiased single-cell technologies is critical to understanding the genetics of disease.

led to Jennifer Doudna and Emmanuelle Charpentier being awarded the 2020 Nobel Prize in Chemistry — have opened up new opportunities and techniques for investigating and validating the genetics of rheumatic diseases.

At its root, CRISPR–Cas technology exploits the programmable specificity of RNA-guided nucleases[14,15]. Unlike previous iterations of programmable protein nucleases, CRISPR–Cas systems are guided to their target with easily tunable guide RNAs (gRNAs). Different versions of Cas proteins exist, with different functions and specifications. The most commonly used versions include Cas9, an RNA-guided DNA endonuclease, Cas12a, another RNA-guided DNA endonuclease that is more sensitive to A-T rich regions than Cas9, and Cas13, an RNA-guided RNA endonuclease. Countless other Cas proteins exist in nature and have been studied. The ease of specifying target sites practically anywhere in the genome along with the rapid democratization of these tools by the scientific community has made CRISPR–Cas easily accessible and highly versatile. Some applications of this technology include the deletion of particular genes, targeted homology-directed repair (HDR), precise nucleotide conversions and recruitment of transcriptional repressors and activators[16,17]. The basic principles of CRISPR–Cas editing have been reviewed in depth elsewhere[16].

Author addresses

[1]Center for Data Sciences, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA.
[2]Department of Medicine, Brigham and Women's Hospital, Boston, MA, USA.
[3]Program in Medical and Population Genetics, Broad Institute, Cambridge, MA, USA.
[4]Gladstone Institutes, San Francisco, CA, USA.
[5]Department of Medicine, University of California, San Francisco, CA, USA.
[6]Department of Microbiology and Immunology, University of California, San Francisco, CA, USA.
[7]Innovative Genomics Institute, University of California, Berkeley, CA, USA.
[8]Rosalind Russell/Ephraim P. Engleman Rheumatology Research Center, University of California, San Francisco, San Francisco, CA, USA.
[9]Parker Institute for Cancer Immunotherapy, San Francisco, CA, USA.
[10]Chan Zuckerberg Biohub, San Francisco, CA, USA.
[11]Division of Immunology, Boston Children's Hospital, Department of Pediatrics, Harvard Medical School, Boston, MA, USA.
[12]Department of Biomedical Informatics, Harvard Medical School, Boston, MA, USA.
[13]Faculty of Medical and Human Sciences, University of Manchester, Manchester, UK.

In this Review, we provide a detailed look at using genome editing to study the genetics of rheumatic diseases, with a focus on autoimmune rheumatic diseases. We discuss how computational and genomic editing approaches can be used to discover loci and genes associated with autoimmune conditions, identify regulatory regions, define causal variants and characterize critical cell types and cell states.

Dissecting risk loci

Traditional approaches. Prior to the widespread utilization of GWAS, traditional approaches to understanding the genetics of rheumatic diseases focused on the investigation of candidate genes thought likely to alter immune function. Most of these candidate genes emerged from model systems, such as painstakingly created gene knockout mice. These models implicated a critical role for various protein-coding genes such as *Stat4*, *Bach2*, *Ptpn22*, *Zap70*, *Foxp3* and *Tnfaip3* (encoding A20), to name but a few, in the development of, or protection against, autoimmunity[18–23]. Some examples include *Tfnaip3* knockout animals, which die prematurely because of uncontrolled and widespread inflammation, *Ptpn22* knockout mice, which have increased levels of T cell activation, and *Stat4* knockout mice, which are protected from arthritis induction[18,20]. Other subtler mouse models used cross-breeding of inbred strains to create congenic animals. In these studies, animal strains that were prone to the spontaneous development of autoimmunity were carefully bred with other strains. The resulting progeny were then genotyped to identify regions associated with the autoimmune phenotype, as has been done with New Zealand Black and White (NZB/W) mice in the investigation of systemic lupus erythematosus and non-obese diabetic (NOD) mice in the investigation of type 1 diabetes[24,25]. Although these early mouse studies were indispensable in discovering regions and genes that might contribute to disease, investigators recognized that not all findings translate to human disease.

To gain a comprehensive understanding of the human genetics of rheumatic disease, researchers have turned to large-scale GWAS to pinpoint variants and loci associated with autoimmunity. For example, an analysis of patients with ankylosing spondylitis, Crohn's disease, psoriasis, primary sclerosing cholangitis or ulcerative colitis revealed extensive pleiotropy (shared genetic risk) among these diseases and identified 244 independent disease-associated loci[26]. Another meta-analysis of ten paediatric autoimmune disorders identified 27 disease-associated loci of genome-wide statistical significance that were enriched for loci implicated in T helper cell signalling[27]. Finally, in a study of 42 disorders that used data from the Biobank Japan project, researchers discovered 276 risk loci across 27 diseases, including some loci unique to the East Asian population[7]. Taken together, GWAS in rheumatic diseases have identified hundreds of loci that might be linked to disease and provide important genetic clues as to the mechanism of disease progression. However, despite the plethora of identified signals, associations from GWAS rarely identify the genes that are causal[28].

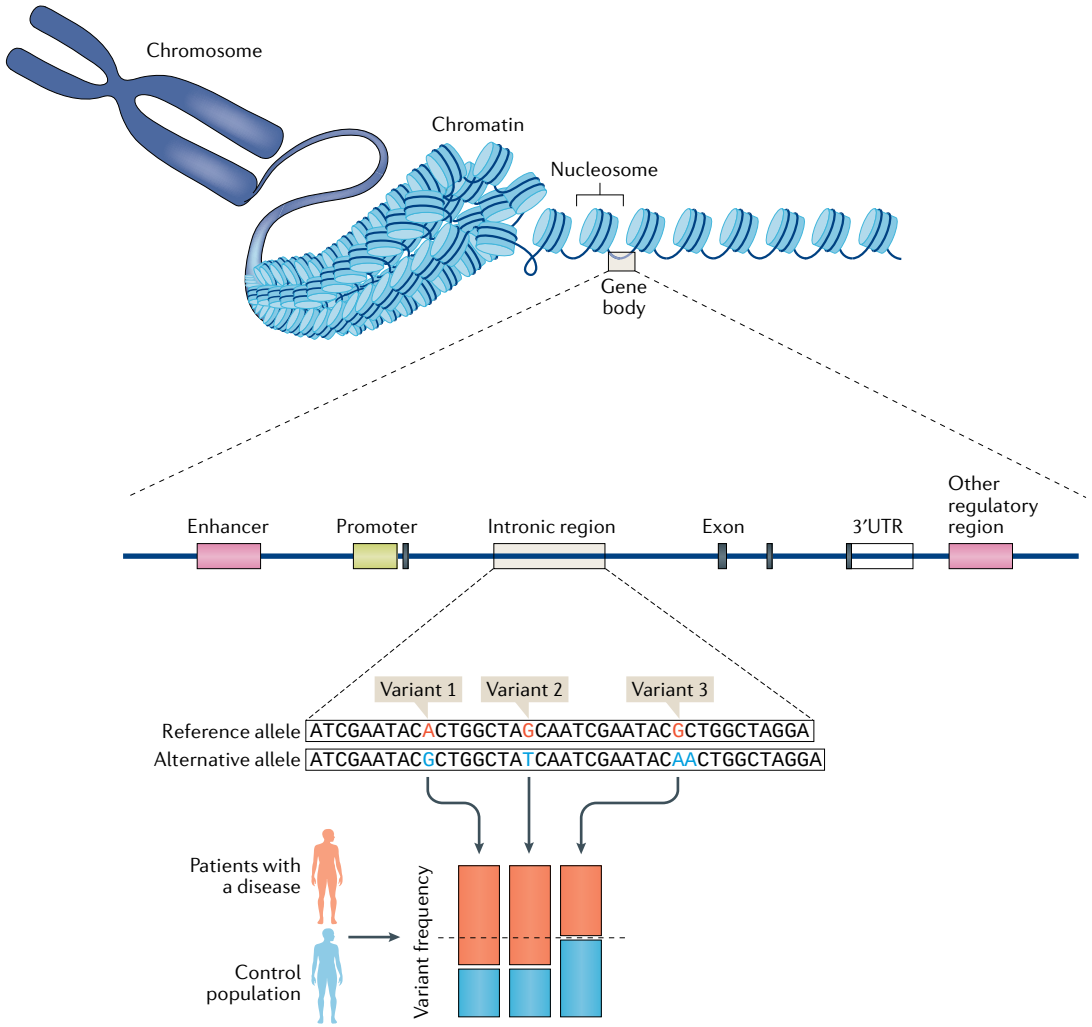

Fig. 1 | **Studying the genetics of rheumatic diseases.** The human genome is made up of billions of pairs of DNA and harbours thousands of protein-coding genes and well as other regulatory non-coding regions. DNA sequences are wrapped around histones, forming nucleosomes, for easy storage; an array of nucleosome form chromatin, which chain together to form chromosomes. Chromatin accessibility varies across the genome (depending on whether the chromatin has an open or closed conformation), governing the transcription and expression of the contained genes. Genes also have promoter, regulator and enhancer elements that control their expression. Particular variations or single nucleotide polymorphisms in DNA, located in both coding and non-coding regions, are linked to the development of rheumatic diseases. Genome-wide association studies identify potential causal variants and loci by comparing the frequency of particular variants in patients with a disease with that in a control population (such as in healthy individuals). In the example shown, variants 1 and 2, but not variant 3, are enriched in patients with the disease (implicating these variants in disease); here, two variants are found in linkage disequilibrium, meaning that they are generally inherited together as a block.

Additionally, rare (minor allele frequency <1–5%) variants that affect gene expression or function might also be missed by GWAS, as GWAS mainly analyse common variants[29]. Genomic editing can complement our discovery of genes involved in autoimmunity using both unbiased genome-wide knockout screens as well as targeted editing to elucidate function.

Genome editing of protein-coding regions. To test the function of every known protein-coding gene and its possible contribution to autoimmune pathways in an unbiased fashion, researchers have employed CRISPR–Cas genome-wide screening assays. In these types of experiments, a pooled library of thousands of gRNAs is used to induce gene knockouts via the generation of indels (insertions and deletions), gene silencing with CRISPR interference (CRISPRi), or gene activation with CRISPR activation (CRISPRa) (FIG. 2). Libraries are virally packaged and transduced into cells followed by perturbation such as T cell receptor (TCR) stimulation, immune cell differentiation or drug treatment. After cell selection on the basis of cell surface expression, survival or another carefully chosen response condition, genomic DNA is extracted from the selected and non-selected (or the input) cell populations to sequence the integrated gRNAs and identify the targets that affect the cellular phenotype. This unbiased screening approach has the potential to discover and investigate the function of genes related to important immune processes such as T cell activation and proliferation, potentially identifying

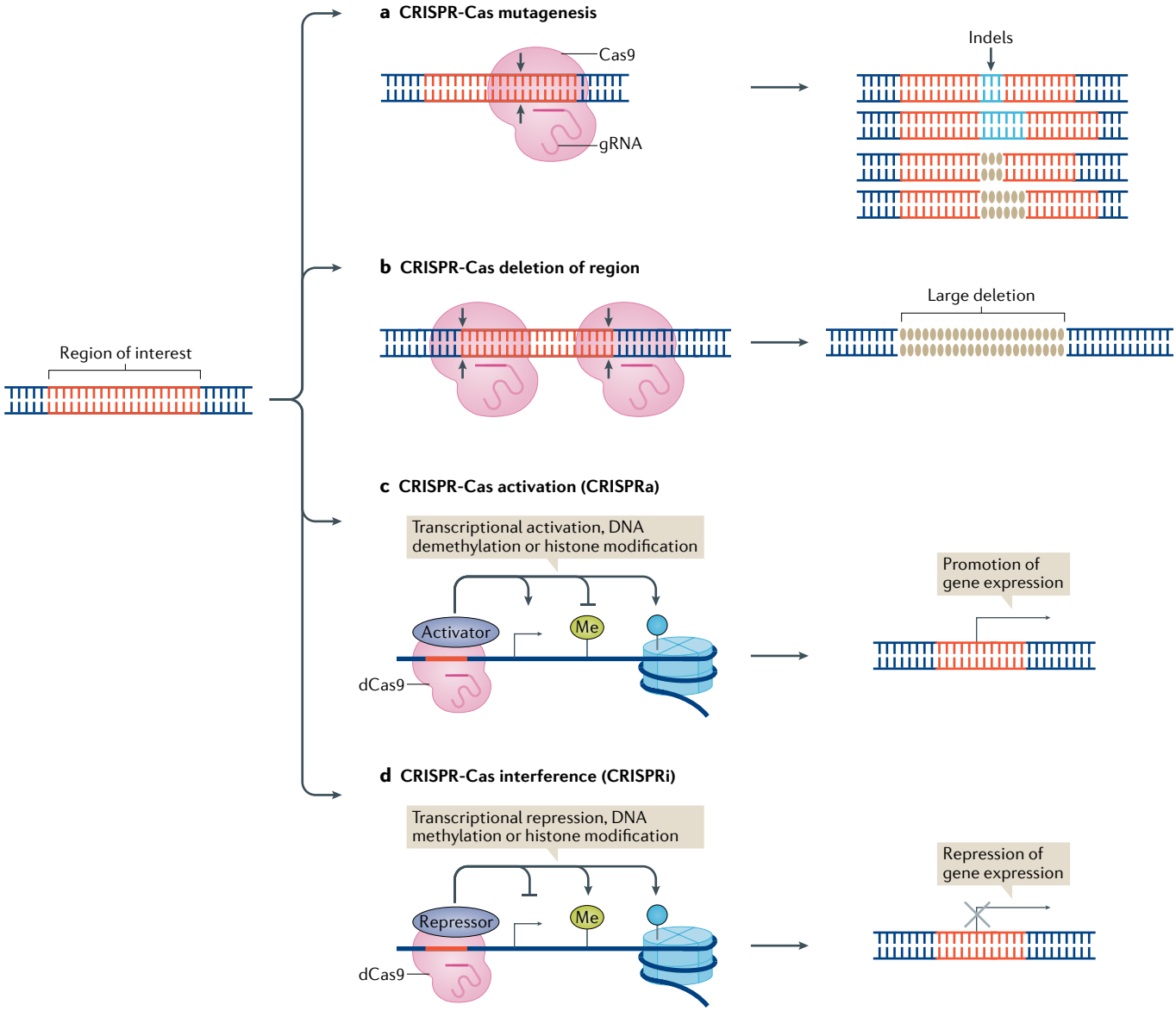

Fig. 2 | **Genomic editing to investigate regions of interest in rheumatic diseases.** A region of interest (for example, a gene or a regulatory non-coding region of interest) can be investigated using a number of genomic editing techniques. **a** | CRISPR–Cas mutagenesis involves the generation of an array of small insertions or deletions (indels) in the region of interest following cleavage by the Cas nuclease. **b** | Cas nuclease-mediated deletion can also be used to delete large sections of the region of interest. **c** | CRISPR–Cas activation (CRISPRa) involves the fusion of proteins that activate gene expression, such as transcriptional activators, enzymes involved in DNA demethylation or histone modifiers that promote DNA accessibility, to a nuclease-deactivated Cas9 protein (dCas9) for targeted promotion of gene expression. **d** | CRISPR–Cas interference (CRISPRi) involves the fusion of proteins that repress gene expression, such as transcriptional repressors, enzymes involved in DNA methylation (Me) or histone modifiers that epigenetically silence the area, to dCas9 for targeted repression of gene expression.

new links and discovering unknown functions. In the study of rheumatic diseases, this approach can be used to better understand the roles of genes implicated in disease by GWAS and discover previously unidentified targets.

Various investigators have used this approach to screen for genes critical in immune cell activation and differentiation in cell lines and mice. For example, using a library of 250,000 gRNAs, one group of researchers investigated which genes can enhance or reduce CD69 expression following TCR stimulation in a cell line, the efforts of which led to the discovery of a previously uncharacterized regulator of TCR activation known as family with sequence similarity 49 member B (FAM49B)[30]. A separate study used a similar approach to study the TNF response following lipopolysaccharide stimulation in primary bone marrow-derived mouse dendritic cells, providing important insight into the genes involved in the regulation of this known autoimmune-related pathway[31]. CRISPR-based genome-wide screening approaches have also been used to detect genes involved in T cell differentiation. For example, in one study, a genome-wide

library of gRNAs was virally transfected into naive T cells from Cas9-expressing transgenic mice that were then stimulated with a TCR agonist and IL-4 (conditions that induced T helper 2 (T_H2) cell differentiation); the cells were then selected on the basis of the expression of GATA3 and other T_H2 markers. This approach led to identification of various genes involved in T_H2 differentiation, such as *Pparg* and *Bhlhe40* (REF.[32]).

More recently, investigators have used *Foxp3* reporter mice and targeted CRISPR screening to uncover the nuclear factors involved in tuning the expression of FOXP3, a critical master regulator of regulatory T (T_{reg}) cells[33]. From this work, two novel modulators, *Usp22* and *Rnf20*, were found to have opposing effects on *Foxp3* expression. Taking this work a step further, the investigators also validated the role of *USP22* in primary human T_{reg} cells by knocking out the gene using CRISPR–Cas technology. Notably, *Usp22* ablation in murine T_{reg} cells exacerbated disease in experimental models of colitis and multiple sclerosis[33]. Together, this work highlights the utility of using CRISPR screening to identify new and relevant targets in autoimmunity.

Excitingly, these genomic screening studies have been extended to primary human immune cells (BOX 1). For example, sequential delivery of a gRNA-encoding library by lentiviral transduction and Cas9 by protein electroporation has been used to conduct a genome-wide screen in human CD8 T cells. By measuring the proliferation of the cells following restimulation with CD3 and CD28, the researchers could identify various genes that affect TCR signalling. Notably, CRISPR–Cas mediated disruption of *UBASH3A* and *TNFAIP3* genes, two genes implicated in autoimmune disease development, resulted in enhanced proliferation of the cells, suggesting that these genes are involved in the regulation of T cell activation in humans and providing potential insight into their role in disease[34].

A similar approach, though limited in scope, can be used to investigate the function of individual genes implicated in autoimmunity. For example, a group of investigators used CRISPR–Cas editing to study the function of *PTPN22*, *PTPN2* and *ZAP70* (all implicated in the development of autoimmunity) in primary human CD4 T cells. The results of this study highlighted the role of these molecules in IL-2 signalling and TCR activation, similar to findings in mouse models[35]. A single-gene knockout approach to autoimmunity-associated genes can also be applied to other primary human lineages, including other T cell subsets, dendritic cells, B cells or natural killer cells[36–39]. For example, one study that used this approach found that knockout of *IRF4*, a gene with a well-established link to autoimmune susceptibility, in primary dendritic cells can enhance cell activation while attenuating inflammatory nuclear factor κB (NF-κB) signalling[38].

Hence, the investigation of gene function in animal models and primary human cells has been revolutionized by genomic editing tools. As the field continues to move forwards, directly studying the function of genes in primary human cells will become easier and more reliable, paving the way for direct and detailed investigations of cells from patients. These types of studies enable us to take an unbiased examination of all genes in diverse human cell types and uncover as yet unknown networks and potential therapeutic targets.

Identifying regulatory regions

Traditional approaches. The studies described so far have broadly helped to find disease-associated genes and identify their roles in critical immune responses such as T cell proliferation and differentiation. Yet, variation between individuals is rarely the result of complete gene deletion but instead reflects SNPs and other genetic variants, such as indels and nucleotide substitutions. Although GWAS have identified more than 10,000 trait-associated variants in hundreds of loci, most loci lie in non-coding regions and are of unknown function[40–42]. Pruning and identifying potentially causal variants can be more effectively achieved by determining which regions have a regulatory function. One strategy is to look to epigenomic data and other functional genomic annotations, such as those being produced by the Encyclopaedia of DNA Elements (ENCODE) project[43]. Data on chromatin configuration, histone modifications and transcription factor-binding can be used to pinpoint functionally active spots in the genome and co-localize variants to these regions.

Examples of techniques that annotate the genome include traditional chromatin immunoprecipitation sequencing, DNase I hypersensitive sites sequencing and chromatin capture assays, which can be used to find regions of transcription factor binding and chromatin configuration[44]. Other more recently developed techniques, such as cleavage under targets and tagmentation (CUT&Tag) sequencing, cleavage under targets and release using nuclease (CUT&RUN) sequencing and assay for transposase-accessible chromatin using sequencing (ATAC-seq), have augmented our ability to annotate the genome with a higher accuracy and lower cell input[45–48]. Computational tools take advantage of this wealth of information to interrogate GWAS signals. Epigenomic annotations have been used to predict causal SNPs and have implicated shared CD4 T cell-related

CUT&Tag
A technique that uses antibodies specific for DNA binding proteins to measure DNA regions bound by these proteins. The antibodies are tethered to a Tn5 transposase fusion protein and, following antibody-binding, activation of the transposase cleaves nearby DNA and generates fragment libraries for sequencing, the data of which are used to identify the bound regions.

CUT&RUN
Similar to CUT&Tag, this technique analyses DNA regions bound by specific proteins using targeted antibodies. Unlike with CUT&Tag, the antibody is tethered to a micrococcal nuclease, which fragments nearby DNA elements.

ATAC-seq
A technique used for assaying areas of open chromatin in the genome; the method relies on unguided Tn5 transposase-induced fragmentation of the genome.

Box 1 | Genomic editing and cell lines

The majority of research cited in this Review has been conducted in immortalized cell lines. This choice of cell lines is one of practicality. Cell lines are easier to use, capable of being propagated at the single-cell level to create clones and are amenable to complex and sequential genetic manipulations; CRISR–Cas editing efficiency is also generally higher in cell lines than in primary cells. However, cell lines also have the disadvantage of often being highly mutated and genomically unstable, reflecting, in many cases, their origin from human cancers. Cell lines are often constitutively activated, and therefore cannot fully recapitulate physiological conditions[118,119]. Fortunately, CRISPR–Cas technologies are largely applicable to primary human immune cells as well as haematopoietic stem cells, and studies in primary immune cells are emerging, as highlighted whenever possible in this Review. We expect that in the near future, CRISPR–Cas-based genomic editing in primary immune cells will become a standard practice.

IMPACT
A computational genome annotation strategy that identifies regulatory elements defined by cell-state-specific transcription factor binding profiles.

Massively parallel reporter assay
A technique used to identify regulatory regions of the genome in a high-throughput assay. Regions of interest are cloned into a minimal reporter with a unique barcode and a promoter to create a large pool of constructs. Constructs are expressed into cells and the RNA and DNA are sequenced to estimate the effects of each regulatory region on barcode gene expression, indicating regulatory capacity.

Fluorescent in situ hybridization
A technique that measures RNA expression by flow cytometry using hybridization and amplification of fluorescent RNA probes.

pathways across many autoimmune disorders[49–53]. Other tools, such as the inference and modelling of the phenotype-related active transcription (IMPACT) annotation tool have attempted to integrate and interrogate all known annotations to better identify cell type-specific causal autoimmune risk variants[40]. However, all of these approaches rely on making inferences and cannot directly confirm functional regions. A better strategy would be to discover the segments within the genome with a regulatory function using direct experimental approaches, such as genome editing.

Genome editing of regulatory regions. Genome-editing techniques now enable us to identify functional regions in non-coding elements experimentally (FIG. 2). Such research can be performed using CRISPR–Cas screening or targeted deletion with Cas nucleases, in a similar fashion to that described in the previous section on "Dissecting risk loci". One of the first uses of pooled CRISPR–Cas screening in a non-coding region was a mutagenic screen that was applied to a *BCL11a* enhancer. The transcription factor BC11A has been reported to control the expression of fetal hemoglobulin and is therefore a potential therapeutic avenue for the treatment of sickle cell disease[54]. In this seminal work, a tiled lentiviral library of gRNA was targeted to this enhancer to systematically create deletions along the entire length of the region in a human cell line. In this way, the investigators could broadly identify nucleotide positions in the enhancer regions that affected gene expression, thereby resolving the region[55]. Similar CRISPR–Cas screening approaches that systematically interrogate a genomic region have been used to uncover regulatory elements in the four major pluripotency genes (*Tdgf1*, *Zfp42*, *Nanog* and *Rpp25)* in mouse embryonic stem cells[56]. In another study, rather than starting with a large, unknown genomic region, the researchers used an alternative method of first computationally identifying all the genome-wide binding sites of p53 and ESR1, and then using a screen to validate these regulatory regions and the downstream genes of interest regulated by these regions[57].

Similar to editing individual genes, CRISPR–Cas9 editing can also be applied in a more targeted fashion to interrogate predicted enhancer regions on a case-by-case basis as opposed to a screening format. For instance, this targeted approach was used to investigate a putative enhancer region around the SNP rs13239597 in close proximity to *IRF5*; the investigators used CRISPR–Cas9 nucleases to delete a 1,000-base pair region around the variant in three different cell lines, which caused cell type-specific changes in *IRF5* and *TNPO3* expression[58].

Although CRISPR–Cas systems were initially used to generate double-stranded DNA breaks to induce indels, researchers quickly adapted the technology by fusing Cas proteins to other modifiers, such as transcriptional activators, repressors and deaminases, to name a few[59]. These methods have greatly expanded the CRISPR–Cas toolkit to enable targeted genome investigation of regulatory regions. For example, in one study, a CRISPRa screen, which fused deactivated Cas9 with transcriptional activators such as VP64, was used to interrogate two autoimmune risk loci, *CD69* and *IL2RA*. In this setup, the binding of the CRISPRa complex results in the recruitment of transcriptional activation machinery, enabling the identification of promoter and enhancer regions. By using a library of gRNA that saturates the genomic region around *CD69* and *IL2RA* in Jurkat T cells, the researchers identified various enhancer areas, including one for *IL2RA* that overlapped with a known autoimmune disease-associated variant. Follow-up research was conducted on the newly recognized *IL2RA* enhancer by investigating mice that had been modified to carry the mutated variant; notably, this modification had a clear effect on T cell stimulation[60]. Hence, this study is a good example of CRISPR screening being applied to an autoimmunity-associated locus to pinpoint causal variants and investigate the functional outcome of these changes on T cells.

A complementary approach to CRISPRa is CRISPRi, which fuses deactivated Cas9 with repressors of gene expression such as the histone methylator KRAB. When the CRISPRi complex is targeted to a region of interest with programmable gRNAs, the associated KRAB proteins effectively silence the region by methylating nearby histones. Similar to CRISPRa, CRISPRi has been used experimentally to investigate autoimmune risk loci. An early example of this approach applied a targeted CRISPRi screen to investigate the functionality of all DNase I sites around the β-globin locus control region and *HER2* (REF.[61]). DNase I sites mark areas of chromatin accessibility, indicating that a region is similarly available to enhancer or repressor proteins and thus potentially important for gene regulation. Some studies have used a combination of both CRISPRa and CRISPRi to explore autoimmune risk loci. For example, in one study exploring the locus *TNFAIP3*, researchers designed a gRNA library that targeted all areas of open chromatin around the gene and performed a CRISPRi screen[62]. A CRISPRa screen of the computationally identified variants was then performed in the region to find regulatory areas of overlap, that is, those areas that resulted in a loss or gain of expression when targeted by both CRISPRa and CRISPRi machinery. In comparison to other techniques, including the in vitro massively parallel reporter assay, CRISPRi had the highest likelihood of detecting true hits[62].

Many of the approaches mentioned so far have relied on reporter cell lines or cell surface markers to measure changes in gene expression and facilitate screening. To move beyond cell lines and target any gene, as opposed to just genes with validated antibodies, investigators have paired CRISPRi screening with fluorescent in situ hybridization. This novel approach enabled the rapid testing of over 3,500 enhancer regions in 30 different genes[63].

CRISPR–Cas screening of thousands of gRNA is a powerful tool for resolving large Mb regions of the genome and generating new hypotheses. However, such scale can be prohibitively expensive and technically challenging. As an alternative, these techniques can also be applied in a more targeted, hypothesis-testing manner to investigate precise regions of interest (<1 kb) of the genome on a case-by-case basis. As an example, to study a potentially causal distal enhancer in a risk locus associated with CD4 T_{reg} cell-mediated suppression of colitis,

Single-cell RNA sequencing
An approach for measuring the expression of RNA in individual cells using droplet or plate-based technology.

Computational fine mapping
A process by which a trait-associated region from a genome-wide association study is further analysed to identify genetic variants that are likely to causally influence the trait, usually through the integration of additional epigenetic or genomic data.

Expression quantitative trait loci
Trait-associated regions that can explain a notable portion of the changes in expression of a gene.

researchers applied targeted CRISPRa in primary human CD4 T cells only to a handful of pre-selected variants in the region of interest[64]. Using this focused approach, they discovered that only gRNAs near *rs11236797* could induce changes in the expression of the nearby gene *GARP*. In this way, the researchers were able to decipher a known colitis-associated region, identify the downstream gene and provide a direct link to T_{reg} cell function.

In the past 5 years, researchers have begun to couple single-cell RNA sequencing (scRNA-seq) with CRISPR screening to rapidly test multiple regions and measure corresponding gene expression in a highly parallelized system. For example, a recent publication used these new methods to test approximately 6,000 putative enhancers with a single cell CRISPRi screen. Using this highly multiplexed technology, the researchers profiled approximately 300,000 single cells and were able to experimentally confirm functional versus non-functional enhancer regions in the genome. This approach allowed the investigators to then discern which genome annotations are most associated with functional enhancer regions, identifying p300, histone H3 acetylation at lysine 27 (H3K27ac) and cell-line-specific transcription factor binding as the most important signatures[65].

Defining non-coding variation is a major challenge in the investigation of rheumatic diseases. In the near future, as these large-scale genomic studies continue to rapidly increase in size and scope, our ability to accurately annotate the human genome will considerably improve. In turn, such advances will enhance our ability to confidently resolve signal from noise in studying the genetics of complex diseases and unveil the full potential of genetics research.

Investigating autoimmune risk variants

The techniques described so far in this Review can identify a relevant immune gene or locus and confirm the location of possible SNPs in a validated regulatory region. But to truly pinpoint and understand the mechanisms of these causal variants, further work is required. This work might include investigation of rare diseases to elucidate critical protein-coding variants and genes, computational fine-mapping studies to narrow down potential hits, traditional molecular biology approaches and genomic editing tools to directly assess variant function in cells of interest.

Rare diseases. For some monogenic autoimmune disorders, researchers have identified causal variants in protein-coding regions, leading to a better understanding of variant and gene function. For example, single mutations in various genes are known to cause Aicardi–Goutières Syndrome, an autoimmune condition characterized by encephalitis with skin involvement, including mutations in *TREX1, RNASEH2A, RNASEH2B* and *RNASEH2C*. All these genes are involved in the regulation of nucleic acid degradation and contribute to excess inflammation[66]. As another example, the monogenic autoimmune disorder familial Mediterranean fever is typified by mutations in *MEFV* that result in aberrant activation of the pyrin inflammasome[66]. Mutations in *WISP3* have also been linked to progressive pseudorheumatoid dysplasia, a progressive skeletal disorder that results in swelling and pain in multiple joints[66]. Other notable examples include autoimmune conditions resulting from mutations in *AIRE*, *FAS* and *BACH2* (REFS[21,67,68]). Although these conditions are rare, they can provide considerable insight into disease pathophysiology and exemplify how a single variant can contribute to disease.

Computational approaches for prioritizing variants. For non-coding variants, particularly common variants identified in GWAS, defining the causal variants can be more difficult than with monogenic disease-associated protein-coding variants. Within a single locus, multiple variants might be present in tight linkage with each other, some or all of which might explain the disease association. Additionally, multiple non-coding SNPs within a locus might work together to yield a particular phenotype, as exemplified by various non-coding SNPs in *STAT4* that are associated with juvenile idiopathic arthritis[69]. Computational fine mapping is one way of identifying the most probable causal variants in complex polygenic rheumatic disorders. Fine mapping can refine the association signals in genotyping data via statistical methods to create credible sets of SNPs with the highest probability of being causal, which can then be investigated in further detail in functional studies[70].

Another common approach to investigating the function of autoimmune risk alleles is expression quantitative trait loci (eQTL) analysis, which uses both genotyping and mRNA data to uncover DNA variants associated with altered gene expression. Large-scale studies of this kind have been conducted in cell lines and tissues to detect cell-specific and tissue-specific variants that might control gene transcription[71–73]. For example, in one eQTL analysis of whole blood, researchers could link 39% of 250 autoimmune disease-associated SNPs identified by GWAS to the expression levels of nearby genes[74]. Taking this approach a step further, another eQTL study found that some SNPs (12% of the eQTL SNPs analysed) had lineage-specific effects, highlighting the importance of studying the appropriate cell types and tissues[75]. Although eQTL studies can identify associations between variants and the expression of genes, linkage disequilibrium can make it difficult to disentangle disease-causing SNPs from incidental SNPs within an inherited block, limiting the interpretation of the results.

Together, fine mapping and eQTL analyses can provide a short list of potentially causal variants in autoimmune risk loci. However, experimental validation remains indispensable, both for proving causality and for deciphering the downstream mechanisms of action that contribute to disease.

Traditional approaches for functional validation. Traditionally, functional validation of causal variants began with the study of individuals with a particular variant of interest. In this type of study, healthy individuals and patients with a particular disease and genotype at a variant of interest would be carefully selected and the relevant immune cells of these individuals profiled for differences in gene expression or other phenotypes[76].

Electromobility shift assays
A molecular biology technique that measures the interaction of DNA and proteins on a protein-binding gel.

Luciferase assays
A technique used to identify regions of the genome that can regulate gene expression. In these assays, the region of interest is cloned upstream or downstream of the gene encoding luciferase and the resultant plasmids are transfected into cells to measure the effect of the modification on luciferase expression.

Affinity precipitation assays
A technique that is similar to electromobility shift assays, with the exception that bound complexes are magnetically pulled down prior to examination on a protein-binding gel.

Unfortunately, in this type of analysis, controlling for other genetic variants as well as differences in cell states is difficult, as such differences might be caused by environmental stimuli. Alternative molecular biology approaches are available that use synthesized oligonucleotides that encompass the variant of interest and the flanking genomic sequences to investigate functionality. These assays include electromobility shift assays that test the interactions of variants with nuclear material containing DNA-binding proteins, luciferase assays that test the ability of a variant to promote the expression of a reporter gene and affinity precipitation assays that similarly test the interaction of variants with DNA-binding proteins in nuclear material following magnetic pulldown[77–79]. Unfortunately, these techniques also have limitations, including a high level of technical noise and an inability to recapitulate the transcription factor interactions, histone modifications and chromatin organization found in the native cell state. To overcome these issues, researchers have developed sophisticated genome editing tools for investigating genetic variants, which can edit specific genomic regions within their native chromatin context (FIG. 3).

Genomic editing for functional variant validation. The simplest application for CRISPR–Cas systems in the study of autoimmune risk variants is the generation of indels in close proximity to the SNP of interest. For example, by using such an approach to investigate six non-coding SNPs in close proximity to an *HLA-DQB1* allele associated with type 1 diabetes, researchers could narrow down this list to a single SNP, *rs71542466*, which had an effect on gene and protein expression of *HLA-DQB1* (REF.[80]). Similarly, CRISPR–Cas indels were used to investigate *rs17622517*, a variant in an enhancer region of a gene associated with autoimmune susceptibility, *IRF1*. Clonal selection of those cells with CRISPR–Cas-mediated deletions around the variant found that such deletions had a clear effect on *IRF1* expression[81]. A similar approach was taken in the study of a *BLK* insertion variant associated with SLE, *rs558245864*; CRISPR–Cas9-mediated 6-bp and 18-bp deletions around this variant led to decreased accessibility of the site to the transcription factor CCCTC-binding factor (CFCF) and decreased *BLK* expression, highlighting the importance of this region and variant[82].

Another major use of CRISPR–Cas genomic editing in variant validation is targeted editing. For this approach, CRISPR–Cas technology and exogenous template material can be used to induce double-strand breaks and HDR. Alternatively, CRISPR–Cas tools that incorporate modified base or prime editors, which have the capacity to directly mutate DNA without inducing double-stranded breaks, can be used.

For example, to validate the effects of the SNP *rs71542466* on *HLA-DQB1* expression, a CRISPR–Cas-guided HDR-based approach was used to mutate the variant from the reference G allele to the disease-associated C allele in a T cell line[80]. The clones with the resultant mutation had altered expression levels of *HLA-DQB1*, providing a causal link between the variant and the induced change[80]. In another study of the autoimmunity-linked gene *TNAIP3*, CRISPR–Cas-guided HDR repair was used to investigate a protein-coding variant associated with rheumatoid arthritis, rs2230926, in a monocyte cell line. Surprisingly, the mutation had no effect on NF-κB signalling but did increase the expression of *PADI4* (REF.[83]). Importantly, CRISPR–Cas HDR methods can also be applied to primary human cells ex vivo. For example, non-viral CRISPR–Cas HDR methods have been used to correct coding mutations in *IL2RA* (encoding the soluble IL-2 receptor-α subunit, also known as CD25) in cells from a family of patients with a rare monogenic immune dysregulation disorder[84]. Amazingly, genomic editing of the primary CD4 T cells from the patients could restore the cell surface expression of CD25.

Base editors consist of Cas proteins fused with deaminases and enable a more precise way of mutating single nucleotides than CRISPR–Cas-mediated HDR. Instead of creating double-stranded breaks in the genome, these systems open the region of interest via Cas complex binding and then promote the mutation of selected nucleotides using the fused proteins. Current iterations of base editing enable both C to T and A to G transitions in a customizable editing window[17,85–87]. Base editors have been used to target pathogenic mutations in a number of disorders. For example, this tool has been used to target mutations in *APOE4*, *MPDU1*, *HGB*, *HBB* and *DMD* in cell lines, primary mouse cells and patient-derived fibroblasts[85–88]. The use of base editors has more recently been applied to human haematopoietic stem cells. For example, by using this approach on haematopoietic stem cells from a patient with sickle cell disease, researchers were able to edit the *BCL11A* enhancer and restore the expression of fetal haemoglobin in differentiated erythrocytes, thereby reducing the number of sickled cells[89]. Although not yet tested for autoimmune risk variants, base editors have also been applied to primary human T cells to introduce multiple simultaneous gene knockouts[90].

Although incredibly effective and powerful, the current version of base editors can only induce a limited set of mutations, either C to T or A to G. To fill this gap and allow for investigation of other transitions, for instance C to G, an alternative approach has been developed, called prime editing. For this approach, Cas proteins are fused to a reverse transcriptase. The resulting complex is programmed with a prime editing guide RNA (pegRNA), which functions as both the targeting gRNA and the donor template for the reverse transcriptase enzyme. This technique has been used to repair pathogenic variants in *HBB*, *HEXA*, *PRNP* and *DNMT1* in various cell lines including HEK293T cells, K562 cells, U2OS cells, HeLa S3 cells and mouse primary cortical neurons[91]. Using this technique, it might be possible to repair practically any variant in the human genome, including small insertions and deletions, although the editing efficiency can be low and variable[91].

Resolving cell heterogeneity

Single-cell assays. Another essential piece of the puzzle in unscrambling the genetics of autoimmune diseases is identifying critical cell populations and states that govern disease risk. Abundant evidence shows that

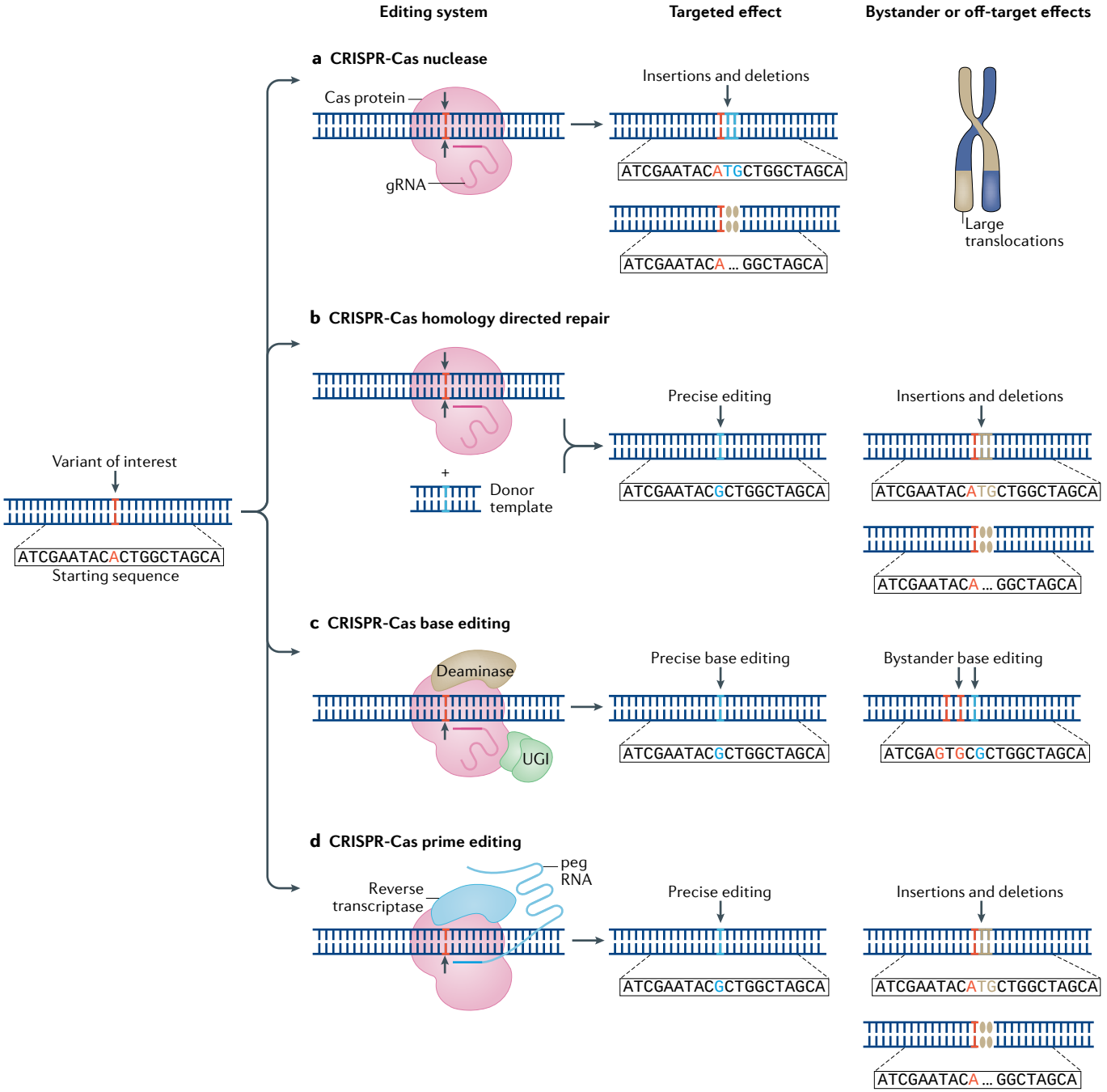

Fig. 3 | **Genomic editing to investigate variants of interest in rheumatic disease.** A variant of interest can be investigated using a number of genomic editing techniques. **a** | The area of interest can be altered with small deletions or insertions using CRISPR–Cas nucleases. **b** | Targeted homology-directed repair with CRISPR–Cas nucleases and a donor template can be used to precisely edit the variant of interest. **c** | CRISPR–Cas base editors (comprising a nuclease-deactivated Cas9 protein (dCas9) or Cas nickase fused with an adenine or cytosine deaminase and a uracil DNA glycosylase inhibitor (UGI)) can be used to directly edit a base of interest. **d** | CRISPR–Cas prime editors (comprising a Cas endonuclease or Cas nickase fused with a reverse transcriptase that is programmed with a prime editing guide RNA (pegRNA)) can be used for precise editing of the target site. For each approach, bystander or unwanted off-target effects can occur (as shown).

Droplet-based RNAseq
A single-cell RNA sequencing method that relies on droplet generation and encapsulation of individual cells.

Mass cytometry
A type of single-cell analysis that tags cells with antibodies conjugated to heavy metals to then analyse staining intensity by time-of-flight mass spectrometry.

immune cells are critical contributors to rheumatic disorders. In the past 10 years, genetic approaches aimed at identifying unique cell-type chromatin and transcription factor signatures, such as co-localizing GWAS hits with ATAC-seq, have been applied to decode which cell types and states might contribute to disease[49,92–96]. However, these genetic approaches rely on data collected from cell lines or bulk sorted samples and therefore fall short in capturing the true heterogeneity in the immune compartment of these disorders. Hence, more recently, large-scale unbiased single-cell investigations have begun to characterize relevant disease cells and states.

Single-cell technologies, such as droplet-based RNAseq and mass cytometry, provide a comprehensive view of the

HyPR-seq
A droplet-based targeted single-cell sequencing technique that involves hybridizing DNA probes to selected RNA to measure the expression of genes.

main cellular states within diseased tissue and are often able to produce tens to thousands of readouts per cell[97]. In one single-cell analysis of synovial tissue from patients with rheumatoid arthritis or osteoarthritis, the heterogeneity of the $CD4^+$ T cell compartment was resolved at a single-cell level, resulting in the discovery of a new lineage of cells, known as T peripheral helper cells, associated with disease[98]. In a parallel single-cell analysis of renal biopsy samples from patients with SLE, researchers were able to characterize an interferon and fibrotic mRNA signature in tubular cells that was associated with failure to respond to treatment[96]. A single-cell atlas of the human colon during ulcerative colitis has also been generated using colon mucosa biopsy samples, which identified 51 distinct cell types[99]. In this analysis, inflammatory fibroblasts, inflammatory monocytes, microfold-like cells and T cells that co-express *CD8A* and *IL17A* were all expanded in the colons of patients with ulcerative colitis compared with the colons of healthy individuals. Interestingly, mapping the various risk alleles associated with ulcerative colitis to particular cell types enabled the researchers to discover that many of the risk alleles were cell type-specific[99]. In general, the discovery of a cell population within a diseased tissue supports a potential role for that cell type in pathogenesis, and thereby implicates underlying genetic mechanisms in that cell type, although further experiments are required to confirm causality. Once such causal cell populations are determined, assessing the molecular phenotypes within these cell populations is an important next step.

With the ever-expanding scope and scale of scRNA-seq, single-cell eQTL analysis represent a promising approach to relate heterogeneous features of the immune cell compartment to variants of interest. In this regard, a new consortium was formed in 2020 (the single-cell eQTLGen consortium) that is aimed at identifying the cellular contexts in which disease-causing variants affect gene expression[100].

Genomic editing in single cells. Genomic editing can be paired with scRNA-seq to simultaneously assess the effects of perturbation on the heterogeneous immune cell compartments. As an example of this approach, researchers have used CRISPR–Cas screening in combination with scRNA-seq to assess the response of mouse bone marrow-derived dendritic cells to lipopolysaccharide at a single-cell level. By targeting 24 known transcription factors through CRISPR-based genetic perturbations, the researchers discovered four distinct groups of transcription factors that uniquely affected cell function and differentiation[101]. An extension of this approach paired CRISPR–Cas screening with ATAC-seq to measure the effects of CRISPR perturbation (using either CRISPR knockout or CRISPRi-based approaches) of transcription factors, chromatin-modifying factors, and non-coding RNAs on chromatin accessibility in human B cell lines and primary keratinocytes[102]. The researchers discovered how these transcription factors and other elements regulated B cell and keratinocyte cell states and might alter the chromatin accessibility of disease-associated variants. In a different example of paired CRISPR–Cas screening and scRNA-seq, this method was applied to TCR-stimulated Jurkat T cells to assess the function of six regulators and 23 transcription factors. The investigators discovered that CRISPR-mediated targeting of *ETS1*, *RUNX1* and *GATA3* reduced the viability of the cells and that CRISPR-mediated targeting of *LCK* and *ZAP70* prevented TCR stimulation[103]. Finally, this approach has been cleverly modified and extended to primary human T cells through sequential use of gRNA-expressing lentiviruses and nucleofection of Cas proteins. Using this technique, the investigators characterized gene programmes controlled by important regulators of human T cell proliferation; notably, the ablation of some of these programmes in T cells results in enhanced killing of cancer cells in vitro[34].

Although profiling total RNA or chromatin accessibility can be informative in elucidating changes in total gene expression in an unbiased fashion, this approach can also be costly. An alternative approach, hybridization of probes to RNA for sequencing (HyPR-seq), involves targeted scRNA quantification in combination with CRISPR–Cas screening. In this approach, a library of gRNA is transfected into cells that are then fixed and hybridized with RNA probes, before being processed into droplets for library generation and sequencing. By using targeted RNA probes, this approach allows for the highly sensitive detection of selected genes of interest and identification of the gRNA. Using HyPR-seq, researchers have been able to target regulatory regions around *GATA1* and detect corresponding changes in gene expression[104].

Finally, researchers have also developed a CRISPR knockin targeting approach that was combined with single-cell analysis to look at the effect of overexpressing various gene constructs in primary human T cells[105]. In this approach, a library of knock-in donor material was integrated into the genome with CRISPR–Cas to assess the effects of each knock-in DNA construct on T cell function. The correctly integrated gene constructs were identified by sequencing unique molecular barcodes introduced from the donor DNA, and the effects on T cell function could be analysed using single-cell RNA sequencing in the same cells. These experiments helped to discover a chimeric TGFBR2-41BB receptor that can promote clearance of a xenotransplant solid tumour model when knocked into human T cells along with an appropriate TCR. For rheumatic diseases, this approach can be used to overexpress candidate genes implicated in autoimmunity, complementing the previously described gene knockouts, to directly study function.

Overall, these single-cell approaches help to provide a full picture of the immune response in rheumatic disease. Paired with genomic editing, these techniques enable the investigation of rheumatic disease genes and variants at-scale in heterogeneous immune cell populations.

Future directions and limitations

Additional advances in genomic editing have helped to propel the potential and applicability of these tools. Cas proteins require particular protospacer adjacent motif (PAM) sites to initiate binding of the editing complex. Hence, several groups have focused on discovering and

Table 1 | **Limitations of CRISPR-Cas editing systems**

Application	Genomic editing technique	Advantages	Disadvantages
Identifying and investigating autoimmune-associated loci and genes	CRISPR–Cas-mediated genome wide screening[30–34]	All annotated genes can be targeted	Limited to cell lines
		Can discover cell type-specific effects	Requires a functional outcome (for example, proliferation)
	CRISPR–Cas-mediated knockout of individual genes[37,38]	Function can be assessed	Low throughput
		Amenable to primary immune cells	
	CRISPR–Cas-mediated activation (CRISPRa) or interference (CRISPRi) of gene expression[61,64]	Easily multiplexable	Currently limited to cell lines
Identifying autoimmune regulatory regions	CRISPR–Cas-mediated activation (CRISPRa) or interference (CRISPRi) of regulatory region[60,62,63,65]	Can test any region of the genome	Effects might be context-dependent and require multiple cell lines for verification
	CRISPR–Cas-mediated deletion or mutagenesis of regulatory regions[55]		
Identifying causal variants	CRISPR–Cas-mediated mutagenesis of causal variants[58]	Amenable to primary immune cells	Induced deletions, insertions and substitutions are random and do not recapitulate variant changes
			Some variants cannot be directly targeted
	CRISPR–Cas-mediated homology directed repair[80,83]	Can directly change reference allele to an alternative allele	Bystander mutations
	CRISPR–Cas-mediated base editing[85–90]		Not all mutations are possible
	CRISPR–Cas-mediated prime editing[91]		Low efficiency
Linking variants to causal immune cell types	CRISPR–Cas-mediated editing paired with single-cell technologies[101–105]	Can be used to directly identify cell populations of interest in rheumatic disorders	Expensive and prone to drop-out in gene expression
		Can assess the function of a particular variant in a broad range of cell populations simultaneously	

characterizing alternative Cas proteins with different PAM restrictions to broaden the range of possible targets[106]. Researchers have even been able to engineer nearly PAM-less Cas proteins through directed evolution[107–111].

A promising alternative to DNA editing is RNA editing with Cas13 nucleases. Unlike the other variants of Cas, these proteins target RNA and not DNA, making it possible to target RNA molecules without potentially causing dangerous genomic mutations[15,112,113]. An interesting adaptation of this approach fuses deactivated Cas13 nucleases with adenosine deaminase acting on RNA type 2 (ADAR2), which enables direct mutagenesis of RNA, similar to the base editors discussed that target DNA. This approach has been applied in a cell line to correct two pathogenic G to A mutations in *AVPR2* and *FANCC*, with modest efficiency[112]. Alternatively, CRISPR-free systems might represent a different route all together; indeed, a study in 2020 unveiled the newly developed CRISPR-free, transcription activator-like effector (TALE)-based editing system that was used to base-edit mitochondrial DNA for the first time[114].

Genomic editing has various limitations; in particular, these tools can have off-target and bystander effects that might convolute results (TABLE 1). For example, cleavage by Cas nucleases induces a wide range of somewhat predictable mutations around the target site, but Cas binding and cleavage can also occur in off-target regions. Base and prime editors similarly have off-target effects but are also prone to both bystander-editing (editing of nearby non-target nucleotides) and, less frequently, unwanted insertions and deletions. As one group reported, the BE3 cytosine-targeted base editor, but not Cas9 or ABE7.10 adenosine-targeted base editors, was 20-fold more likely to induce random single nucleotide mutations in the genome than the spontaneous mutation rate[115]. Another important consideration is that base-editors edit a window of C or A nucleotides in the target site, which could make correcting a single nucleotide impossible, depending on the flanking sequences and the variant of interest. Finally, the discussed Cas13 nucleases that directly target RNA molecules are also reported to often induce transcriptome-wide off-target RNA mutations[113]. Optimization of Cas proteins, intelligent gRNA design and the use of multiple gRNA can offset some of these effects; however, the limitations and challenges of these technologies are important to keep in mind as we move towards their therapeutic application.

Directed evolution
A process of protein engineering that mimics biological evolution. A library of mutated genes is expressed in cell lines and a phenotype is selected; the process is repeated with new mutations and harsher selection conditions until a desired outcome is achieved.

Conclusions

The ease of use, customizability and accessibility of genomic editing technology has expedited its use in a number of applications, particularly for cell therapies in cancer and rare monogenic disorders. However, in the study of polygenic autoimmune diseases and associated variants, successful application remains scarce. The studies highlighted in this Review showcase how genomic editing can be used to identify and validate autoimmune disease-associated loci, genes and variants.

Practically, for rheumatologists and patients with rheumatic disease, genomic editing has potential in

the development of new cellular therapies. Promising applications include the editing of stem cells to promote tissue regeneration and re-shape cytokine responses, the induction and strengthening of regulatory responses via autologous T_{reg} cell therapies and the correction of pathogenic mutations in patients with monogenic diseases[116,117].

As the field continues to grow, the applicability, scale and precision of genome editing technologies should continue to improve. All these advances in genomic editing will undoubtedly continue to resolve the causal genes, regions and SNPs responsible for complex polygenic autoimmune diseases. In the future, we anticipate that genomic editing directly in primary immune cells will become a major focus for proving causality and a powerful step towards defining associations between genotype and phenotype in immune cells of interest. Additionally, a renewed emphasis on multiplexed and multi-omic analyses should enable the simultaneous investigation and experimental validation of multiple variants or regions at the same time. Understanding how these variants interact individually and together to contribute to the development of complex and polygenic disorders represents the next frontier in genetics and genomic editing.

Overall, genomic editing has shown great promise in the study of the genetics of rheumatic diseases and remains the ideal approach for rapidly experimentally validating findings directly in primary human immune cells. One way or another, genomic editing tools are here to stay and will only become more accessible with time as researchers continue to adopt and expand these approaches in future studies.

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Current and future therapies for primary Sjögren syndrome

Raphaèle Seror[1,2], *Gaetane Nocturne*[1,2] *and Xavier Mariette*[1] ✉

Abstract | Primary Sjögren syndrome (pSS) is a systemic autoimmune disease that is characterized by a triad of symptoms that affect all patients (dryness, pain and fatigue). In addition, systemic involvement can affect between one-third and one-half of patients. The management of patients with pSS has been negatively affected by a lack of effective treatments; however, knowledge of the epidemiology of pSS has increased, and advances in developing classification criteria, systemic disease activity scoring and patient-reported outcomes have been made during the past decade. Progress has also been made in understanding the mechanisms that underlie the pathogenesis of pSS, which has enabled a more targeted therapeutic approach to be taken. At present, therapeutic decisions rely on the evaluation of symptoms and systemic manifestations and are mostly formed on the basis of experience rather than evidence, and on similarities with other autoimmune diseases, although the 2019 management recommendations from EULAR are now being used to inform clinical management of pSS. This Review summarizes the available evidence for systemic treatments for pSS and includes discussions of advances in outcome assessment, the current evidence for DMARD use and an overview of promising future therapeutics.

Xerostomia
Oral dryness.

Xerophthalmia
Ocular dryness.

Primary Sjögren syndrome (pSS) is a systemic autoimmune disease characterized by lymphocytic infiltration of the salivary and lachrymal glands that leads to xerostomia and xerophthalmia. Dryness is a cardinal symptom of pSS that is present in almost all patients and is frequently associated with fatigue and pain. In addition, 30–50% of patients with pSS will present with systemic complications during the course of their disease[1,2].

Great progress has been made in unravelling the pathophysiology of pSS over the past 20 years[3], and it is now established that innate immunity and particularly types I, II and (probably) III interferons have an important role in the onset of the disease. Environmental triggers might be involved in initiating immune activation in susceptible individuals, particularly in those with risk factors in genes involved in interferon signalling pathways. These first steps could activate salivary gland epithelial cells and cause them to function as part of the immune response; notably, these cells are able to produce the cytokine B cell-activating factor (BAFF) which, along with other factors such as crosstalk between T cells and B cells, can promote B cell activation. In turn, B cells produce autoantibodies that form immune complexes that can maintain and perpetuate the immune response[3]. These advances in our understanding of the underlying pathology of pSS have led to the identification of several promising new therapeutic avenues that are currently under investigation (FIG. 1).

At present, the management of patients with pSS is impeded by a lack of evidence about the efficacy of treatments. Thus, treatment decisions are often made on the basis of experience rather than evidence. Treatment choices rely on the evaluation of both glandular symptoms and systemic (extra-glandular) manifestations; symptomatic treatment is usually appropriate for glandular symptoms, whereas immunosuppressive agents are reserved for systemic manifestations. The publication of the EULAR recommendations for the therapeutic management of pSS in 2019 represented a great step towards a more homogeneous and consensual management of pSS, particularly for non-specialist health-care professionals[4]. Nevertheless, these recommendations rely, for the most part, on expert opinion and the extrapolation of treatment effects from other systemic autoimmune diseases, rather than on evidence of treatment efficacy in pSS[5]. As newer targeted therapies are evaluated in clinical trials, it is hoped that the dream of being able to offer personalized care according to the pathophysiological mechanism that predominates in each patient[6] will move closer to reality.

In this Review, we discuss advances in outcome assessment in pSS and how these advances have changed the way that clinical trials are conducted, detail the available evidence on the efficacy of systemic treatments for pSS and provide an overview of promising agents that are currently under investigation.

[1]*Department of Rheumatology, Université Paris-Saclay, INSERM U1184: Centre for Immunology of Viral Infections and Autoimmune Diseases, Assistance Publique-Hôpitaux de Paris, Hôpital Bicêtre, Le Kremlin Bicêtre, Paris, France.*

[2]*These authors contributed equally: Raphaèle Seror, Gaetane Nocturne.*

✉*e-mail: xavier.mariette@aphp.fr*

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Key points

- Currently available classic immunosuppressive drugs might be effective for treating some systemic manifestations of primary Sjögren syndrome (pSS), as is the case for other connective tissue diseases.
- Following the failure of the first randomized controlled trials (RCTs) in pSS, efforts are ongoing to define new therapeutic targets and new outcome measures.
- For the first time, two agents have met their primary outcome of improvement in systemic disease activity in RCTs in pSS: anti-B cell-activating factor receptor and anti-CD40 antibodies.
- Targeting B cells remains the most promising therapeutic approach for pSS.
- New outcome measures for RCTs aim to combine end points to assess all disease manifestations, including systemic activity, saliva and tears function, patient-reported outcomes and biological features.

Therapeutic objectives and outcomes

Although almost all patients with pSS have dryness, pain and fatigue, only some will develop systemic manifestations during the disease course. Consequently, the current therapeutic objectives are twofold: to relieve patients of their main symptoms and to treat severe systemic manifestations. Patients with systemic complications often present with biomarkers of B cell activity after diagnosis and have the highest risk of lymphoma[7–11]. Thus, in addition to the two main treatment objectives, experts agree that another objective when managing these patients is to prevent the emergence of systemic complications, and especially lymphoma. However, at present, this objective remains an unmet need.

In the past few decades, advances made in the understanding of the pathogenesis of pSS, together with the development of targeted therapies and biologic agents, have opened up new avenues for the treatment of this disease. B cell-targeted therapies have been the most promising agents to be investigated in pSS in the past decade[12]. Nevertheless, the first attempts at assessing these therapies in large-scale clinical trials failed to demonstrate their efficacy, despite promising results in open-label studies[13,14] (TABLE 1). The reasons for these initial failures were varied and included the therapeutic target, the inclusion criteria (particularly the inclusion of patients with inactive disease), the heterogeneity of presentation and the outcome measures used[15]. Effectively, in the first trials to assess the efficacy of conventional synthetic DMARDs (csDMARDs) and biologic DMARDs (bDMARDs) in pSS, the primary end points were often improvements in symptoms such as dryness, fatigue and pain. Although improving these manifestations is a valuable target, csDMARDs and bDMARDs might also affect systemic disease, suggesting that improvement in systemic manifestations could be a valuable outcome to measure. Thus, in parallel to the development of classification criteria for pSS[16], validated consensual outcome measures were required to stimulate clinical research and better identify efficient drugs.

The development of standardized pSS outcomes measures by EULAR in 2010 considerably changed how patients with pSS are assessed and how research in this disease is conducted. Two tools were developed: the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) for systemic activity[17] and the EULAR Sjögren's Syndrome Patient Reported Index (ESSPRI) as a disease-specific patient-reported outcome measure[18] (BOX 1). The ESSDAI includes 12 domains that cover all possible systemic manifestations and is a reliable and sensitive tool[19,20]. The ESSDAI is also clinically relevant, as a high score correlates with biomarkers of B cell function[21,22], and is predictive of major outcomes of the disease such as lymphoma[8,23,24] and death[25,26]. To help both physicians and researchers, ESSDAI scores for levels of disease activity and for minimal clinically important improvement (MCII) have been determined[18] (BOX 1). To date, the ESSDAI has been used and evaluated in many studies and has become the reference standard for evaluating systemic disease activity in patients with pSS. By contrast, the ESSPRI was developed to be a pSS-specific patient-reported outcome measure that includes the main symptoms of dryness, fatigue and musculoskeletal pain. The ESSPRI has excellent reliability and construct validity and, compared with existing patient-reported outcome measures (the Sicca Symptoms Inventory and the Profile of Fatigue and Discomfort questionnaire), the ESSPRI is shorter and has a higher responsiveness[20]. As for the ESSDAI, relevant thresholds have been determined for MCII and the patient acceptable symptom state with ESSPRI scores (BOX 1). The simplicity and validity of ESSPRI scoring has enabled the ESSPRI to emerge as the consensus tool for the evaluation of patient symptoms, and it is now widely used in both clinical practice and in clinical trials. Thus, these new tools have helped to better define inclusion criteria and primary outcomes in clinical trials in pSS. The ESSDAI is now one of the inclusion criteria and the primary outcome in almost all ongoing trials (TABLE 2), with many trials recruiting patients with moderate disease activity (an ESSDAI score ≥5) and using either the total change in ESSDAI score from baseline or MCII as the primary end point.

Management of pSS

Historically, the management of patients with pSS has been hindered by a lack of effective treatments. At present, no single treatment is able to target both a patient's glandular symptoms and any systemic complications, suggesting that management of these manifestations requires different approaches. Treatment of the main symptoms of pSS mostly relies on the use of symptomatic agents (including tear and saliva substitutes, analgesics and saliva-stimulating agents such as pilocarpine or cevimeline), and steroids with immunosuppressants for severe systemic involvement, but evidence demonstrating the efficacy of these drugs from evidence-based medicine studies is scarce[5]. The decision to intensify treatment in pSS is dependent on disease activity and the organ system involved, and treatment decisions are often formed on the basis of experience with related rheumatic diseases, such as rheumatoid arthritis or systemic lupus erythematosus (SLE). The current approach to treating pSS was described in detail in the 2019 EULAR recommendations for the management of pSS[4] and is outlined in BOX 2. Interestingly, the EULAR recommendations provide decision trees for the treatment of each of the possible pSS manifestations (glandular or systemic).

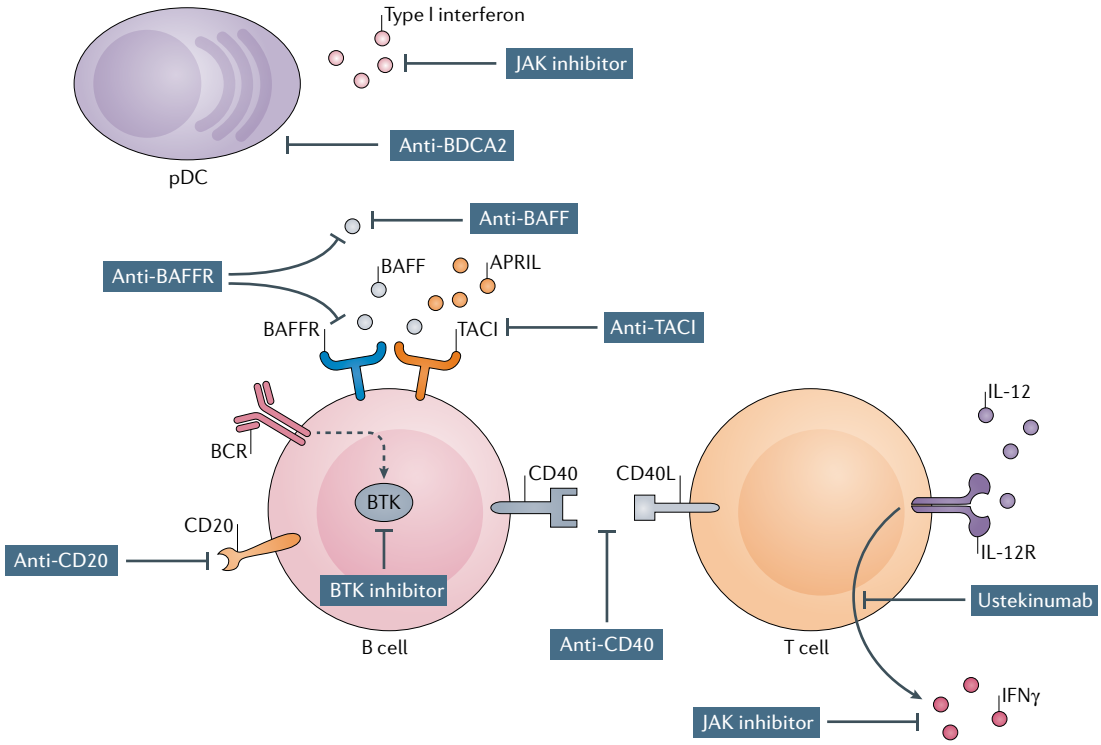

Fig. 1 | **New therapeutic targets in primary Sjögren syndrome.** Advances in understanding the pathogenic processes involved in primary Sjögren syndrome (pSS) have led to new therapeutic targets being evaluated in patients with pSS. B cells are being targeted by new anti-CD20 antibodies and by antibodies that target the B cell-activating factor (BAFF) signalling pathway. Anti-CD40 antibodies are also being evaluated, which can block the crosstalk between T cells and B cells. Direct targeting of Bruton's tyrosine kinase (BTK), an important molecule in B cell receptor (BCR) signalling, is another approach that is being assessed. In addition, interferons seem to be a relevant therapeutic target. Interferons could be blocked by antibodies that target plasmacytoid dendritic cells (pDCs), which secrete type I interferons, by Janus kinase (JAK) inhibitors that block downstream type I and type II interferon signalling pathways, and by ustekinumab, which inhibits the IL-12 signalling pathway and the induction of T helper 1 cells, which secrete type II interferons. APRIL, a proliferation-inducing ligand; BAFFR, BAFF receptor; BDCA2, blood dendritic cell antigen 2; CD40L, CD40 ligand; IL-12R, IL-12 receptor; TACI, transmembrane activator and CAML interactor.

In general, treatment of systemic complications needs to be discussed with specialized centres in order to define the best strategy.

Conventional synthetic DMARDs

Similar to other systemic autoimmune diseases, csDMARDs have been used empirically in pSS on the basis of experience rather than evidence-based medicine. Although no csDMARD has been approved for the treatment of pSS, some could be efficient for treating specific manifestations of this complex and heterogeneous condition. For example, methotrexate might be useful in the management of arthritis and myositis. Similarly, other csDMARDs might be indicated for systemic complications; for example, hydroxychloroquine might be useful in treating purpura, or mycophenolate mofetil in treating interstitial lung disease. However, until 2020, the few randomized controlled trials (RCTs) that had evaluated the use of csDMARDs (or bDMARDs) in patients with pSS did not provide conclusive evidence supporting their efficacy[5].

Hydroxychloroquine is the immunomodulatory drug that is most often used to treat pSS[27]. From a pathophysiological point of view, hydroxychloroquine interferes with Toll-like receptor (TLR) signalling and inhibits the type I interferon pathway[28]. Hydroxychloroquine is usually prescribed in patients with fatigue, arthralgia or myalgia, rather than in those with severe systemic manifestations; however, despite its wide use in clinical practice, evidence regarding the efficacy of hydroxychloroquine in the treatment of pSS is limited. Before the JOQUER RCT was conducted[29], published data were mostly derived from open or retrospective studies[30,31] and from one crossover trial that included 19 patients[32]. In the JOQUER trial, 120 patients with no systemic complications were randomly allocated to receive either placebo or hydroxychloroquine 400 mg daily[29]. After 6 months of treatment, no difference was found between patients receiving placebo and those receiving hydroxychloroquine for the primary composite end point of a ≥30% reduction in dryness, pain and fatigue, or for any of the clinical secondary end points. However, hydroxychloroquine is known to have an effect on B cell biomarkers, including causing decreases in IgG concentrations and in the interferon signature[33] and, in clinical practice, hydroxychloroquine is used to treat conditions such as purpura (particularly when linked to hypergammaglobulinaemia), cutaneous lesions or inflammatory

Table 1 | **Randomized controlled trials in patients with primary Sjögren syndrome that used non-validated outcomes**

Agent	Target	Comparator	Number of participants	Primary end point	Was the primary end point met?	Ref.
Etanercept	TNF	Placebo	14	A ≥20% improvement for two of three domains (subjective or objective measures of dry mouth, subjective or objective measures of dry eyes and IgG level or ESR) from baseline to week 12	No	36
Infliximab	TNF	Placebo	103	A ≥30% improvement in scores on two of three VAS (measuring joint pain, fatigue and dryness (buccal, ocular, skin, vaginal or bronchial)) at week 10	No	35
Anakinra	IL-1	Placebo	26	Difference in Fatigue Severity Scale and fatigue VAS scores between groups at week 4	No	38
Rituximab	CD20	Placebo	17	A 20% reduction in fatigue VAS scores at week 24	No	48
Rituximab	CD20	Placebo	30	Improvement in SWSFR at weeks 5, 12, 24 and 48	Yes; significant improvements at week 5 ($P = 0.018$) and week 12 ($P = 0.004$)	49
Rituximab	CD20	Placebo	120	A 30 mm improvement in scores on two of four VAS (measuring global disease, pain, fatigue and dryness) at week 24	No, but there were significant improvements in scores on the fatigue VAS at week 6 ($P < 0.001$) and week 16 ($P = 0.012$) (secondary end point)	43
Rituximab	CD20	Placebo	133	A 30% improvement in scores on the fatigue VAS or oral dryness VAS at week 48	No, but modest effect on unstimulated saliva flow rate (secondary end point)	14
Baminercept	Lymphotoxin	Placebo	72	Change in SWSFR from baseline to week 24	No, and no significant effect on ESSDAI score (secondary end point)	84

Non-validated outcomes are mainly patient-related outcomes or salivary flow, which have never been validated as pertinent outcomes. ESR, erythrocyte sedimentation rate; ESSDAI, EULAR Sjögren's Syndrome Disease Activity Index; SWSFR, stimulated whole saliva flow rate; VAS, visual analogue scale.

arthralgia. The discrepancies between the effectiveness of hydroxychloroquine for treating certain manifestations and the results of clinical trials thus illustrate the difficulty in choosing a good target population and an adequate clinical end point in therapeutic trials in pSS.

In 2020, the results of an RCT were published in which the combination of leflunomide 20 mg and hydroxychloroquine 400 mg daily was compared with placebo in 29 patients with pSS[34] (TABLE 2). Using this combination of treatments, the primary end point of change in ESSDAI score from baseline to week 24 was met, with a mean difference between the active treatment and placebo groups of −4.35 points (95% CI −7.45 to −1.25 points, $P = 0.0078$). These encouraging results require replication in a larger number of patients, but clearly show that the evolution in clinical trial design (with the use of the ESSDAI as a primary end point) can lead to a positive outcome.

Biologic DMARDs

TNF inhibition. TNF inhibitors have been assessed in pSS in two RCTs, both of which failed to demonstrate efficacy (TABLE 1). The first RCT was the TRIPSS study, in which infliximab was compared with placebo[35], and the second RCT compared etanercept with placebo[36]. In the TRIPSS study, the primary end point was improvement in the scores on visual analogue scales (VASs) for fatigue, pain and sicca, and in the study assessing etanercept, the primary end point was improvement in two items from among ocular dryness, oral dryness, erythrocyte sedimentation rate and IgG concentration. No statistically significant improvements were found in

these two studies[35,36]. The negative results of these trials suggest that TNF inhibition might not be a relevant treatment approach for pSS. This outcome might be a result of the balance between TNF and type I interferon; TNF can decrease the production of IFNα by plasmacytoid dendritic cells (pDCs), and inhibition of TNF leads to sustained IFNα secretion[37]. Thus, therapeutic inhibition of TNF could potentially promote the interferon signature, which has a pathological role in pSS[3].

IL-1 inhibition. Fatigue is one of the main symptoms of pSS that is burdensome to patients. Working on the hypothesis that IL-1 might participate in the development of fatigue, a double-blind RCT was conducted to assess the efficacy of anakinra (IL-1 receptor antagonist) in pSS[38] (TABLE 1). The primary outcome of change in two different fatigue scores at week 4 was not achieved. Adding to this result, another study showed that fatigue is paradoxically negatively associated with the interferon signature (IFNγ-induced protein 10 and IFNγ)[39], suggesting that IL-1 and type II interferon might not directly mediate fatigue, and that bDMARDs that target these cytokines might not be effective in treating this symptom.

B cell depletion. Given the important role of B cells in the pathogenesis of pSS, rituximab (which targets CD20 on B cells) has emerged as a promising therapy and has been tested in several open-label studies to assess its effects on fatigue, dryness and pain[21,40–42]. These first studies showed promising results, although the results were mixed with regard to the longevity of the improvement in symptoms and quality of life[43–45]. Open-label studies focused on systemic manifestations of pSS have also shown efficacy for rituximab. In 78 patients from the French AIR registry, rituximab was effective in 69% of individuals and had a steroid-sparing effect[21,46]. In studies that specifically focused on patients with pSS who had low-grade lymphoma[40] or peripheral neuropathies as a result of either cryoglobulinaemia or vasculitis[47], rituximab therapy was effective, lending support to the idea that rituximab is beneficial for at least some systemic manifestations of pSS.

On the basis of these promising preliminary results, four RCTs of rituximab in pSS have been conducted (TABLE 1). All of the RCTs evaluated one of, or a combination of, sicca, fatigue and pain as the primary outcome. The first trial, published in 2008, included 17 patients with pSS from the UK[48]. In this study, the level of fatigue significantly decreased in patients treated with rituximab compared with those treated with placebo, but the primary end point was not met. The next trial, which was published in 2010, was a Dutch study that included 30 patients with pSS (10 who received placebo and 20 who received rituximab)[49]. In this study, oral dryness decreased following rituximab infusions. Following these two studies, two larger multicentre trials were conducted, one in France (the TEARS trial with 120 patients)[13] and one in the UK (the TRACTISS trial with 133 patients)[14]. In the TEARS trial, the primary end point of improvement of at least 30 mm on two of four VASs (including pain, fatigue, dryness and global disease activity) was not met, but improvements in the secondary outcome measures of unstimulated whole saliva flow rate and salivary gland ultrasonographic features (decrease in hypoechoic zones in parotid and submandibular glands) were found in those receiving rituximab[13]. In the TRACTISS trial, no significant improvements were found in any outcome measures, except unstimulated whole saliva flow rate[14].

Belimumab, a monoclonal antibody that targets BAFF and that has been approved for use in SLE, has also been assessed in pSS in an open-label study[50]. In the BELISS trial, 30 patients with pSS were included who were positive for anti-SSA/Ro antibodies and had one of the following: current systemic complications or salivary gland enlargement; early disease (<5 years); or biomarkers of B cell activation. The primary outcome was improvement in two of five items (pain, fatigue, dryness, systemic disease activity assessed by the physician and B cell activity biomarkers) at week 28, and was met by 60% of the participants[50]. No RCT has yet been conducted, but evaluation of new strategies in which

Box 1 | **EULAR outcome measures for primary Sjögren syndrome**

The EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI)

Purpose

- To measure systemic disease activity.

Content

- Twelve domains: glandular, constitutional, lymphadenopathy, articular, cutaneous, respiratory, renal, muscular, peripheral nervous system, central nervous system, haematological and biological.
- The weight of each domain reflects its relative importance to disease activity.
- Each domain includes three or four levels, with zero indicating no activity and three or four indicating high activity.

Scoring

- Score for each domain = level of activity × weight of the domain.
- Final score = sum of all domain scores.
- The theoretical range of scores is 0–123.
- The score rates only active manifestations, not damage features.

Score interpretation

- Disease activity levels are determined as:
  - No activity (ESSDAI score 0)
  - Low activity (ESSDAI score <5)
  - Moderate activity (ESSDAI score ≥5 but ≤13)
  - High activity (ESSDAI score ≥14)
- Minimal clinically important improvement is defined as an improvement in ESSDAI score of ≥3 points.

The EULAR Sjögren's Syndrome Patient Reported Index (ESSPRI)

Purpose

- To assess patient-reported outcomes in primary Sjögren syndrome.

Content

- Three domains (dryness, fatigue and musculoskeletal pain) are each assessed on a numerical scale of 0–10.

Scoring

- Total score = mean of the three domain scores.
- The range of scores is 0–10.

Score interpretation

- The patient acceptable symptom state is defined as an ESSPRI score of <5.
- Minimal clinically important improvement is defined as an improvement in ESSPRI score of ≥1 point or ≥15%.

Table 2 | **Randomized controlled trials in patients with primary Sjögren syndrome that use validated outcomes**

Trial identifier	Agent(s)	Target	Comparator	Number of participants	Inclusion criteria	Primary end point	Results	Refs
EUCTR2014-003140-12-NL	Leflunomide and hydroxychloroquine	Cell metabolism, autophagy and TLR signalling	Placebo	29	ESSDAI score ≥5; positive labial salivary gland biopsy	Change in ESSDAI score from baseline to week 24	Significant improvement in ESSDAI score ($P = 0.0078$)	34,100
NCT01782235 (ETAP)	Tocilizumab	IL-6R	Placebo	110	ESSDAI score ≥5; positive for anti-SSA/Ro antibodies	Improvement in ESSDAI score of ≥3 points from baseline to week 24	No difference in primary end point between active treatment and placebo	54,101
NCT02631538	Belimumab and rituximab co-administration	BAFF and CD20	Placebo	70	ESSDAI score ≥5; positive for anti-SSA/Ro or anti-SSB/La antibodies; UWSFR >0	Number of participants with SAEs at week 68	Completed, results not available	61
NCT02149420	Ianalumab (VAY736)	BAFFR	Placebo	27	ESSDAI score ≥6; ANA (titre ≥1:160); positive for RF or anti-SSA/Ro or anti-SSB/La antibodies; SWSFR >0	Change in ESSDAI score from baseline to week 12	Predefined criteria for primary end point not met, but showed a trend towards positive effect versus placebo	62,102
NCT04078386	RC18	TACI	Placebo	42	ESSDAI score ≥5; positive for anti-SSA/Ro antibodies	Change in ESSDAI score from baseline to week 24	Active, not recruiting	64
NCT02291029	Iscalimab (CFZ533)	CD40	Placebo	12 (cohort 1) and 32 (cohort 2)	ESSDAI score ≥6; positive for anti-SSA/Ro antibodies or ANA (titre ≥1:160) and RF positive; SWSFR >0	Change in ESSDAI score from baseline to week 12	Significant improvement in ESSDAI score ($P = 0{\cdot}009$) with i.v. iscalimab	67,103
NCT03905525 (TWINSS)	Iscalimab (CFZ533)	CD40	Placebo	260 (split over two cohorts)	Positive for anti-SSA/Ro antibodies (both cohorts); SWSFR >0 (both cohorts); ESSDAI score and ESSPRI score ≥5 (cohort 1); ESSDAI score <5 and ESSPRI (fatigue or dryness) score ≥5 (cohort 2)	Change in ESSDAI score from baseline to week 24 (cohort 1); change in ESSPRI score from baseline to week 24	Recruiting	104
NCT04572841	SAR441344	CD40L	Placebo	88	Positive for anti-SSA/Ro antibodies; SWSFR ≥0.1; ESSDAI score ≥5; disease duration ≤7 years	Change in ESSDAI score from baseline to week 12	Recruiting	68
NCT04035668	LOU064	BTK	Placebo	252 (dose-ranging study)	ESSDAI score ≥5; ESSPRI score ≥5; positive for anti-SSA/Ro antibodies; UWSFR >0	Change in ESSDAI score from baseline to week 24	Recruiting	74

Table 2 (cont.) | **Randomized controlled trials in patients with primary Sjögren syndrome that use validated outcomes**

Trial identifier	Agent(s)	Target	Comparator	Number of participants	Inclusion criteria	Primary end point	Results	Refs
NCT02610543	Seletalisib (UCB5857)	PI3K	Placebo	58 (aim), only 27 enrolled	ESSDAI score ≥5; positive for anti-SSA/Ro or anti-SSB/La antibodies; UWSFR >0; salivary gland biopsy	Change in ESSDAI score from baseline to week 12	Study terminated early owing to enrolment issues (data from 20 patients analysed); trend for improvement in ESSDAI and ESSPRI scores	78,105
NCT02334306	MEDI5872 (AMG557)	ICOSL	Placebo	42 (aim), only 32 enrolled	ESSDAI score ≥6; positive for anti-SSA/Ro or anti-SSB/La antibodies and IgG titre >16 g/l or positive for RF	Change in ESSDAI score from baseline to week 12	No significant improvement in ESSDAI score compared with placebo	87,106
NCT02067910 (ASAP-III)	Abatacept	CTLA4	Placebo	80	ESSDAI score ≥5; disease duration ≤7 years; positive parotid gland biopsy	Change in ESSDAI score from baseline to week 24	No significant improvement in ESSDAI score compared with placebo or in secondary end points (except IgG and RF concentrations)	91,107
NCT02915159	Abatacept	CTLA4	Placebo	187	ESSDAI score ≥5; positive for anti-SSA/Ro antibodies	Change in ESSDAI score from baseline to week 24	No significant improvement in ESSDAI score compared with placebo	92,108
NCT04496960	Tofacitinib	JAK1 and JAK3	Placebo	30	ESSDAI score between 2 and 13; SWSFR >0	Safety and tolerability	Recruiting	95

Validated outcomes are EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) or EULAR Sjögren's Syndrome Patient Reported Index (ESSPRI) scores, which have been developed and validated as pertinent outcomes. ANA, antinuclear antibody; BAFF, B cell-activating factor; BAFFR, BAFF receptor; BTK, Bruton's tyrosine kinase; CD40L, CD40 ligand; CTLA4, cytotoxic T lymphocyte-associated protein 4; ICOSL, inducible T cell co-stimulatory ligand; IL-6R, IL-6 receptor; i.v., intravenous; JAK, Janus kinase; PI3K, phosphatidylinositol 3-kinase; RF, rheumatoid factor; SAE, serious adverse event; SWSFR, stimulated whole saliva flow rate; TACI, transmembrane activator and CAML interactor; TLR, Toll-like receptor; UWSFR, unstimulated whole saliva flow rate.

belimumab and rituximab are combined is currently ongoing (see section on enhancing B cell depletion).

Future approaches

Novel strategies to target B cells

Several lines of evidence support a role for B cells in the pathogenesis of pSS[12], making this cell subset a promising therapeutic target (FIG. 1). Methods of inhibiting B cells are diverse and include targeting cytokines (such as anti-IL-6 receptor antibodies), directly targeting B cell survival (such as anti-CD20, anti-BAFF or anti-BAFF receptor (BAFFR) antibodies), inhibition of co-stimulation (such as anti-CD40 antibodies) or targeting small molecules such as kinases.

Targeting new cytokines. Hopes have been raised by the possibility of targeting IL-6, which has important roles in terminal B cell differentiation, in B cell activation and in supporting the production of IgG[51]. Moreover, IL-6 concentrations are increased in the serum, saliva and tears of patients with pSS[52,53]. The safety of tocilizumab, a monoclonal anti-IL-6 receptor antibody has been evaluated in patients with pSS in an RCT[54]. In the ETAP study, 110 patients were randomly allocated at a ratio of 1:1 to receive a monthly infusion of placebo or tocilizumab 8 mg/kg. The number of individuals achieving the primary end point (improvement in the ESSDAI score of ≥3 points) did not differ significantly between those receiving tocilizumab (52.7%) and those receiving placebo (63.6%), and no safety concerns were detected. Despite the puzzling very high rate of response in the placebo group, these results suggest that B cell activation is probably not mediated by IL-6 in most patients with pSS.

Enhancing B cell depletion. As previously discussed, rituximab failed to demonstrate efficacy in RCTs in pSS and belimumab has not yet been assessed in an RCT in this disease; nevertheless, these treatments are still being pursued in pSS. New anti-CD20 antibodies are now available that induce a more profound B cell

Box 2 | **EULAR recommendations for the management of primary Sjögren syndrome**

Overarching principles

- Patients with Sjögren syndrome should be managed at, or in close collaboration with, centres of expertise following a multidisciplinary approach.
- The first therapeutic approach for dryness should be symptomatic relief using topical therapies.
- Systemic therapies may be considered for the treatment of active systemic disease.

Individual recommendations

- Baseline evaluation of salivary gland function is recommended before starting treatment for oral dryness.
- The preferred first therapeutic approach for oral dryness according to salivary gland function may be:
  - Non-pharmacological stimulation for mild dysfunction
  - Pharmacological stimulation for moderate dysfunction
  - Saliva substitution for severe dysfunction
- The first-line therapeutic approach to ocular dryness includes the use of artificial tears and ocular gels or ointments.
- Refractory or severe ocular dryness may be managed using topical immunosuppressive-containing drops and autologous serum eye drops.
- Concomitant diseases should be evaluated in patients presenting with fatigue or pain, whose severity should be scored using specific tools.
- Consider analgesics or other pain-modifying agents for musculoskeletal pain, considering the balance between potential benefits and adverse effects.
- Treatment of systemic disease should be tailored to organ-specific severity using the EULAR Sjögren's Syndrome Disease Activity Index definitions.
- Glucocorticoids should be used at the minimum dose and length of time necessary to control active systemic disease.
- Immunosuppressive agents should be mainly used as glucocorticoid-sparing agents, with no evidence supporting the choice of one agent over another.
- B cell-targeted therapies may be considered in patients with severe, refractory systemic disease.
- The systemic organ-specific therapeutic approach may follow, as a general rule, the sequential (or combined) use of glucocorticoids, immunosuppressive agents and biologic agents.
- Treatment of B cell lymphoma should be individualized according to the specific histological subtype and disease stage.

depletion than rituximab, such as obinutuzumab, which is currently being assessed in lupus nephritis with promising preliminary results[55]. A new strategy for the treatment of pSS combining rituximab with belimumab is currently being investigated. This strategy is supported by several lines of evidence. First, serum BAFF concentrations increased in patients with pSS after B cell depletion induced by rituximab[46,56,57]. Second, a slight increase in the number of $CD27^{+}IgD^{-}$ switched memory B cells occurred after exposure to belimumab in the BELISS study[50]. Third, individuals who did not respond to therapy in the TEARS study had higher serum BAFF concentrations at baseline than those who did respond to therapy[58]. These findings all highlight the potential utility of combining rituximab and belimumab to treat pSS. Two open-label trials assessing this combination of treatments in patients with lupus nephritis[59,60] have found contradictory results (one positive and the other negative). No safety concerns were reported in these two trials. In pSS, a double-blind RCT has been conducted[61] and first results should be published soon.

Another way to combine targeting the BAFF signalling pathway with B cell depletion is to use an anti-BAFFR antibody. Because this antibody is a depleting antibody and BAFFR is exclusively expressed on B cells, it induces direct B cell depletion. Moreover, anti-BAFFR antibodies inhibit the BAFF–BAFFR signal that could be present in the remaining non-depleted B cells. A phase IIa study assessing the anti-BAFFR antibody ianalumab (VAY736) in patients with pSS has been conducted with somewhat positive results[62] (TABLE 2). A phase IIb study has also been conducted and the preliminary results reported[63]. In the phase IIb study, 190 patients with pSS were randomly allocated at a 1:1:1:1 ratio to receive monthly doses of ianalumab (at 5 mg, 50 mg or 300 mg) or placebo. The primary outcome was change in ESSDAI score from baseline to week 24, which was met with the 300 mg dose of ianalumab compared with placebo[63]. A trend for improvement in the stimulated whole saliva flow rate was also noted for ianalumab 300 mg compared with placebo at week 24. However, no difference was found for secondary end points such as the ESSPRI or the Functional Assessment of Chronic Illness Therapy Fatigue. Adverse events were limited to mild to moderate infusion reactions. These studies suggest that targeting BAFFR to cause both B cell depletion and BAFF–BAFFR pathway inhibition is a promising option in pSS, and phase III studies are awaited. Still focused on targeting the BAFF signalling pathway, a phase II trial in pSS evaluating a monoclonal antibody that targets transmembrane activator and CAML interactor, a receptor for BAFF and a proliferation-inducing ligand (APRIL), which thus inhibits both BAFF and APRIL, is currently underway[64].

Targeting co-stimulation. CD40–CD40 ligand (CD40L) interaction is important for B cell development, antibody production, germinal centre formation and optimal T cell-dependent antibody responses[65]. In patients with pSS, expression of CD40L by $CD4^{+}$ T cells is increased compared with that in healthy individuals[66], suggesting that CD40–CD40L interactions might be a viable target in pSS. Indeed, positive results were published in 2020 for a trial of the anti-CD40 antibody iscalimab (CFZ533) in patients with pSS[67]. This phase II study evaluated iscalimab in two cohorts: the first cohort included 12 patients who received either placebo or iscalimab 3 mg/kg subcutaneously at weeks 0, 2, 4 and 8, and the second cohort included 32 patients who received either placebo or iscalimab 10 mg/kg intravenously at weeks 0, 2, 4 and 8 (REF.[67]). The primary outcomes were safety and change in ESSDAI score from baseline to week 12. No safety concerns were reported. Treatment with intravenous iscalimab resulted in a significant improvement in the ESSDAI score compared with placebo, with a mean decrease of 5.21 points in those receiving intravenous iscalimab (TABLE 2). These results suggest that inhibition of CD40–CD40L interaction is a promising option, and phase III studies are awaited. In addition, SAR441344, an anti-CD40L antibody, is currently being assessed in pSS in a phase II trial that is still recruiting participants[68].

Targeting kinases. Two kinase inhibitors have also been assessed in pSS: a Bruton's tyrosine kinase (BTK) inhibitor and a phosphatidylinositol 3-kinase (PI3K) inhibitor. BTK participates in intracellular signalling in B cells, particularly following B cell receptor (BCR) stimulation[69], and inhibitors of BTK have been successfully developed for use in B cell malignancies[70,71]. BTK is also involved in autoimmune diseases, as exemplified in mice that overexpress BTK, which develop lupus-like disease characterized by kidney and lung involvement, as well as salivary gland involvement, similar to that observed in pSS[72]. These mice also have increased germinal centre formation, increased numbers of plasma cells and are positive for antinuclear antibodies. Moreover, a 2020 study showed that in vitro inhibition of BTK and PI3K can reduce overactivation of B cells induced by salivary gland epithelial cells derived from patients with pSS[73]. The BTK inhibitor LOU064 is currently being assessed in pSS in a phase II double-blind RCT[74]. The primary outcome will be change in ESSDAI score from baseline to week 24, and recruitment is ongoing.

The PI3K family is involved in several aspects of immunity involving the PI3K–RAC serine/threonine protein kinase–serine/threonine protein kinase mTOR pathway and comprises three classes, of which PI3Kα, PI3Kβ and PI3Kδ belong to class IA[75]. In contrast to PI3Kα and PI3Kβ, which are ubiquitously expressed, PI3Kδ expression is restricted to leukocytes, and particularly to B cells[75]. Similar to BTK inhibitors, PI3Kδ inhibitors were first assessed in the B cell malignancies chronic lymphocytic leukaemia and non-Hodgkin lymphoma[76,77]. In pSS, the PI3Kδ inhibitor seletalisib (UCB5857) has been assessed in a 12-week proof-of-concept study[78]. Change in ESSDAI score from baseline to week 12 was the primary end point; however, the study was marked by enrolment challenges and only 27 patients were randomly allocated to the drug out of the target of 58 patients, and only 20 patients completed the study owing to an increased frequency of adverse effects in those receiving seletalisib. A trend for improvement in ESSDAI and ESSPRI scores was found in those receiving seletalisib compared with those receiving placebo, but this trend did not achieve statistical significance[78], possibly owing to a loss of statistical power (estimated at 36% instead of 80%). These results do not support the use of seletalisib in pSS, but also do not exclude the possible development of other, more selective, PI3Kδ inhibitors.

Germinal centre-like structures

Germinal centre-like structures are present in all sites of chronic inflammation, such as synovial tissue in rheumatoid arthritis and salivary glands in pSS[79]. These structures share similarities with bona fide germinal centres, including morphology (having a light zone and a dark zone) and function, as these structures are where somatic hypermutation, BCR editing and immunoglobulin class switching occur[80]. In pSS, these structures support autoantibody production (particularly anti-SSA/Ro antibodies)[81] and are associated with lymphoma occurrence[82]. Major advances have been made in understanding the mechanisms that support the formation and persistence of germinal centre-like structures, which have helped to identify new therapeutic targets[83]. Nevertheless, as discussed in this section, these new targeted therapies have so far failed to demonstrate efficacy in pSS, which does not encourage further studies targeting this pathway in pSS.

Lymphotoxin. Lymphotoxin promotes the recruitment of B cells and T cells to germinal centre-like structures, as well as the formation of high endothelial venules within these structures[83]. Baminercept, a lymphotoxin-β receptor IgG1 fusion protein, has been assessed in a phase II RCT that included 52 patients with pSS[84] (TABLE 1). Despite evidence of a significant decrease in serum CXCL13 concentrations in those who received baminercept compared with those who received placebo, the primary end point (improvement in stimulated whole saliva flow rate at week 24) was not achieved[84]. In addition, no significant improvements in ESSDAI scores were observed.

CD4⁺ T follicular helper cells. T follicular helper (T_{FH}) cells are the main protagonists in the persistence of germinal centre-like structures, as they sustain B cells and promote the generation of high-affinity antibodies[85]. Blockade of the T_{FH} cell pathway via inhibition of the inducible T cell co-stimulator (ICOS)–ICOS ligand (ICOSL) axis or IL-21 signalling seems promising in pSS[86]. Membrane expression of ICOS by T_{FH} cells determines their survival and controls their anatomical localization within the B cell follicle. An anti-ICOSL antibody (MEDI5872) has been assessed in pSS in a phase II trial, the preliminary results of which have been reported[87]. Similar to the results for baminercept, despite the demonstration of efficacy on several biological outcomes (titres of IgA, IgG and IgM rheumatoid factor), the primary outcome of change in ESSDAI score from baseline to week 12 was not met[87]. No safety concerns were reported.

T cell co-stimulation

Abatacept (a cytotoxic T lymphocyte-associated protein 4–Fc fusion protein) modulates co-stimulatory signals between CD80 and CD86, and CD28, that are required for full T cell activation[88]. Abatacept has been assessed in pSS in two open-label studies. In one study, 11 patients with pSS were treated with eight infusions of abatacept over 24 weeks[89]. Treatment was associated with a reduction in glandular inflammation and an increase in saliva production[89]. In the other study (ASAP), which included 15 patients with pSS, ESSDAI and ESSPRI scores improved and serum rheumatoid factor and IgG concentrations decreased significantly during treatment, but salivary and lacrimal gland function was not modified[90]. Two phase III RCTs with abatacept have been conducted[91,92]. In both studies, despite biological efficacy (decreases in serum IgG and rheumatoid factor concentrations), abatacept did not meet the primary efficacy outcome of improvement in ESSDAI score from baseline to week 24. No safety concerns were detected. Thus, with the current available outcomes, abatacept does not seem to improve the clinical condition of patients with pSS.

Light zone
An area of the germinal centre in which B cells depend on T cell help and are selected by competing for antigens presented by follicular dendritic cells.

Dark zone
An area of the germinal centre that contains large centroblasts that are rapidly proliferating and undergoing somatic mutation.

Box 3 | **A core set of outcome measures for future clinical trials in primary Sjögren syndrome**

Domains that could be part of a future responder index include:

- Systemic disease activity (measured by the Clinical EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) or ESSDAI)
- Patient-reported symptoms (measured by the EULAR Sjögren's Syndrome Patient Reported Index or by individual visual analogue scales for pain, fatigue and dryness (oral and ocular))
- Tear gland function (measured by the ocular staining score or Schirmer's test)
- Salivary gland function (measured by whole saliva flow (unstimulated or stimulated) or by salivary gland ultrasonography)
- Biological domain (measured by serum rheumatoid factor or complement concentration, or by serum IgG concentration)

Type I interferons

The interferon signature is one of the main immunological characteristics of pSS. In one study, around two-thirds of patients with pSS presented with a high level of interferon activity that was associated with hypergammaglobulinaemia, autoantibody positivity (antinuclear antibodies and anti-SSA/Ro antibodies), and a high focus score[93]. Thus, targeting interferons is a promising strategy for the treatment of pSS (FIG. 1). Three approaches to blocking interferons are currently being assessed. First, a monoclonal antibody (BIIB059) that targets blood dendritic cell antigen 2 on pDCs, the professional producers of IFNα, has been assessed in a phase II study in patients with active cutaneous lupus erythematosus. The preliminary results of this study were promising and showed a statistically significant dose-related improvement in disease activity compared with placebo[94]. Given that the interferon signature is shared by SLE and pSS, targeting pDCs could also be of interest in pSS. Second, the Janus kinase inhibitor tofacitinib, which inhibits both type I and type II interferons is being assessed in an RCT in patients with pSS, which is currently recruiting[95]. Third, ustekinumab, an anti-IL-12p40 antibody that inhibits IL-12 and IL-23, and thus inhibits the IFNγ pathway, is being assessed in a phase I open-label trial that is also currently recruiting[96].

Advances in trial design

Work is still underway to improve the treatment of patients with pSS, and clinical trial design in this field is still evolving. Adoption of the current trial design that uses ESSDAI scores as an inclusion criterion and as an outcome measure has meant that, for the first time, some (but not all) RCTs have succeeded in demonstrating treatment efficacy (TABLE 2). However, experience acquired from these trials with the new design suggests that outcomes other than the ESSDAI might also improve with treatment, such as the ESSPRI, whole saliva flow rate and biological components such as IgG and rheumatoid factor concentrations. Different selection criteria can now be used to identify those patients who are most likely to respond to treatment: for example, patients with recent-onset disease, those with moderate-to-high disease activity (an ESSDAI score of ≥5), those with some specific systemic manifestations, those with B cell activation biomarkers or those with residual glandular function. However, the more criteria that are added, the more difficult it becomes to recruit participants[97,98]. The new design also excludes a large proportion of patients who have no systemic complications, but who do have a high disease burden owing to high levels of fatigue, pain and dryness. In addition, patients with the most active form of pSS cannot currently be included in RCTs, as it is unethical to expose such individuals to placebo.

Research is currently focused on the development of a disease-specific responder index and on ways to extend recruitment to patients with no active systemic complications (the majority of patients with pSS) (BOX 3). A first step has been the development of a composite score based on the results of the ASAP-III study to demonstrate that abatacept is superior to placebo[91]. This score, Composite of Relevant Endpoints for Sjögren's Syndrome (CRESS), has the advantage of including five domains that encompass all of the major disease features (systemic activity, saliva function, tears function, patient-reported outcomes and biological features (BOX 3)), but has the disadvantage of being developed from a single study[99]. The international European Innovative Medicines Initiative 2 programme NECESSITY project aims to develop a new consensual outcome, the Sjögren's Syndrome Tool for Assessing Response to Treatments (STAR), which will be a responder index. In the first steps of STAR development, the same five domains included in CRESS were confirmed by expert and patient consensus (Delphi process) to represent the core set of measures to be included in STAR. STAR will be developed using data from nine existing trials but also expert and patient opinion. A preliminary version of STAR will be validated in a new prospective trial conducted by the NECESSITY consortium. The objective is that this tool could be used in future clinical trials involving all types of patients with pSS — those with systemic manifestations (an ESSDAI score of ≥5), but also those with symptoms but no systemic complications, for whom there is an important unmet need.

Conclusions

Progress in understanding the pathogenesis of pSS has enabled the development of several new targeted therapies for this disease, of which those targeting B cells are the most promising. In addition to the identification of valuable therapeutic targets, methodological advances in how clinical trials are conducted has helped demonstrate for the first time treatment efficacy in pSS; however, work is still in progress to improve clinical trial design and refine outcome assessment in patients with pSS. Finally, several RCTs are currently underway to evaluate the efficacy and safety of drugs that target the newly identified pathways, the results of which should be available in the next 2 years. One can expect that the combination of new targeted therapies, new outcome measures and better phenotyping of patients will help improve the management of disease in patients with pSS.

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Focus score
The number of foci (dense aggregates of ≥50 mononuclear cells) per 4 mm² over the whole glandular area of a salivary gland.

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Insights into the biology and therapeutic implications of TNF and regulatory T cells

Benoit L. Salomon

Abstract | Treatments that block tumour necrosis factor (TNF) have major beneficial effects in several autoimmune and rheumatic diseases, including rheumatoid arthritis. However, some patients do not respond to TNF inhibitor treatment and rare occurrences of paradoxical disease exacerbation have been reported. These limitations on the clinical efficacy of TNF inhibitors can be explained by the differences between TNF receptor 1 (TNFR1) and TNFR2 signalling and by the diverse effects of TNF on multiple immune cells, including FOXP3$^+$ regulatory T cells. This basic knowledge sheds light on the consequences of TNF inhibitor therapies on regulatory T cells in treated patients and on the limitations of such treatment in the control of diseases with an autoimmune component. Accordingly, the next generation of drugs targeting TNF is likely to be based on agents that selectively block the binding of TNF to TNFR1 and on TNFR2 agonists. These approaches could improve the treatment of rheumatic diseases in the future.

Tumour necrosis factor (TNF) is an inflammatory cytokine that is detected in the blood within minutes after an injury and has a major protective role in infectious diseases. In the late 1980s, TNF was detected in the joints of patients with rheumatoid arthritis (RA)[1,2]. A few years later, overexpression of TNF in transgenic mice was shown to induce autoimmune arthritis[3]. Agents that block this cytokine, termed TNF inhibitors, include monoclonal antibodies (mAbs) and soluble TNF receptors. Anti-TNF therapy was first tested in patients with sepsis without clear success and then repurposed for the treatment of RA in the early 1990s[1,2]. TNF inhibitors are now widely used and have greatly improved the medical care of patients with RA, juvenile idiopathic arthritis, psoriasis, psoriatic arthritis and ankylosing spondylitis. Five original TNF inhibitors and numerous biosimilars have been approved, mostly for the treatment of arthritis, psoriasis or ankylosing spondylitis (TABLE 1). However, not all patients respond to TNF inhibitor treatment. One-third of patients with RA have to stop taking these drugs within the first year because of insufficient efficacy or adverse events[4]. About 20% of patients with psoriasis do not respond to treatment with a TNF inhibitor and around one-third of initial responders lose response over time[5]. Similar efficacy profiles are observed for patients with inflammatory bowel disease (IBD)[6]. Although this Review focuses on the effects of TNF inhibitors in rheumatic diseases, particularly RA, I also discuss their effects and use in the treatment of other autoimmune and inflammatory diseases to illustrate the role and mechanisms of these agents in general.

Treatment with TNF inhibitors is also associated with adverse effects, such as infections, which are explained by the intrinsic anti-inflammatory effects of these agents. More intriguing (and counterintuitive) is the paradoxical exacerbation of pre-existing autoimmune disease or the development of new-onset autoimmune disease following TNF inhibitor therapy. Rarely, treated patients can develop lupus-like syndrome, vasculitis, antiphospholipid syndrome or sarcoidosis. For example, the reported prevalence of systemic lupus erythematosus among recipients of TNF inhibitor therapy is 0.1–0.2%[7–9]. A few patients develop organ-specific autoimmune conditions, such as interstitial lung disease, optical neuritis, demyelinating neuropathy, multiple sclerosis (MS), psoriasis or autoimmune hepatitis, with the highest prevalence (2.00–5.00%) reported for psoriasis and the lowest (0.05–0.20%) for demyelinating disease. Several reviews have discussed in depth the spectrum of autoimmune diseases occurring in patients treated with TNF inhibitors[7–10], among which MS is of particular interest. In the late 1990s, before the increased risk of demyelinating neuropathy associated with TNF inhibitor treatment was known, two clinical trials investigated the efficacy of TNF inhibitors in MS. However, these drugs induced unexpected disease exacerbations that led to the worldwide contraindication of these drugs in these patients[11,12]. These observations sparked intense interest in elucidating why not all patients respond to

Sorbonne Université, INSERM, CNRS, Centre d'Immunologie et des Maladies Infectieuses (CIMI-Paris), Paris, France.

e-mail: benoit.salomon@inserm.fr

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Key points

- Tumour necrosis factor (TNF) is a major inflammatory cytokine that has deleterious effects in several rheumatic and autoimmune diseases as attested by the success of TNF inhibitor therapy.
- Some patients do not respond to TNF inhibitors and others develop paradoxical autoimmune exacerbations that can be explained by the immunoregulatory role of TNF.
- The pro-inflammatory and anti-inflammatory properties of TNF are largely segregated by the capacity of this cytokine to bind to TNF receptor 1 (TNFR1) and TNFR2, respectively.
- The anti-inflammatory effects of TNF are explained by its capacity to increase the proliferation, stability and suppressive function of FOXP3$^+$ regulatory T cells via TNFR2 signalling.
- Antagonists of TNFR1 and agonists of TNFR2 constitute a new generation of drugs that might be more effective and have fewer adverse effects than classical TNF inhibitors.

TNF inhibitor therapy, in the development of biomarkers to predict response and in understanding why some patients develop paradoxical autoimmunity.

This Review focuses on the effects of TNF on inflammation and immunity. The pro-inflammatory and regulatory roles of TNF (both of which are now well established) are described and the effects of this cytokine on diverse aspects of regulatory T (T_{reg}) cell biology, including their expansion, differentiation and suppressive function, are addressed. Finally, the effect of TNF inhibitors on T_{reg} cells is described and the potential candidates for the next generation of drugs that target TNF or its receptors are explored. Although TNF also plays important roles in organogenesis, development of lymphoid organs, protection of tissues in the nervous system, heart and joints[13–15], and inhibition of tumorigenesis[16], these topics are outside the scope of the present Review and will not be considered.

The two Janus faces of TNF

TNF has complex regulatory and pro-inflammatory effects in diseases with an autoimmune component[13–15] such as RA[17]. This cytokine is produced under various inflammatory conditions by multiple cell types and exists in two forms: a soluble form that acts as a ligand and a membrane-bound form that can act as either a ligand or a receptor[18–20]. Furthermore, TNF can induce multiple downstream signalling pathways[9] as a result of binding to two different receptors, TNF receptor 1 (TNFR1) and TNFR2, which are structurally related but have divergent biological properties. TNFR1 is broadly expressed, whereas TNFR2 is expressed mostly by T cells, certain myeloid and endothelial cells, and some cells of the central nervous system[21,22].

The next sections describe the distinct functions of TNFR1 and TNFR2 and discuss the pro-inflammatory and anti-inflammatory effects of TNF on innate immune cells and lymphocytes as well as presenting information on the cellular source of TNF.

TNFR1 and TNFR2

TNF is one of the most potent pro-inflammatory cytokines[23], which explains the success of TNF inhibitor therapy in diseases with an inflammatory component. However, the paradoxical development or exacerbation of autoimmune disease in some patients treated with these drugs reveals the anti-inflammatory aspect of this cytokine, which is partly explained by effects downstream of TNFR2. Polymorphisms in *TNFRSF1B*, which encodes TNFR2, are frequently observed in patients with rheumatic diseases (RA, systemic lupus erythematosus, ankylosing spondylitis and systemic sclerosis) or IBD[24]. These mutations seem to alter the binding kinetics between TNF and TNFR2 and lead to the inhibition of downstream NF-κB signalling, which suggests that TNFR2 signalling plays a protective role in these diseases[25]. A single-nucleotide polymorphism in *TNFRSF1A*, which encodes TNFR1, is specifically associated with an increased risk of MS. This allele results in the expression of a novel soluble form of TNFR1 that binds to and blocks TNF and therefore mimics the MS-exacerbating effect of TNF inhibitor therapy[26]. Other mutations in *TNFRSF1A* that cause TNFR1 misfolding and endothelium reticulum stress are found in patients with periodic fevers[27].

The differential functions of TNFR1 and TNFR2 in rheumatic and autoimmune diseases have been defined in mouse models. Generally, *Tnfrsf1a*-knockout mice

Table 1 | **Clinically approved TNF inhibitors in the USA and Europe**

Drug	Molecule	Biosimilars	Approved rheumatic disease indications[a]
Etanercept	Human TNFR2–IgG1 Fc fusion protein	Benepali, Erelzi, Nepexto	RA, JIA, psoriatic arthritis, plaque psoriasis, AS
Infliximab	Humanized chimeric anti-TNF IgG1/κ mAb	Remsima, Inflectra, Flixabi, Ixifi, Zessly, Avsola	RA, psoriatic arthritis, plaque psoriasis, AS
Adalimumab	Fully human anti-TNF IgG1/κ mAb	Exemptia, Adfrar, Amjevita, Cyltezo, Amgevita, Solymbic, Imraldi, Halimatoz, Hefiya, Hyrimoz, Hulio, Idacio, Kromeya, Hadlima, Abrilada, Amsparity	RA, JIA, psoriatic arthritis, plaque psoriasis, AS, hidradenitis suppurativa, non-infectious uveitis
Certolizumab pegol	PEGylated human Fab fragment of anti-TNF mAb	NA	RA (Europe only), psoriatic arthritis
Golimumab	Fully human anti-TNF IgG1/κ mAb	NA	RA, psoriatic arthritis, AS

AS, ankylosing spondylitis; JIA, juvenile idiopathic arthritis; mAb, monoclonal antibody; NA, not applicable; RA, rheumatoid arthritis; TNF, tumour necrosis factor; TNFR, TNF receptor. [a]Disease indications for biosimilars can differ from those of the original drug and depend on the countries where they are registered.

Table 2 | **Pathogenic and protective roles of TNFR1 and TNFR2 in models of rheumatic and autoimmune diseases**

Mouse model	*Tnfrsf1a* knockout	*Tnfrsf1b* knockout	TNFR1 antagonist	TNFR2 agonist	Refs
Collagen-induced arthritis	Attenuated	Exacerbated	Attenuated	Attenuated	119,121,184,200,201
Antigen-induced arthritis	ND	Exacerbated	ND	ND	201
DTHA	ND	Exacerbated	ND	ND	115
Arthritis in TNF-transgenic mice	Attenuated	Exacerbated	ND	ND	202
EAE	Attenuated	Exacerbated	Attenuated	Attenuated	28,132,187,191,203–208

DTHA, delayed-type hypersensitivity arthritis; EAE, experimental autoimmune encephalomyelitis; ND, not determined; TNF, tumour necrosis factor; TNFR, TNF receptor.

have reduced disease severity, whereas *Tnfrsf1b*-knockout mice develop exacerbated disease (TABLE 2). In addition, treatment with either TNFR1 antagonists or TNFR2 agonists suppresses disease symptoms in mouse models of arthritis and in mice with experimental autoimmune encephalomyelitis (EAE), a model of MS, further supporting a pathogenic role of TNFR1 and a protective role of TNFR2 (TABLE 2). A pathogenic role of TNFR1 and a protective role of TNFR2 have also been observed in mouse models of IBD, at least during the chronic phase of the disease[15,28]. Thus, TNFR1 and TNFR2 seem to be pathogenic and protective, respectively, in some autoimmune and chronic inflammatory diseases.

Effects of TNF on innate immunity

Pro-inflammatory effects. The pro-inflammatory effects of TNF on innate immunity involve several distinct mechanisms (FIG. 1). TNF is one of the main drivers of acute inflammation because it activates endothelial cells, induces chemokine release, and promotes intense and early (within hours) recruitment of neutrophils and monocytes via both TNFR1 and TNFR2 (REFS[29,30]). Acute inflammation is also attributed to the TNF-mediated activation of canonical NF-κB signalling, which leads to the early induction of inflammatory cytokines, including TNF itself, IL-6, IL-8 and IL-1β[14]. TNF also sustains inflammation through the activation of receptor-interacting protein kinase 1 (RIPK1) and RIPK3, which promote necroptosis and the release of inflammatory compounds termed damage-associated molecular patterns[31]. In addition, via TNFR1 signalling, TNF promotes innate immunity by favouring the maturation of dendritic cells[32,33].

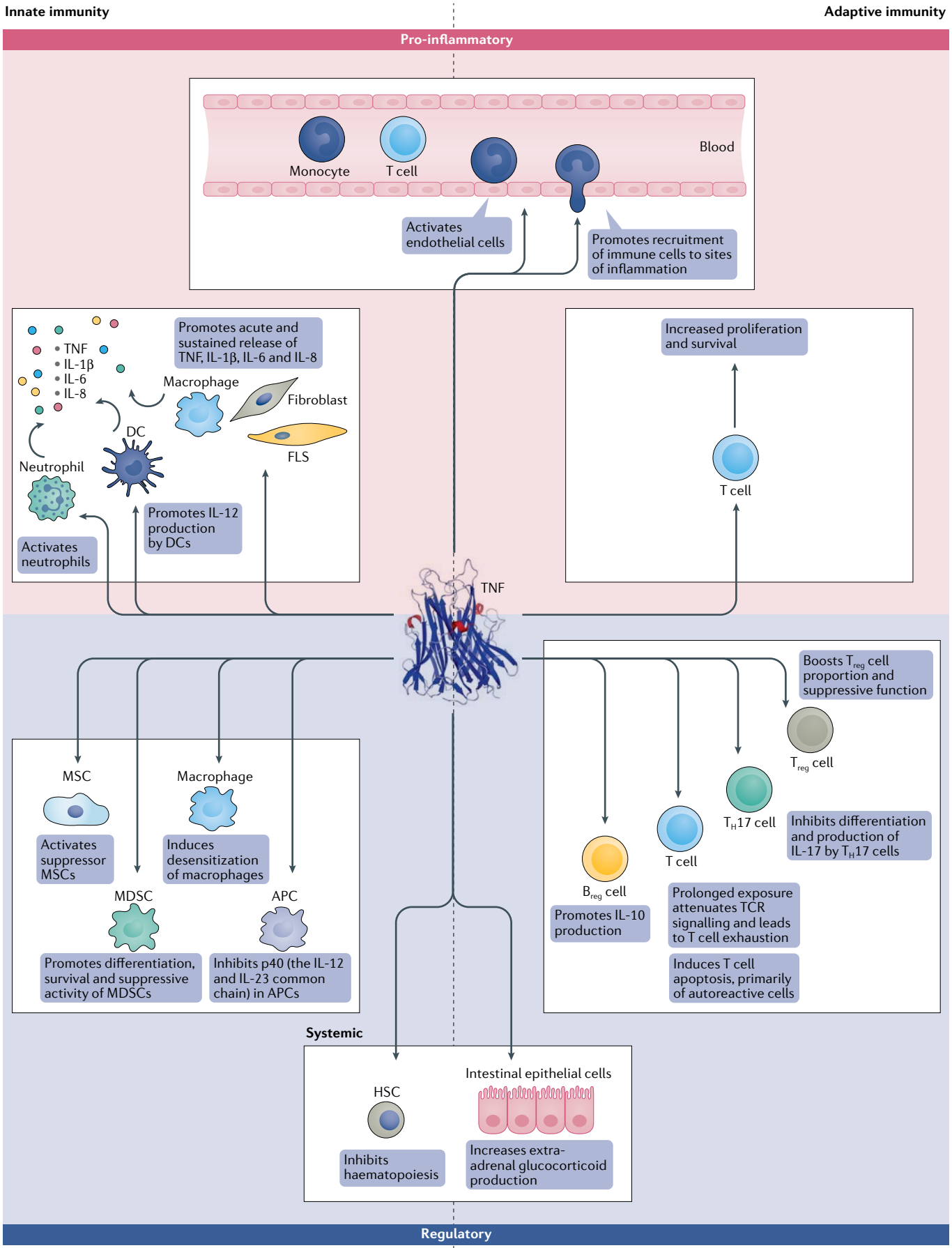
Regulatory effects. The immunoregulatory functions of TNF are likely to involve multiple mechanisms (FIG. 1). TNF might promote the extra-adrenal production of immunoregulatory glucocorticoids[34] and inhibit haematopoiesis[35]. TNF also stimulates innate immunosuppressive cells (via TNFR2) and activates mesenchymal stem cells, which produce increased levels of immunosuppressive prostaglandin E2 (PGE_2), as has been shown in synovial fluid from patients with RA[36,37]. TNF also promotes immunosuppression by favouring either the differentiation or the suppressive function of myeloid-derived suppressor cells through an increase in their production of reactive oxygen species, arginase 1 and inducible nitric oxide synthase[38–42].

Effects on dendritic cells, monocytes and macrophages. Although TNF seems to favour the production of T-bet and IL-12 by dendritic cells[43], other studies suggest that the presence of TNF inhibits the production of p40 (the common chain of IL-12 and IL-23) by dendritic cells, macrophages and monocytes[44–46]. These divergent findings could be explained by differential actions of TNF depending on the maturation stage and type of both antigen-presenting cells and dendritic cell subsets.

In addition, TNF can either promote or inhibit macrophage activation, effects that are both probably mediated by TNFR1 (reviewed elsewhere[14]). The early response of macrophages to incubation with TNF, observed after a few hours, is dependent on both NF-κB and MAPK and involves the expression of genes encoding various inflammatory molecules and cytokines. This initial response is followed (after 24 hours) by a state of desensitization, also called cross-tolerance or endotoxin tolerance. Desensitized (also termed tolerized) macrophages are unable to produce inflammatory factors when stimulated by potent activators such as Toll-like receptor ligands. The mechanism of desensitization involves NF-κB inhibition following the activation of glycogen synthase kinase 3 (GSK3) and TNF-induced protein 3 (TNFAIP3)[47]. Tolerized macrophages have a transiently reduced capacity to produce IL-12 and IL-23, which are pro-inflammatory[46,48]. The physiological role of cross-tolerance is probably the prevention of life-threatening inflammation in the context of overwhelming macrophage activation by pathogens and Toll-like receptor ligands. Ultimately, after prolonged incubation with TNF, specifically in the presence of type I interferons, macrophages overcome this desensitized state and recover their inflammatory function by modifying their metabolism and epigenetic status[49,50].

Effects of TNF on lymphocytes

Pro-inflammatory effects. TNF can either promote or suppress immunity through its differential effects on lymphocytes (FIG. 1). The pro-inflammatory effects of TNF result from the co-stimulation of T cells, mainly via TNFR2. TNF activates the NF-κB and AKT signalling pathways that lead to increased T cell proliferation and survival, which are associated with increased levels of BCL-2, BCL-XL, IL-2 and survivin[51–56]. However, the co-stimulatory effect of TNF binding to TNFR2 on conventional T cells seems to be of marginal importance


Innate immunity
Adaptive immunity
Pro-inflammatory
Blood
Monocyte
T cell
Activates endothelial cells
Promotes recruitment of immune cells to sites of inflammation
TNF
IL-1β
IL-6
IL-8
Promotes acute and sustained release of TNF, IL-1β, IL-6 and IL-8
Macrophage
Fibroblast
DC
FLS
Neutrophil
Promotes IL-12 production by DCs
Activates neutrophils
Increased proliferation and survival
T cell
TNF
Boosts T_{reg} cell proportion and suppressive function
T_{reg} cell
T_H17 cell
Inhibits differentiation and production of IL-17 by T_H17 cells
T cell
B_{reg} cell
Promotes IL-10 production
Prolonged exposure attenuates TCR signalling and leads to T cell exhaustion
Induces T cell apoptosis, primarily of autoreactive cells
MSC
Activates suppressor MSCs
Macrophage
Induces desensitization of macrophages
MDSC
APC
Promotes differentiation, survival and suppressive activity of MDSCs
Inhibits p40 (the IL-12 and IL-23 common chain) in APCs
Systemic
HSC
Inhibits haematopoiesis
Intestinal epithelial cells
Increases extra-adrenal glucocorticoid production
Regulatory

◀ Fig. 1 | **The pro-inflammatory and anti-inflammatory activities of TNF are driven by effects on innate and adaptive immunity.** Tumour necrosis factor (TNF) is a major pro-inflammatory cytokine (top panel) that activates both innate (left side) and adaptive (right side) immunity. TNF promotes the recruitment of leukocytes, favours the production of other pro-inflammatory cytokines, activates neutrophils and participates in the co-stimulation of conventional T cells. TNF also has regulatory activities (bottom panel) such as inhibition of haematopoiesis, increased glucocorticoid production, activation of suppressive cells (such as mesenchymal stem cells (MSCs) and myeloid-derived suppressor cells (MDSCs)) or altering the function of dendritic cells (DCs) and macrophages. TNF also regulates immunity by promoting IL-10-producing B cells, inducing T cell apoptosis, altering T cell receptor (TCR) signalling, inhibiting T helper 17 (T_H17) cell differentiation, and boosting numbers and function of regulatory T (T_{reg}) cells. APC, antigen-presenting cell; B_{reg}, regulatory B; FLS, fibroblast-like synoviocyte; HSC, haematopoietic stem cell.

compared with its strong effect on T_{reg} cells[57], which is extensively discussed below.

Regulatory effects. Although one report suggests that TNF promotes the expression of IL-10 by B regulatory cells[58], much more is known about the inhibitory effects of TNF on T cells. Prolonged exposure to TNF attenuates T cell receptor signalling by impairing store-operated calcium influx[59,60] and also favours T cell exhaustion; in one report, TNF blockade during chronic infection with lymphocytic choriomeningitis virus abrogated the inhibitory gene expression signature[61]. TNF is able to induce activation-induced cell death via TNFR1 engagement[62]. Interestingly, TNFR2 signalling also seems to increase T cell apoptosis by interfering with signalling pathways downstream of TNFR1 (REF.[63]). However, TNFR2-dependent cell death might specifically occur in autoreactive T cells, which have altered TNFR2 signalling[25,62,64–68]. Crosstalk between TNFR1 and TNFR2 signalling is discussed in more detail in subsequent sections.

TNF also inhibits the differentiation of T helper 17 (T_H17) cells by increasing IL-2 production[69] and decreases IL-17 production by conventional T cells and effector T_{reg} cells via the activation of TNFAIP3 (REFS[70,71]). This mechanism might explain the increase in numbers of T_H17 cells described in *Tnfrsf1a*-knockout mice or after treatment with TNF inhibitors in mouse models of RA and psoriasis[44–46,72,73]. A similar increase in T_H17 cells has been reported specifically in non-responding patients with RA treated with TNF inhibitors[44,74]. Interestingly, these non-responding patients showed a T_H1-mediated and T_H17-mediated immune response against the TNF inhibitor, which might have precipitated their lack of clinical response[75]. Finally, in the late 2000s, the regulatory properties of TNF were proposed to result from its effects on T_{reg} cells. These mechanisms are extensively discussed below.

Cellular sources of TNF

Multiple cell types are able to produce TNF, but the immune cells that produce this cytokine in the highest amounts are myeloid cells and activated T cells[76]. The role of TNF produced by these two cell types in rheumatic and autoimmune diseases has been investigated using genetically modified mice with conditional knockout of TNF only in myeloid cells or only in T cells. In mice with collagen-induced arthritis, conditional knockout of TNF in myeloid cells leads to reduced disease severity, showing that the TNF produced by these cells contributes to the pathology. By contrast, mice with TNF-deficient T cells have exacerbated arthritis, suggesting a protective role of the TNF produced by T cells[77]. Similarly, mice with EAE and TNF-deficient myeloid cells have attenuated disease, which is either delayed in onset or reduced in severity depending on the model[76,78]. Finally, the role of TNF produced by B cells has been analysed in mice with TNF-deficient B cells. These mice have reduced arthritis and reduced levels of autoantibodies[77].

TNF structure and signalling

Soluble and transmembrane TNF. Crystallographic studies show that trimers of TNF interact with trimers of either TNFR1 or TNFR2 (REFS[79,80]). This trimeric association of the cytokine with its receptor is characteristic of the TNF superfamily and is critical for downstream signalling[63,81].

TNF is initially produced as a transmembrane molecule that can be processed by disintegrin and metalloproteinase domain-containing protein 17 (also known as TNF-converting enzyme (TACE)) encoded by the *ADAM17* gene[82]. Thus, activated myeloid and T cells produce transmembrane TNF and secreted soluble TNF, which are both biologically active[77,83,84]. The role of soluble TNF in the pathophysiology of rheumatic and autoimmune diseases has been assessed in mice engineered to express a TNF protein that cannot be cleaved by TACE. Thus, these mice produce normal levels of transmembrane TNF but no soluble TNF[83]. Importantly, such mice do not develop EAE or arthritis, showing that soluble TNF but not transmembrane TNF contributes to these diseases[77,83,84]. By contrast, mice with global deletion of *TNF* (full knockout) still develop EAE, which suggests that transmembrane TNF has protective effects in the disease[83].

Despite a similarly high binding affinity for its two receptors, trimeric soluble TNF triggers TNFR1 signalling much more efficiently than it does TNFR2 signalling[85]. Although this observation requires further confirmation, TNFR1 signalling is usually considered to be triggered by both soluble and transmembrane TNF, whereas TNFR2 signalling is preferentially triggered by transmembrane TNF[86]. These observations suggest that soluble TNF (notably, that produced by myeloid cells at the onset of a rheumatic or autoimmune disease) binds to TNFR1 to promote inflammation and precipitate the disease, whereas transmembrane TNF (probably that expressed by both myeloid and T cells) has regulatory effects mostly derived from triggering TNFR2. These observations might have implications for the use of TNF inhibitors. For example, etanercept (a TNFR2–Fc fusion protein) can efficiently block soluble TNF (as well as α3 and α2β1 lymphotoxins) but not transmembrane TNF, whereas anti-TNF mAbs block both soluble and transmembrane TNF[82]. This concept also has implications for the design of next-generation TNF inhibitors, as discussed below.

TNFR1 and TNFR2 signalling pathways. The TNFR1 and TNFR2 signalling pathways are complex and have been extensively reviewed elsewhere[14,15,63,86]. Accordingly,

only the pathways most relevant to this Review are outlined here. Most of the available knowledge has been obtained in cell lines and non-immune cells and deserves further investigation to confirm its relevance in immune cells.

Upon binding of trimeric TNF to TNFR1, the cytoplasmic tail of the receptor recruits the adaptor protein TNFR1-associated death domain (TRADD) via its death domain. TRADD can then interact with other adaptor proteins, such as TNF receptor-associated factor 2 (TRAF2), and kinases such as RIPK1 or cellular inhibitor of apoptosis 1 (cIAP1) and cIAP2. The resulting molecular complex, named complex 1, is able to phosphorylate and ubiquitylate several other molecules, ultimately leading to potent activation of the canonical NF-κB and MAPK pathways. Members of these pathways, such as JUN N-terminal kinase (JNK) and p38, in turn activate the AP1 complex[14,15,63,86]. This complex 1-dependent signalling pathway favours cell proliferation and survival. Alternatively, TNFR1 and TRADD interact with the Fas-associated death domain (FADD) adaptors RIPK1 and RIPK3, forming the complex 2 interactome, which is able to induce cell death either by apoptosis (via caspase 8 activation) or necroptosis (via mixed lineage kinase domain-like (MLKL) protein activation)[14,15,63,86].

Complex 1 and complex 2 are downstream effectors of TNFR1 signalling. Complex 1 is probably involved in most of the effects of TNF on dendritic cells and macrophages, including the activation of inflammatory target genes and the production of inflammatory cytokines. Complex 2 is involved in TNF-dependent, activation-induced cell death and in the formation of inflammation-dependent damage-associated molecular patterns.

The signal transduction pathway downstream of TNFR2 lacks a death domain and involves different adaptors. The binding of transmembrane TNF to TNFR2 recruits TRAF1 or TRAF2 adaptors to this receptor, leading to the activation of cIAP1 or cIAP2 kinases and of the canonical and non-canonical NF-κB, JNK and AKT pathways that promote cell proliferation and survival[14,15,63,86–88]. These pathways are likely to be involved in the TNF-dependent activation of mesenchymal stem cells and myeloid-derived stem cells as well as in T cell co-stimulation. TRAF2 recruitment to TNFR2 also decreases the amount of cytoplasmic TRAF2, which interferes with TNFR1 signalling by favouring the formation of (cell death-promoting) complex 2 to the detriment of (survival-promoting) complex 1 (REF.[63]). This crosstalk between the TNFR1 and TNFR2 signalling pathways seems to be responsible for TNFR2-dependent T cell death[89].

TNF reverse signalling. Reverse (extracellular to intracellular) signalling induced by transmembrane TNF has been described but remains poorly documented. This phenomenon is only outlined here as it has been reviewed elsewhere[18–20]. In this context, TNFR1 or TNFR2 can act as ligands for transmembrane TNF, which can function as a cell receptor transducing a signal in several different situations. For example, TNFR2-expressing T cells promote the increased expression of TNF in monocytes and/or macrophages via transmembrane TNF, a phenomenon that has been observed in the joints of patients with RA. Additionally, TNFR1-expressing endothelial cells induce cross-tolerance in monocytes and/or macrophages via transmembrane TNF. Finally, TNF inhibitors are also able to bind to transmembrane TNF and thereby induce the apoptosis of transmembrane TNF-expressing cells; this phenomenon has been observed, for instance, in T cells and synovial macrophages from patients with RA[90,91]. The mechanism of TNF reverse signalling involves increased intracellular levels of calcium and TGFβ and activation of the MAPK–ERK pathway. However, the in vivo relevance of reverse signalling is difficult to assess because this phenomenon has been poorly described. I consider that reverse signalling might contribute to the spectrum of effects of TNF and might play an important role in inducing cross-tolerance of macrophages and in the death of transmembrane TNF-expressing cells induced by the administration of TNF inhibitors.

Integrative view of TNF functions

Here, I present a simplified and integrated view of what I believe is the major role of TNF in immunity (FIG. 1). TNF is one of the most potent inflammatory cytokines owing to its capacity to activate endothelial cells, neutrophils, macrophages and dendritic cells, leading to leukocyte recruitment and massive release of inflammatory cytokines at sites of inflammation. Most of these pro-inflammatory phenomena are mediated by TNFR1 signalling. Besides its pro-inflammatory functions, TNF also has anti-inflammatory (regulatory) functions, although their role and mechanisms in immunity are yet to be clarified. The regulatory functions involve TNF-dependent activation of suppressive cells such as mesenchymal stem cells, myeloid-derived stem cells and, of course, T_{reg} cells (which are extensively discussed below). TNF might also promote the death or exhaustion of T cells and inhibit pathogenic T_H17 cells. Most of these regulatory phenomena are mediated by TNFR2 signalling.

The end result of TNF blockade depends on the type of autoimmune disease present and the timing of treatment. Blocking the interaction between TNF and TNFR1 led to increased numbers of pathogenic T_H1 and T_H17 cells in mouse models of arthritis and psoriasis[44–46,72,73]. This increase was associated with exacerbation of psoriasis (as might logically be expected) but surprisingly with attenuation of arthritis because this treatment also blocked the migration of pathogenic T cells to the joints[46]. Similarly, patients with RA treated with TNF inhibitors have increased numbers of circulating T_H1 and T_H17 cells[44,74,75], which could explain some of the paradoxical inflammation observed in a subset of these patients. The effects of TNF blockade could also depend on the timing of treatment in relation to the course of disease. To investigate the role of TNF signalling via TNFR2 in a model of collagen-induced arthritis, *Tnfrsf1a*-knockout mice were treated with TNF either on days 2–20 or on days 22–40 after disease induction[92]. Interestingly, early TNF treatment led to disease exacerbation, whereas late TNF treatment led to the

attenuation of arthritis. An opposite effect of TNF that is similarly dependent on the stage of disease progression has been documented in non-obese diabetic mice; TNF seems to exacerbate diabetes in young mice by activating dendritic cells and to attenuate it in adult mice by inhibiting conventional T cells and promoting T_{reg} cell activation[59,93–95].

Effects of TNF on T_{reg} cells

T_{reg} cells are master regulators of autoimmune diseases. Mice and humans that are genetically deficient in T_{reg} cells die soon after birth from a massive and systemic autoimmune syndrome, which reveals the critical role of these cells in the suppression of autoimmunity[96]. Functional or quantitative defects of T_{reg} cells have been reported in many human autoimmune diseases[97]. Other indirect evidence supports the concept that T_{reg} cells contribute to human autoimmune diseases. For instance, some biomarkers of disease activity, such as C-reactive protein levels, are inversely correlated with the proportion of T_{reg} cells in patients with RA[98] or IBD[99,100]. Moreover, T_{reg} cell transfer seems to have beneficial effects in patients with various autoimmune diseases[101].

Transcriptomic analyses that compared T_{reg} with conventional T cells in lymphoid tissues showed that several members of the TNFR superfamily, including TNFR2, TNFR superfamily member 4 (OX40), TNFR superfamily member 9 (4-1BB) and TNFR superfamily member 18 (GITR) are included in the T_{reg} cell signature[102]. These molecules are further upregulated upon stimulation of either the T cell receptor (TCR) or T cell-specific surface glycoprotein CD28 and are therefore preferentially expressed by effector T_{reg} cells rather than by resting T_{reg} cells[103,104]. At steady state, 30% of T_{reg} cells express TNFR2 and most of this subset are effector T_{reg} cells that have a stronger suppressive function in vitro than do $TNFR2^{-}$ resting T_{reg} cells[105,106]. Thus, TNFR2 belongs to the T_{reg} cell signature and is a marker of highly suppressive T_{reg} cells.

Effects on T_{reg} cell expansion

Expansion is defined as an increase in cell numbers and results from a combination of increased proliferation, prolonged survival and phenotypic stability. TNFR2 signalling seems to expand T_{reg} cells by increasing all three of these factors.

Initially, TNF and/or TNFR2 co-stimulation were shown to increase T_{reg} cell proliferation in mice[22,107]. Our group and others showed that effector T cells, in particular T_H17 cells, are a major source of the TNF that induces this increase in the T_{reg} cell population in vivo[108–110]. Similar findings were obtained for human T_{reg} cells[84,111,112]. TNF can also substantially prolong T_{reg} cell survival[103]. Indirect evidence indicates that TNFR2 signalling also maintains *FOXP3* expression, which increases the phenotypic stability of T_{reg} cells and therefore their long-term expansion[112–115].

In many of these in vitro studies, soluble TNF was capable of boosting T_{reg} cell expansion. Although transmembrane TNF has a stronger effect than soluble TNF on the induction of TNFR2 signalling[85], strong evidence indicates that soluble TNF can indeed stimulate the expansion of T_{reg} cells by binding to TNFR2. Furthermore, TNFR1 expression has not been detected on T_{reg} cells (unlike TNFR2 expression)[22]. The expansion-promoting effect of soluble TNF on T_{reg} cells was lost in TNFR2-deficient T_{reg} cells and when blocking TNFR2 but not TNFR1 (REF.[113]). Finally, treatment with TNF or TNFR2 agonists induced similar co-stimulation of T_{reg} cells[111]. The capacity of soluble TNF to efficiently induce TNFR2 signalling could be explained by the use of high concentrations of this cytokine or the presence of TNF aggregates with crosslinking properties in the preparations. TNFR2 agonists, which are either multimers of mutated TNF or mAbs that bind only to TNFR2 (discussed in more detail below), strongly co-stimulate T_{reg} cells in both mice and humans[57,103,111,116–118]. In a study of pre-activated T cells, TNFR2 co-stimulation strongly increased the proliferation of T_{reg} cells but had no effect on conventional T cells[57]. The capacity of TNFR2 co-stimulation to promote T_{reg} cell expansion was confirmed in vivo in animals treated with TNFR2 agonists[86,117,119–121].

Although very little is known about TNFR2 signal transduction in T_{reg} cells, transcriptomic analyses showed that the binding of TNF to TNFR2 on purified mouse or human T_{reg} cells induced a gene expression signature indicative of NF-κB pathway activation[103,122]. More precisely, TNFR2 signalling induced nuclear translocation and binding of RelA to its DNA target sequence, which suggests that the canonical NF-κB pathway is activated by TNFR2 signalling in T_{reg} cells. Importantly, the increased proliferation and prolonged survival of T_{reg} cells induced by TNFR2 triggering was severely attenuated in RelA-deficient T_{reg} cells[103,104]. Some evidence also suggests that the non-canonical NF-κB pathway is also activated by TNFR2 signalling in T_{reg} cells, but this observation has to be treated with caution because these assays were conducted on a cell population with a low T_{reg} cell purity[123]. Other data suggest that TNFR2 signalling induces activation of the MAPK pathway, notably via p38 (REFS[124,125]). TNFR2-mediated co-stimulation of T_{reg} cells also induced a glycolytic switch associated with the activation of mammalian target of rapamycin complex 1 (mTORC1) signalling via phosphoinositide-3 kinase (PI3K), although the signalling pathway connecting TNFR2 to PI3K was not identified[57]. Overall, strong evidence indicates that the boost in T_{reg} cell numbers induced by TNFR2 signalling involves activation of the canonical NF-κB pathway. The role of the other signalling pathways mentioned here requires further documentation.

Effects on T_{reg} cell suppressive function

The effects of TNF on the suppressive function of mouse and human T_{reg} cells have been assessed in vitro (TABLE 3). The first of these studies showed no effect of low-dose (≤5 ng/ml) TNF in human cells[98]. Five subsequent reports showed that treatment with TNF, usually at a high dose (50 ng/ml), reduced the suppression of conventional T cell activation by human T_{reg} cells[122,126–129]. By contrast, in vitro studies performed in mouse cells showed that the presence of high amounts of TNF either had no effect or even increased T_{reg} cell-mediated

Table 3 | **Effect of TNF on T_{reg} cell function in vitro**

T_{reg} cell population	Culture conditions	*n*	TNF added	Assay type	Effect of TNF on T_{reg} cell suppression	Ref.
Human $CD4^+CD25^{hi}$	Soluble anti-CD3 and anti-CD28 mAbs	6	Before or during	Cytokine FACS	No change	98
Human $CD4^+CD25^{hi}$	Coated anti-CD3 mAbs	6	Before	Proliferation ^3H	Decreased	128
Human $CD4^+CD25^+$	HBV e-antigen	7	None	Proliferation ^3H	Decreased	127
Human $CD4^+CD25^+$	Coated anti-CD3 mAbs	NR	During	Proliferation FACS, cytokine ELISA	Decreased	129
Human $CD4^+CD25^{hi}$	Coated anti-CD3 mAbs	3	Before or during	Proliferation FACS	Decreased	122
Human $CD4^+CD25^{hi}CD127^{low}$	Coated anti-CD3 and anti-CD28 mAbs	5?	Before	Proliferation FACS	Decreased	126
Human $CD4^+CD25^{hi}CD127^{low}$ $CD45RA^-$	Coated anti-CD3 and anti-CD28 mAbs; APC and soluble anti-CD3 mAbs; APC and coated anti-CD3 mAbs	28	Before or during	Proliferation FACS	No change or increased	133
Mouse $CD4^+CD25^+$	APC and soluble anti-CD3 mAbs	6	Before or during	Proliferation FACS	No change or increased	22
Mouse $CD4^+CD25^+$	APC and soluble anti-CD3 mAbs	3	Before	Proliferation FACS	Increased	107

APC, antigen-presenting cells; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; HBV, hepatitis B virus; mAbs, monoclonal antibodies; *n*, number of healthy individuals; NR, not reported; TNF, tumour necrosis factor; T_{reg}, regulatory T.

suppression of conventional T cell activation[22,107]. Moreover, other evidence also suggests that TNF does not inhibit T_{reg} cell-mediated suppression of conventional T cells and might even increase it. For instance, administration of a TNFR2 agonist to mice with graft-versus-host disease (GvHD) or collagen-induced arthritis promoted T_{reg} cell expansion and had a therapeutic effect[117,119,121]. Additionally, treatment of cultured T_{reg} cells with TNF increased their capacity to suppress colitis or GvHD after transfer[103,130], whereas TNFR2-deficient T_{reg} cells had a reduced capacity to suppress colitis or GvHD[114,131]. However, these observations provide only indirect evidence that TNF either had no effect on or increased T_{reg} cell suppressive function in mice. Indeed, this cytokine might influence other parameters of T_{reg} cell biology (such as proliferation, survival, functional stability or migration). Interestingly, EAE was exacerbated in genetically modified mice in which TNFR2 was ablated only in T_{reg} cells. The ablation of TNFR2 in T_{reg} cells seems to decrease their suppressive function specifically in the inflamed central nervous system[132]. In this context, the expression of TNFR2 by T_{reg} cells might be essential for their suppressive function and their capacity to control EAE.

My research group also performed an analysis of the suppressive capacity of T_{reg} cells from several human donors under three different T cell activation conditions and consistently found that TNF (added either before or during the suppression assay) either had no effect on or even slightly increased the suppressive activity of human T_{reg} cells[133]. The preservation of T_{reg} cell suppressive activity after TNFR2 co-stimulation (achieved using a TNFR2 agonist) in humans has also been confirmed[57].

Several factors might account for the contrasting findings in mouse and human cells. First, as none of the available markers can exclusively characterize the population of human T_{reg} cells, the purified T_{reg} cell populations used in some of these studies might still have some level of contamination by activated conventional T cells, especially when only CD4 and CD25 expression was used to sort the cells[134]. Second, given the high inter-individual variability in T_{reg} cell phenotypes, responses to TNF and suppressive activity, it is important to collect data from a sufficiently large sample of individuals. Last, a T_{reg} cell functional defect identified in a suppression assay could be due either to intrinsic T_{reg} cell dysfunction or to the presence of contaminating conventional T cells that are resistant to T_{reg} cell suppression. This last point is critical with regards to the effects of TNF. Indeed, in addition to its proliferation-promoting effect on T_{reg} cells, TNF not only increases the proliferation of conventional T cells[51,53,133] but also increases their resistance to T_{reg} cell-mediated suppression[135]. In several studies performed in human cells, TNF was present during the suppression assays and might act on any contaminating conventional T cells, which would impair the evaluation of T_{reg} cell-suppressive function (TABLE 3). Accordingly, the pre-incubation of T_{reg} cells with TNF is appropriate before evaluating their capacity to suppress conventional T cells.

Another critical point is the choice of parameter used to assess the activation of conventional T cells. As TNF strongly increases T_{reg} cell proliferation (and possibly also cytokine production), measuring the activation of only the conventional T cells within the population is critical. This measurement can be done by analysing fluorescent marker dilution or assessing intracellular cytokine production using flow cytometry techniques such as fluorescence-activated cell sorting (FACS). Researchers should not use thymidine incorporation or enzyme-linked immunosorbent assays (ELISA) to measure the proliferation or cytokine production of the whole cell population, which includes both conventional T cells and T_{reg} cells. For this reason, to accurately determine whether TNF alters the suppressive function of T_{reg} cells, we recommend that TNF is added only during the pre-incubation phase (that is, before the suppressive assay) and that activation of only the conventional T cells is measured by FACS. The absence of these two precautionary measures in some of the reports claiming that TNF inhibits T_{reg} cell suppressive activity in humans undermines their conclusions (TABLE 3).

To conclude, weak evidence indicates that TNF is able to either inhibit or increase the suppressive activity of T_{reg} cells. After careful analyses of the data from in vitro assays, I would say that TNF has no or only a minor effect on T_{reg} cell suppressive function in this context. However, this cytokine seems to play an essential role in the stimulation of T_{reg} cell function in some conditions associated with inflammation.

The data derived from in vitro studies of mechanisms underlying the suppressive activity of T_{reg} cells reflect only the tip of the iceberg as only two or three suppressive mechanisms have been analysed in these studies to date. However, it is now well established that T_{reg} cells in vivo are able to use a wide range of suppressive mechanisms depending on their tissue localization and the type of inflammation present[136,137]. The suppressive activity of T_{reg} cells also involves many different effector molecules. Some have been thoroughly studied and shown to be essential for aspects of T_{reg} cell suppression such as cytotoxic T lymphocyte protein 4 (CTLA4) and IL-10 (REF.[138]). FOXP3 expression is also critical because its loss leads to the loss of T_{reg} cell function[138], but no single marker has been shown to easily quantify the level of T_{reg} cell suppression.

Several mechanisms have been suggested to explain how TNF might increase the suppressive function of T_{reg} cells in mice. TNF promotes the full differentiation of effector T_{reg} cells by stimulating NF-κB, which might increase some of the suppressive functions of these cells[103,104]. TNF also synergizes with IL-2 to increase the expression of CD25 (the IL-2 receptor α-chain) and FOXP3 (REFS[22,133]). Moreover, TNF increases the IL-2-induced phosphorylation of STAT5 (REF.[22]) and limits the loss of FOXP3 expression in cultured cells by preventing re-methylation of the *Foxp3* promoter[113,115]. Thus, TNF might increase T_{reg} cell suppression and stability by favouring both STAT5 phosphorylation and FOXP3 expression, which are key determinants of these T_{reg} cell features[139,140]. Finally, TNF limits IL-17 production by T_{reg} cells by activating TNFAIP3 (REF.[71]).

Other mechanisms have been proposed to explain how TNF might decrease T_{reg} cell function. TNF decreases FOXP3 expression by increasing the expression of deleted in breast cancer 1 (DBC1) and microRNA 34a (miR-34a), which respectively promote FOXP3 degradation and reduce *FOXP3* transcription and translation[128,141,142]. Alternatively, TNF might increase the expression of serine/threonine-protein phosphatase PP1, which dephosphorylates FOXP3, thereby decreasing its effect on T_{reg} cell suppressive function[126].

Effects on T_{reg} cell differentiation

The population of $FOXP3^+$ T_{reg} cells is composed of thymic T_{reg} cells, which acquire their T_{reg} cell state during their development in the thymus, and peripheral T_{reg} cells, which acquire their T_{reg} cell state during the peripheral differentiation of mature naive conventional T cells. Finally, induced T_{reg} cells can be differentiated in vitro from naive conventional T cells by TCR stimulation in the presence of IL-2 and TGFβ. Thus, induced T_{reg} cells are the in vitro counterpart of peripheral T_{reg} cells.

However, although TNF alone has no effect on thymic T_{reg} cell differentiation, experiments in mice show that TNF inhibits the differentiation of induced T_{reg} cells, whereas treatment with TNF inhibitors increases the differentiation of induced T_{reg} cells[143,144]. This inhibitory effect of TNF was also observed on peripheral T_{reg} cells in vivo. In mice with EAE, the injection of anti-TNF or anti-TNFR2 mAbs at the time of disease induction led to reduced disease severity, which was associated with an increased proportion of T_{reg} cells and evidence of increased peripheral T_{reg} cell differentiation[144]. Two other papers do not support this observation and even suggest that the TNF–TNFR2 axis promotes the differentiation of both induced and peripheral T_{reg} cells[28,73]. However, the design of these two studies meant that contaminating natural T_{reg} cells were present in the starting inoculum and thus treatment with TNF might boost the expansion of these contaminating cells rather than increase the differentiation of induced T_{reg} cells[28,73]. TNF does not seem to affect thymic T_{reg} cell differentiation at steady state, because mice lacking TNFR2 have normal thymic T_{reg} cell numbers. However, the ablation or neutralization of TNFR2 combined with the ablation or neutralization of two other members of the TNFR superfamily (namely, OX40 and GITR) led to the reduced differentiation of thymic T_{reg} cells[145]. Overall, whereas the effect of TNF on T_{reg} cell differentiation is still open to discussion, an excess of TNF seems to impair the differentiation of induced T_{reg} cells and peripheral T_{reg} cells in mice.

In humans, the inhibition of T_{reg} cell differentiation by TNF was first observed in patients with RA. TNF inhibitor treatment increased the in vitro differentiation of induced T_{reg} cells derived from patients with RA but not of those from healthy controls[146]. This observation explained why blood samples from patients with RA treated with infliximab had an increased proportion of T_{reg} cells, which might result from the increased differentiation of peripheral T_{reg} cells[98,146]. Other members of the TNF family, such as 4-1BB, OX40 or TNFR superfamily member 25 (also known as death receptor 3 (DR3)), can

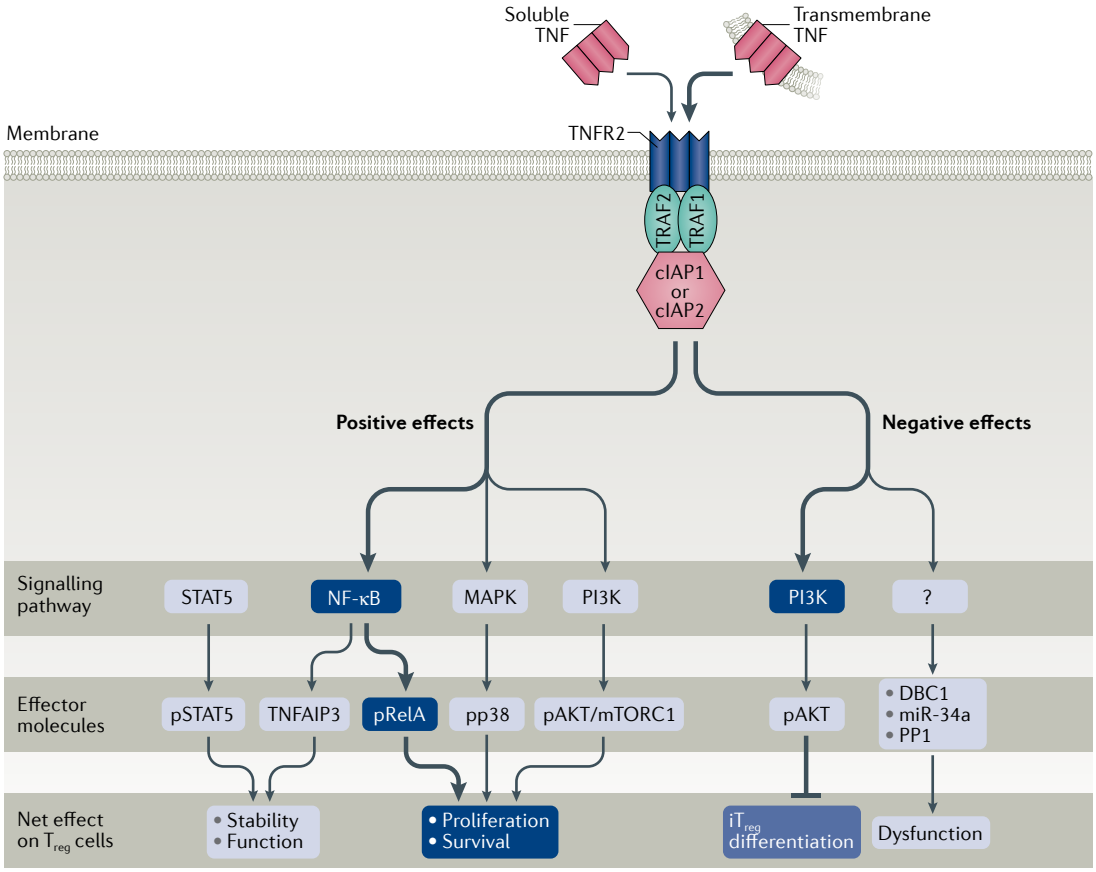


Fig. 2 | **The overall effects of TNF on T_{reg} cells.** Most of the effects of tumour necrosis factor (TNF) on regulatory T (T_{reg}) cells are due to induction of TNF receptor 2 (TNFR2) signalling, which is probably preferentially mediated by transmembrane TNF rather than soluble TNF. Signal transduction downstream of TNFR2 that does not involve kinase activity involves TNF receptor-associated factor (TRAF) adaptor proteins. Multiple downstream signalling pathways lead to positive (left) and negative (right) effects on T_{reg} cell biology. TNFR2 signalling strongly induces T_{reg} cell proliferation and has a moderate survival-promoting effect on T_{reg} cells; both of these effects depend on RelA and probably also on the activation of p38, AKT and mammalian target of rapamycin complex 1 (mTORC1) by phosphorylation (p). Weak evidence indicates that TNF also promotes the stability and suppressive function of T_{reg} cells, perhaps via the TNF-induced protein 3 (TNFAIP3) and signal transducer and activator of transcription 5 (STAT5) signalling pathways. In addition to these positive effects of TNF, the negative effects of this cytokine are clear in relation to the inhibition of induced T_{reg} (iT_{reg}) cell differentiation (which involves phosphoinositide 3-kinase (PI3K) and/or pAKT pathway activation). Weak evidence suggests that TNF induces T_{reg} cell dysfunction, perhaps via a mechanism involving deleted in breast cancer 1 (DBC1), microRNA 34a (miR-34a) and serine/threonine-protein phosphatase PP1. Arrow thickness and box shading intensity are proportional to the importance of the effect or the likelihood that a given molecule is involved in the pathway.

also inhibit the differentiation of induced T_{reg} cells[147–149]. These observations suggest that a shared mechanism is involved, perhaps implicating the NF-κB, PI3K or MAPK pathways. IFNγ produced by T cells following TNFR co-stimulation has also been proposed to inhibit the differentiation of induced T_{reg} cells. Alternatively, the increased activation of the PI3K–AKT pathway resulting from TNFR signalling could lead to the reduced activation of phosphorylated SMAD3, which transactivates *Foxp3* expression in mouse induced T_{reg} cells[144].

Overall effects of TNF on T_{reg} cells

In summary, TNF has multiple negative and positive effects on T_{reg} cell biology, most probably resulting from TNFR2 rather than TNFR1 signalling (FIG. 2). The best characterized of the positive effects of TNF are increased T_{reg} cell proliferation and expansion. TNF also seems to promote T_{reg} cell survival in vitro, although the relevance of this effect in vivo is difficult to evaluate. The TNF-dependent increases in T_{reg} cell proliferation and survival are at least partially dependent on RelA and activation of the canonical NF-κB pathway. The involvement of p38 and PI3K–AKT pathway activation has also been suggested but requires further investigation. Finally, weak evidence indicates that TNF increases the stability and suppressive function of T_{reg} cells, a phenomenon that might be partially due to TNF signalling synergizing with IL-2 signalling and with phosphorylation of STAT5. Other reports suggest a negative effect of TNF on T_{reg} cell biology in vitro. Whether this cytokine truly has a negative effect on T_{reg} cell function is questionable. By contrast, TNF seems to increase the suppressive function of T_{reg} cells in vivo, at least in some inflammatory contexts. However, the evidence of an inhibitory effect

of TNF on the differentiation of induced T_{reg} cells is fairly solid and might involve the PI3K–AKT pathway (FIG. 2).

T_{reg} cells in RA

As T_{reg} cells play an important role in the suppression of autoimmunity, numerous studies have attempted to identify whether these cells have a quantitative or functional defect in patients with autoimmune diseases. Major drawbacks of these studies include the use of sample sizes too small to account for interindividual variability and the absence of a specific marker for human T_{reg} cells, which has led to the utilization of different combinations of markers. As a result, the literature is full of conflicting data.

I present here the main findings on T_{reg} cell proportion and function in patients with RA. This disease is particularly interesting as T_{reg} cells can be obtained from both blood and joints (the target tissue of the disease), which are easily accessible for analysis. Data obtained in other autoimmune diseases are also included where relevant.

T_{reg} cell proportion

Contrasting findings have been reported for the proportion of T_{reg} cells in the blood of patients with RA receiving conventional immunosuppressive treatment (the effects of TNF inhibitors are discussed below). Among studies that compared patients with RA with healthy control individuals, four described a decreased T_{reg} cell proportion[75,150–152], five found no difference[98,126,146,153,154] and one found an increased T_{reg} cell proportion in patients with RA[155].

Most studies that have analysed both blood and synovial fluid of patients with RA concluded that the proportion of T_{reg} cells was higher in synovial fluid than in blood and remained stable over time in individual patients[152,155–157]. The T_{reg} cells isolated from synovial fluid seem to be bona fide T_{reg} cells because they exhibit *FOXP3* promoter demethylation. Additionally, the phenotype of these T_{reg} cells indicates that they have an activated status[157]. The synovial fluid of patients with RA contains high amounts of IL-6, TNF and IFNγ, low levels of IL-17A, IL-10 or IL-13, and does not contain IL-1 (REFS[126,157]). Which of these factors is responsible for the increased proportion and activation of synovial T_{reg} cells remains unclear. However, IL-6 is not likely to be involved because this cytokine (which is produced by joint fibroblasts) induces the transdifferentiation of T_{reg} cells into highly pathogenic T_H17 cells in a mouse model of autoimmune arthritis, a phenomenon that might also take place in patients with RA[158]. IL-6 also induced the proteasomal degradation of FOXP3 and loss of the suppressive activity of T_{reg} cells[159,160]. We do not know much about the effect of IFNγ on T_{reg} cells. Therefore, the activation and/or expansion of T_{reg} cells in the synovial fluid of patients with RA is likely to be caused by high local levels of TNF.

T_{reg} cell function

Compared with T_{reg} cells obtained from the blood of healthy control individuals, T_{reg} cells isolated from the blood of patients with RA were shown to have similar suppressive activity in one study[155] and decreased suppressive activity in another[128]. In a third study, the capacity of these cells to suppress conventional T cell proliferation was maintained but their cytokine production was reduced[98]. Contrasting findings have also been reported for the suppressive activity of T_{reg} cells isolated from the synovial fluid of patients with RA. Several studies showed that synovial fluid T_{reg} cells from patients with RA were as active, or more so, than blood T_{reg} cells from either patients with RA or healthy control individuals in terms of the suppression of proliferation or IFNγ production[152,155–157]. Another publication reported that synovial fluid T_{reg} cells from patients with RA had decreased suppressive activity[126]. Importantly, these studies noted considerable variation between patients, with T_{reg} cells from some individuals but not from others showing a high level of suppression[157]. This observation might explain the contrasting results and further emphasizes the importance of generating data from at least 7–10 different patients, which was not the case for most of these studies.

Firm conclusions are difficult to draw because the available evidence does not provide a clear picture of whether T_{reg} cells in the blood of patients with RA have similar proportions and functions to those of healthy control individuals. The situation is a little bit clearer for synovial fluid T_{reg} cells, which seem to be present at an increased proportion in patients with RA.

Effects of TNF inhibitors

T_{reg} cell proportion. The proportion of T_{reg} cells in the blood of patients with RA has been analysed in many studies at 3–6 months (typically 3 months) after the initiation of TNF inhibitor treatment. In studies of patients with RA treated with infliximab, the T_{reg} cell proportion increased[75,98,115,146,151] after treatment (TABLE 4). In studies of patients with RA treated with either adalimumab or etanercept, the T_{reg} cell proportion was either increased[150,154,161] or unchanged[153,154,162] (TABLE 4). This T_{reg} cell increase was more often observed in responding than in non-responding patients.

Moreover, in studies of patients with Crohn's disease or IBD treated with infliximab (Supplementary Table 1), the T_{reg} cell proportion was also either unchanged[99,163] or increased[100,115,163–168]. Some of the studies in patients with IBD or Crohn's disease also analysed the kinetics of this treatment-related increase in T_{reg} cell proportion. In a study of patients with Crohn's disease, the increase was transient and only occurred after the first injection[165]. In two studies of patients with IBD, the increase occurred 2 weeks after the first injection and was maintained for ≥22 weeks[100,166], whereas in another study in patients with Crohn's disease, no increase was detected after 1 week but an increase was detected at week 24 in patients who had low T_{reg} cell proportions before treatment[99] (Supplementary Table 1).

Two studies in patients with uveitis[169,170] and one study in patients with ankylosing spondylitis[171] showed an increase in the T_{reg} cell proportion after TNF inhibitor therapy. However, one study in patients with juvenile idiopathic arthritis observed no difference[172] and one study in patients with sarcoidosis observed a decrease in the

Table 4 | T_{reg} cell proportions in blood before and after TNF inhibitor therapy

Study population	TNF inhibitor (concomitant medications)	Sampling time points[a]	T_{reg} cells			Ref.
			Cell population	Pre-treatment (proportion)[b]	Post-treatment (proportion)	
27 patients with RA; 8 healthy controls	Infliximab (NSAIDs, methotrexate)	Baseline, 1.5 and 3.0 months	$CD4^+CD25^{hi}$	Same	Increased from baseline; increased in responders vs non-responders	98
17 patients with RA; 15 healthy controls	Infliximab (NSAIDs, methotrexate)	Baseline and 3.0 months	$CD4^+CD25^{hi}$	Decreased[c]	Increased[c] from baseline	151
31 patients with RA; 20 healthy controls	Infliximab (NSAIDs, methotrexate)	Baseline and 4.0–6.0 months	$CD4^+FOXP3^+$	Same	Increased from baseline	146
40 patients with RA; 10 healthy controls	Infliximab (methotrexate, salazopyrin, hydroxychloroquine, steroids)	NR	$CD4^+CD25^+FOXP3^+$	Decreased	Increased from baseline and in responders vs non-responders	75
10 patients with RA; 10 healthy controls	Adalimumab (NSAIDs methotrexate, steroids)	Baseline and 3.0 months	$CD4^+CD25^{hi}$	Same	No change from baseline	153
50 patients with RA; 15 healthy controls	Adalimumab or etanercept (NR)	NR	$CD4^+FOXP3^+$	Same	Increased from baseline; increased in responders vs non-responders to adalimumab; no change from baseline with etanercept	154
48 patients with RA	Adalimumab or etanercept (methotrexate, leflunomide)	Baseline, 1.5 and 3.0 months	$CD4^+FOXP3^+$, $CD25^{hi}CD127^{low}$	ND	No change from baseline; no difference between responders and non-responders	162
20 patients with RA; 10 healthy controls	Etanercept (methotrexate)	Baseline and 3.0 months	$CD4^+CD25^{hi}FOXP3^+$	Decreased	Increased from baseline	150
33 patients with RA	Etanercept (methotrexate)	Baseline, 3.0 and 6.0 months	$CD4^+CD25^+FOXP3^+$	ND	Increased from baseline	161
16 patients with RA	Infliximab or etanercept[d] (NR)	Baseline and 3.0 months	$CD4^+CD25^+CD127^{low}$ $FOXP3^+$	ND	Increased from baseline	115
7 patients with JIA	Etanercept (NSAIDs, methotrexate)	Baseline and 1.0–5.0 months	$CD4^+FOXP3^+$	ND	No change from baseline	172
222 patients with AS; 68 healthy controls	Infliximab or etanercept (NSAIDs)	Baseline and 6.0 months	$CD4^+CD25^{hi}FOXP3^+$	Decreased	Increased from baseline; increased in responders versus non-responders	171
46 patients with sarcoidosis; 26 healthy controls	Infliximab (NR)	Baseline, 3.5 and 6.0 months	$CD4^+CD25^{hi}$	Increased	Decreased from baseline	173
12 patients with uveitis	Adalimumab (NR)	Baseline, 1.0 and 6.0 months	$CD4^+CD25^{hi}CD127^{low}$ $FOXP3^+$	ND	Increased from baseline	169
16 patients with uveitis; 15 healthy controls	Infliximab (NR)	Baseline, 4.0–27.0 months	$CD4^+FOXP3^+$	Same	Increased from baseline[e]	170

AS, ankylosing spondylitis; JIA, juvenile idiopathic arthritis; ND, not determined; NR, not reported; NSAID, non-steroidal anti-inflammatory drug; RA, rheumatoid arthritis; TNF, tumour necrosis factor; T_{reg}, regulatory T. [a]Baseline (before initiation of TNF inhibitor treatment). [b]In patients versus controls. [c]Absolute number. [d]Three patients also received golimumab, adalimumab or certolizumab. [e]Versus patients treated only with cyclosporine or colchicine.

T_{reg} cell proportion[173] following TNF inhibitor therapy (TABLE 4).

Some general conclusions can be drawn from these data. Most publications described an increase in the proportion of T_{reg} cells in blood after TNF inhibitor therapy. Discrepancies between some studies could be due to the following factors: first, infliximab seems to induce an increase in the T_{reg} cell proportion more consistently than either adalimumab or etanercept. Second, a T_{reg} cell increase seems to be more consistent among patients who responded to TNF inhibitor treatment. The type of concomitant medications might also matter. For instance, although methotrexate monotherapy induces an increase in T_{reg} cell proportion[150], combination therapy with methotrexate and a TNF inhibitor provided an optimal increase in T_{reg} cells in vitro[174]. Additionally, steroid treatment might increase T_{reg} cell proportion and function[175,176]. Last, as discussed above, technical factors related to the way that T_{reg} cells were purified might influence the conclusions of these studies. Some activated conventional T cells (which also express CD25) are likely to contaminate the population identified as T_{reg} cells. Thus, the findings of these studies have to be considered carefully because the level of conventional T cell contamination could differ between healthy control individuals and patients with rheumatic or autoimmune diseases or before and after TNF inhibitor treatment. Use of the CD45RA (naive T cell) or CD45RO (memory T cell)

markers, in addition to CD25 or CD127, would help to limit the risk of such contamination[134].

Several mechanisms by which T_{reg} cells might increase after TNF inhibitor treatment are supported by experimental evidence. First, T_{reg} cells might increase because treatment with TNF blockers, such as infliximab, favours the differentiation of peripheral T_{reg} cells[146]. Second, T_{reg} cells might increase because treatment with anti-TNF mAbs, such as adalimumab, augments the expression of transmembrane TNF on monocytes, which then triggers T_{reg} cell expansion via TNFR2 signalling. Thus, anti-TNF mAbs that are intended to inhibit TNF might paradoxically increase its activity[84]. The preferential expansion of activated T_{reg} cells rather than resting T_{reg} cells in patients receiving anti-TNF mAbs supports this hypothesis[100]. In patients with RA[151] or IBD[168], T_{reg} cells that are more sensitive to spontaneous apoptosis than those of healthy control individuals might be present at an increased proportion in patients treated with TNF inhibitors because they are protected from cell death by this therapy. In patients with IBD, TNF inhibitor therapy blocks T_{reg} cell migration to inflamed tissues, which results in increased T_{reg} cell levels in blood and decreased levels in the intestinal mucosa[166]. Last, TNF inhibitor therapy leads to a decrease in inflammatory cytokine levels and pathogenic T cells while sparing T_{reg} cells in patients with Crohn's disease[161] or ankylosing spondylitis[165,171]. Therefore, this treatment might target conventional T cells in preference to T_{reg} cells, thereby explaining the relative increase in the proportion of T_{reg} cells within the population of $CD4^+$ T cells. As the increased proportion of blood T_{reg} cells following TNF inhibitor treatment is an in vivo phenomenon that occurs over a long period, determining which of the above-described mechanisms is most relevant remains a challenge.

Suppressive function. Treatment with mAb TNF inhibitors affects not only the T_{reg} cell proportion but also their suppressive function. Early work showed that T_{reg} cells from patients with RA obtained before the initiation of TNF inhibitor therapy had a poor capacity to suppress cytokine production by conventional T cells and that the suppressive activity of these T_{reg} cells was restored following anti-TNF treatment[98]. These functional T_{reg} cells resulted from either the generation of new peripheral T_{reg} cells following infliximab treatment[146] or from the expansion of differentiated T_{reg} cells following adalimumab treatment[84]. These restored T_{reg} cells were even able to suppress pathogenic T_H17 cells, unlike the T_{reg} cells of healthy control individuals[154]. Dysfunction of T_{reg} cells obtained from the blood or synovial fluid of patients with RA and restoration of their suppressive function after TNF inhibitor treatment (infliximab) were also confirmed in two other studies[126,128]. Restoration of functional blood T_{reg} cells after TNF inhibitor treatment has also been described in patients with IBD[164].

TNF inhibitors based on mAbs seem to act, at least in part, by restoring the functional T_{reg} cell compartment. By contrast, etanercept is likely to act by suppressing conventional T cells and/or rendering them sensitive to the suppressive effects of T_{reg} cells[172,177]. In another study, T_{reg} cells obtained from patients with Crohn's disease were shown to be functional even before the initiation of infliximab treatment[178]. However, the T_{reg} cell purification strategy used in this paper meant that activated conventional T cells might have contaminated the population of T_{reg} cells, thereby resulting in an inaccurate measurement of the suppressive activity of genuine T_{reg} cells. Therefore, the conclusions of this report have to be interpreted with caution.

In summary, the beneficial effects of TNF inhibitor therapies could be due either to the restoration of fully functional T_{reg} cells or to an increased susceptibility of conventional T cells to the suppressive effects of T_{reg} cells.

T_{reg} cell biomarkers of response. The development of biomarkers to identify the 20–30% of patients with RA or IBD who will not respond to TNF inhibitor therapy is highly desirable and some T_{reg} cell-related biomarkers are potential candidates. In some studies, an increase in the T_{reg} cell proportion after TNF inhibitor treatment was observed only in patients who responded to this therapy (TABLE 4). Thus, the T_{reg} cell proportion before TNF inhibitor treatment has been proposed as a predictive biomarker of treatment response. However, patients with IBD who respond to TNF inhibitors could have T_{reg} cell proportions before therapy that are either higher[99,100] or lower[178] than those of non-responding patients. Moreover, in patients with ankylosing spondylitis[171,174] or RA[171,174], the T_{reg} cell proportion before TNF inhibitor therapy was not predictive of treatment efficacy.

As discussed above, a possible mechanism for the observed increase of T_{reg} cells upon mAb TNF inhibitor treatment is binding of the mAb to transmembrane TNF on myeloid cells, leading first to its increased expression and then to a boost in T_{reg} cell numbers mediated by TNFR2 signalling[84]. As the expression of transmembrane TNF on monocytes can be readily assessed by flow cytometry, the capacity of adalimumab to provoke an increase in T_{reg} cell numbers in a 3-day culture has been used to identify which patients with RA would respond to this treatment[174].

In summary, the pre-treatment T_{reg} cell proportion does not seem to be a reliable biomarker of response to anti-TNF therapies. The expression of transmembrane TNF on myeloid cells as a biomarker of treatment response deserves to be confirmed in other studies.

Next-generation drugs targeting TNF

The putative mechanisms underlying non-response and paradoxical autoimmunity to TNF inhibitor treatment could be explained by the regulatory aspect of TNF. Blocking TNF is associated with an increased risk of impairing the activity of some suppressor cells, including T_{reg} cells, or increasing the activation of autoreactive T cells. The overall effect of these treatments is likely to depend on the specific autoimmune disease present, its stage and severity, and on genetic and environmental factors unique to each patient. At the time of treatment, if TNF has a dominant inflammatory and pathogenic role, TNF inhibitors will be beneficial. By contrast, if TNF has a dominant regulatory and protective role, TNF inhibitors will be detrimental.

Table 5 | **Therapeutic effects of drugs targeting TNFRs in autoimmune disease models**

Agent	Structure	Therapeutic efficacy	Refs
Antagonists of TNFR1			
DMS5540	Bispecific anti-TNFR1 and anti-albumin mAb	Arthritis (CIA)	184
Atrosab	Humanized anti-TNFR1 IgG1 mAb, mutated in the Fc fragment to abrogate complement and immune complex activation	EAE	185
TROS	Trivalent nanobody comprising two mAb domains binding to TNFR1 and one mAb domain binding to albumin	EAE	186
HM1097	Hamster IgG	EAE	187
XPro1595	Dominant-negative PEGylated TNF muteins that interact with soluble TNF to form inactive heterotrimers, which have low binding and signalling activity	Arthritis (CIA) and EAE	188,190,192
R1antTNF	PEGylated TNF mutein that binds specifically to TNFR1 without signalling activity	Arthritis (CIA) and EAE	189,191
Agonists of TNFR2			
MR2-1	Mouse mAb against human TNFR2	Increased expansion and stability of T_{reg} cells; not tested in vivo	57,118
Unnamed	Mouse mAb against human TNFR2	Increased expansion and stability of T_{reg} cells; not tested in vivo	111
TNF07	Human TNF mutein trimer	Increased expansion of T_{reg} cells; not tested in vivo	116
TNC-scTNF$_{R2}$	Human TNF mutein trimer	Not tested in vitro or in vivo	193
STAR2	Mouse TNF mutein nanomer	Increased expansion, survival and function of T_{reg} cells; effective in CIA, EAE and GvHD	103,117,120,121,132
EHD2-sc-mTNF$_{R2}$	Mouse TNF mutein hexamer	Increased expansion of T_{reg} cells; effective in EAE and CIA	119,208

CIA, collagen-induced arthritis; EAE, experimental autoimmune encephalomyelitis; GvHD, graft-versus-host disease; mAb, monoclonal antibody; TNF, tumour necrosis factor; TNFR, TNF receptor; T_{reg}, regulatory T; TROS, TNFR1 silencer.

Given that most of the pro-inflammatory properties of TNF are due to TNFR1 signalling induced by soluble TNF and most of the regulatory properties are due to TNFR2 signalling induced by transmembrane TNF, the next generation of TNF inhibitors might preferentially target TNFR1 or TNFR2 (REFS[14,25,82,86,88,179,180]). Two types of TNFR-specific agents have been proposed: mAbs and the so-called TNF muteins, which are forms of this cytokine harbouring mutations in the receptor-interacting domains[181–183].

Selective TNFR1 antagonists

The capacity of TNFR1 antagonists to block the pro-inflammatory interaction of TNF with TNFR1 has been investigated in mouse models of autoimmune diseases.

Both mAbs and TNF muteins have been developed that have potent TNFR1 antagonist activity and a strong therapeutic effect in mouse models of autoimmune diseases (TABLE 5). For example, the mAb DMS5540 was as effective as etanercept in the treatment of collagen-induced arthritis. In addition, the effects of DMS5540 on immune cells were superior to those of etanercept as DMS5540 induced T_{reg} cell activation and reduced the activation of conventional T cells, a phenomenon not observed with etanercept[184]. Several anti-TNFR1 mAbs (namely, atrosab, the trivalent nanobody TNFR1 silencer (TROS) and HM1097) were able to suppress EAE[185–187]. Finally, the muteins XPro1595 and R1antTNF had therapeutic effects in arthritis or EAE; when these agents were compared with etanercept, they sometimes demonstrated improved efficacy[188–192].

TNFR2 agonists

As TNFR2 signalling stimulates the expansion of T_{reg} cells, TNFR2 agonists, such as the mAbs MR2-1 and another unnamed version, are interesting candidates for improving T_{reg} cell therapy in autoimmune diseases (TABLE 5). When added to human T_{reg} cell cultures, these mAbs promote the expansion and improve the stability and purity of T_{reg} cells over time[111,118].

Two additional TNF muteins with human TNFR2 agonist activity (TNF07 and TNC-scTNF$_{R2}$) have been generated[116,193] and TNF07 has been shown to promote T_{reg} cell activation in vitro. In the future, mAbs or TNF muteins with TNFR2 agonist activity might be used to improve cell culture methods used to generate T_{reg} cell preparations for use in cell therapy. This notion is supported by mouse studies showing that the addition of TNF or a TNFR2 agonist to T_{reg} cell cultures increased the capacity of these cells to suppress colitis[103,130] or GvHD[103,130] after their reintroduction in vivo[103,130].

The capacity of TNFR2 agonists to stimulate T_{reg} cells in vivo has been tested in mouse models. Treatment with

either of two TNF muteins with TNFR2 agonist activity (STAR2 and EHD2-sc-mTNF$_{R2}$)[194,195] induced in vivo T_{reg} cell activation and expansion[117,120] that was associated with the prevention or amelioration of arthritis[119,121], EAE[132] or GvHD[117]. These agents also protected the central nervous system of treated animals from inflammation and neuronal injury induced by chronic nerve constriction or drug treatment, respectively[195,196].

Whereas classical immunosuppressive drugs aim to suppress autoimmunity by neutralizing pathogenic cells, an alternative approach is based on increasing the expansion or suppressive capacity of T_{reg} cells. A prototype of this new class of drugs is IL-2 (REF.[197]). My research group showed that administration of low-dose IL-2 boosts the proliferation of T_{reg} cells and induces remission of type 1 diabetes mellitus in non-obese diabetic mice[198,199]. Low-dose IL-2 is now being investigated as a treatment for other autoimmune diseases in multiple clinical trials. One study has investigated this treatment in 14 different autoimmune diseases, including RA and ankylosing spondylitis (NCT01988506). TNFR2 agonists are another type of drug able to boost the number or function of T_{reg} cells. No clinical trial has so far investigated the use of TNFR2 agonists to treat an autoimmune disease. However, the bacillus Calmette–Guérin vaccine can induce TNF release without secondary effects, thereby providing an indirect way to trigger TNFR2 signalling; this vaccine has been tested for efficacy in type 1 diabetes mellitus (NCT00607230 and NCT02081326).

Conclusions

TNF has a long and fascinating yet chaotic history. This cytokine was discovered in the mid-1970s and named for its effect as a tumour cell killer. Major milestones in its history include its cloning in the mid-1980s, the discovery that TNF binds to two receptors, that its signalling transduction is highly complex (and remains to be fully explored), and that it has multiple effects at steady state.

TNF is now known to be one of the most important inflammatory cytokines. Although TNF is critical for beneficial immune responses, the realization that it is also harmful in many autoimmune diseases led to the great success of TNF inhibitors and ultimately to the flowering of research into other biological therapies. The regulatory role of this cytokine is also important to consider. Here again, the mechanisms underlying the immunosuppressive activity of TNF are complex. However, one of its main features seems to involve the expression of transmembrane TNF on myeloid or T cells, which interacts with TNFR2 on T_{reg} cells to boost their proliferation and maybe also their stability and suppressive function.

The inflammatory and regulatory roles of TNF are both essential to consider in the design of future generations of TNF inhibitors. Preclinical studies have shown that selective antagonists of TNFR1 inhibit the inflammatory action of TNF, whereas selective agonists of TNFR2 boost T_{reg} cell numbers and potentially also improve their function. Therefore, TNFR1 antagonists and TNFR2 agonists could be beneficial in the treatment of several diseases with an autoimmune component. In the future, biotechnology and pharmaceutical companies are expected to work hand in hand with academic laboratories towards the successful translation of these fascinating observations into the clinic.

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Competing interests
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TNF inhibition for immune checkpoint inhibitor-induced irAEs: the jury is still out

Karijn P. M. Suijkerbuijk and Rik J. Verheijden

We congratulate Chen and colleagues on their elegant Review discussing the complex interactions between TNF inhibition and immune checkpoint inhibition (Chen, A. Y., Wolchok, J. D. & Bass, A. R. TNF in the era of immune checkpoint inhibitors: friend or foe? *Nat. Rev. Rheumatol.* **17**, 213–223 (2021))[1]. Supported by preclinical data, the authors conclude that short-term TNF inhibition to treat immune checking inhibitor (ICI)-induced toxicity should not compromise ICI efficacy. The authors refer to our study examining the effect of immunosuppressive management of ICI-induced toxicity on survival among patients with grade ≥3 immune-related adverse events (irAEs)[2]. In this study, we reported a shorter overall survival in patients who received anti-TNF therapy compared with patients treated with corticosteroids only.

Chen and colleagues advocate that this observation might have been biased owing to the use of overall survival as an outcome measure, as some severe irAEs (such as colitis) have a higher mortality than others (such as endocrine toxicity). However, the overall survival did not differ notably between patients with colitis and patients with other irAEs (adjusted HR 1.15; 95% CI 0.82–1.60)[2]. Moreover, there was no difference in toxicity-related mortality between those patients who received anti-TNF therapy and those who did not[2]. Similarly, melanoma-specific survival was shorter in patients who received TNF inhibition[2].

Chen and colleagues validly comment that we did not account for time to irAE onset, which could have biased the results if the onset of irAEs occurred earlier in patients who received TNF inhibition than in the patients who only received corticosteroids. However, given that these analyses were performed in patients with grade ≥3 irAEs only, we do not expect the differences in time to irAE onset to be notable.

In their Review, the authors mention two small studies that seemingly contradict our findings[3,4]. In 27 ICI-treated patients with melanoma who received anti-TNF therapy for colitis, Lesage et al. reported a median progression free survival (PFS) of 3 months[3], which was comparable to the PFS reported for patients being treated with a CTLA4 inhibitor in the CheckMate 067 trial[5]. However, one-third of the patients reported on by Lesage et al.[3] received a PD1 inhibitor or combination ICI therapy, for which the median PFS in the CheckMate 067 study was considerably higher (7 and 11.5 months, respectively)[5]. In the second study, among patients with ICI-induced colitis, Wang and colleagues observed no difference in overall survival in the patients treated with corticosteroids and an anti-TNF therapy ($n = 23$) and the patients treated with corticosteroids alone ($n = 38$)[4]. Remarkably, preliminary results of a study of 150 patients with ICI-induced colitis from the same institution showed a worse overall survival in those patients who received TNF inhibition compared with those who received vedolizumab (a gut-selective integrin inhibitor; $P = 0.042$)[6].

In conclusion, we support the authors' hypothesis that the net effect of TNF inhibition on tumorigenesis might be positive or negative in different situations. Nevertheless, we think that more research is needed before we can rule out detrimental effects of TNF inhibition for irAEs.

There is a reply to this letter by Bass, A. R & Chen, A. Y. *Nat. Rev. Rheumatol.* https://doi.org/10.1038/s41584-021-00641-y (2021).

Karijn P. M. Suijkerbuijk ✉ *and Rik J. Verheijden*
Department of Medical Oncology, UMC Utrecht, Utrecht, The Netherlands.
✉*e-mail: K.Suijkerbuijk@umcutrecht.nl*

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Competing interests
K.P.M.S. reports receiving speakers bureau honoraria from Novartis and Roche, and is a consultant and/or advisory board member for Novartis, Roche, MSD, Abbvie, Pierre Fabre and Bristol-Myers Squibb. R.J.V. declares no competing interests.

Reply to: TNF inhibition for immune checkpoint inhibitor-induced irAEs: the jury is still out

Anne R. Bass and Allen Y. Chen

We would like to thank Karijn Suijkerbuijk and Rik Verheijden for their correspondence (Suijkerbuijk, K. P. M. & Verheijden, R. J. TNF inhibition for immune checkpoint inhibitor-induced irAEs: the jury is still out. *Nat. Rev. Rheumatol.* https://doi.org/10.1038/s41584-021-00640-z (2021))[1] on our Review (Chen, A. Y., Wolchok, J. D. & Bass, A. R. TNF in the era of immune checkpoint inhibitors: friend or foe? *Nat. Rev. Rheumatol.* **17**, 213–223 (2021))[2], which raises some important issues concerning our analysis of their study assessing the safety of TNF inhibitor treatment for immune-related adverse events (irAEs) from immune checkpoint inhibitors (ICIs).

In our Review we wrote, "the use of overall survival as the end point might have introduced confounders as some high-grade irAEs (such as colitis) have a higher mortality than others (such as high-grade endocrine toxicity, which can be treated with hormone replacement)"[2]. Suijkerbuijk and Verheijden are correct in pointing out that melanoma-specific survival was also shorter in the TNF inhibitor-treated patients, suggesting that toxicity-related deaths did not explain the differences in survival. We agree, this omission was an oversight on our part.

We do believe, however, that unmeasured confounders impacted their analysis of TNF inhibitor-related mortality, particularly as most TNF inhibitor-treated patients in their study received ipilimumab (an anti-CTLA4 therapy)[3]. The study analysed patients

with melanoma treated with a first line ICI therapy from 2012 to 2017, a period that saw many temporal changes in both melanoma treatment and irAE management[4]. Ipilimumab monotherapy was rarely used after 2015, when it was supplanted by anti-PD1 therapy and later by combination ICI therapy[5]. The authors controlled for ICI regimen, but did not control for year of entry into the registry or type of second-line therapy (for example, anti-PD1 therapy versus targeted agents). However, many of the biases inherent in observational data are also shared by the two other cohort studies we cited[6,7].

The recent abstract highlighted by Suijkerbuijk and Verheijden is certainly of interest[8]. In this retrospective study of 150 patients with ICI-induced colitis, overall survival from time of colitis diagnosis was shorter in patients treated with the TNF inhibitor infliximab compared with patients treated with the anti-integrin (gut-specific) therapy vedolizumab. However, the study did not control for multiple confounders, including duration of steroid use (which was longer in the infliximab-treated group) and ICI regimen. Colitis treatment was based on clinician choice, and infliximab might have been used for more severe colitis cases. Colitis following combination ICI therapy, for example, is both more severe and occurs sooner after ICI initiation than colitis after ICI monotherapy[9]. Because severe ICI-induced colitis generally requires ICI discontinuation, the duration of ICI therapy might have been shorter in these individuals.

We certainly do not argue that TNF inhibitors are a magic bullet. Evidence suggests that cancer survival is best in ICI-treated patients who experience only low grade irAEs[10], possibly because they do not require immunosuppression and/or they are able to continue their ICI therapy. A large prospective trial that compares infliximab to vedolizumab for treatment of ICI-induced colitis, or that compares a TNF inhibitor to corticosteroids for the treatment of ICI-associated inflammatory arthritis, would provide much needed answers regarding TNF inhibitor safety.

Anne R. Bass[1,2]✉ *and Allen Y. Chen*[1,2]
[1]*Division of Rheumatology, Hospital for Special Surgery, New York, NY, USA.*
[2]*Department of Medicine, Weill Cornell Medicine, New York, NY, USA.*
✉*e-mail: bassa@hss.edu*

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Competing interests
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