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RHEUMATOID ARTHRITIS

New player in RA pathogenesis brought to light

A novel subset of T cells is responsible for driving autoantibody production by B cells in the synovium of patients with rheumatoid arthritis (RA), according to new research published in *Nature*. These cells, dubbed T ‘peripheral helper’ cells, are expanded in patients with seropositive RA and are thought to home to the inflamed synovium, where they fulfil a similar role to T follicular helper (T_{FH}) cells. “Our strategy was to focus on T cells that express markers that indicate that they have been recently or chronically activated or might be homing into involved tissues,” explains Michael Brenner, corresponding author of the study. “We hypothesized that these T cells would be the most informative in telling us about pathologic T cell functions that drive RA,” he adds.

Autoantibody production is an important factor in driving seropositive RA. “T cells and B cells frequently form aggregates within the synovium in RA, yet which T cell population promotes B cell responses within the synovium has remained unclear,” states Brenner. Within

“the frequency of T peripheral helper cells fell over time in patients with ... RA who responded to ... therapy”

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lymphoid organs, T_{FH} cells interact with B cells, stimulating them to produce antibodies. “The T cells we identified in rheumatoid synovium also drive B cell responses, but do so within inflamed peripheral tissues, rather than within lymphoid tissue,” explains Deepak Rao, first author on the study. “Using mass cytometry, RNA sequencing, and functional studies, we found that this T cell population has a unique phenotype that combines the ability to infiltrate inflamed tissues with the ability to drive B cell responses and antibody production,” he continues, noting that, “this study provides the first detailed description of T cells with this unique combination of features.”

The T peripheral helper cells identified by Rao and colleagues accounted for almost a quarter of all CD4⁺ T cells in the synovium of patients with seropositive RA, but were not expanded in patients with seronegative RA, psoriatic arthritis or juvenile idiopathic arthritis. Importantly, the frequency of T peripheral helper cells fell over time in patients with seropositive

RA who responded to immunosuppressive therapy. “This remarkable disease-specific association with autoantibody-positive RA makes mechanistic sense because the expanded T peripheral helper cell population promotes B cell activation and antibody production,” says Rao.

Phenotypically, T peripheral helper cells share some similarities with T_{FH} cells, with both subsets producing IL-21 and expressing programmed cell death protein 1 (PD1) and inducible T-cell co-stimulator (ICOS). However, T peripheral helper cells do not express CXCR5, a key chemokine receptor expressed by T_{FH} cells, but instead express a range of chemokine receptors known to direct cells towards inflamed tissue, including CCR2, CX3CR1 and CCR5. The differences continue at the transcriptional level, with T peripheral helper cells expressing only low levels of BCL6, a key T_{FH} cell transcription factor, and high levels of BLIMP1, a transcription factor that is downregulated in T_{FH} cells.

“When one thinks about the current targeted immunotherapies for autoimmune diseases like RA, one recognizes that they are ‘blunt’ instruments since they block major cytokines or cytokine receptors, deplete a whole cell type or globally block T cell activation or the homing of cells to major organs,” says Brenner. “A next step that many investigators hope might be possible would be more ‘specific’ immunotherapies. Our discovery of a distinct T peripheral helper cell found only in patients with autoantibody positive RA raises the possibility that such T cell populations might be one of the first windows into the next level of specificity in targeting ‘only the pathologic T cells,’” he concludes.

Joanna Collison

ORIGINAL ARTICLE Rao, D. A. et al. Pathologically expanded peripheral T helper cell subset drives B cells in rheumatoid arthritis. *Nature* <http://dx.doi.org/10.1038/nature20810> (2017)

ACUTE INFLAMMATORY ARTHRITIS

Potential therapies for chikungunya arthritis

Targeting T cells is emerging as a promising strategy for the treatment of chikungunya arthritis. Two independent studies published in *Science Translational Medicine* demonstrated amelioration of disease when targeting pathogenic CD4⁺ T cells in mice infected with chikungunya virus (CHIKV).

CHIKV disease is a mosquito-borne disease that typically results in arthritic manifestation in the joints of infected patients (chikungunya arthritis) that resembles rheumatoid arthritis (RA). Currently, specific treatments for CHIKV arthritis are lacking. “Previously, we found that CD4⁺ T cells, and not CD8⁺ T cells, had a pathogenic role in driving CHIKV-induced joint inflammation,” remarks Laurent Réna, co-corresponding author of the Teo *et al.* study. In this study, the investigators sought to characterize CD4⁺ T cells involved in CHIKV disease pathogenesis to better understand what processes could be targeted therapeutically.

By use of proteome-wide screening, the researchers identified epitopes within nsP1 and E2 viral proteins that were recognised by splenic CD4⁺ T cells from CHIKV-infected

“These therapeutic strategies could also be relevant for the treatment of inflammatory arthritis associated with other infectious diseases”

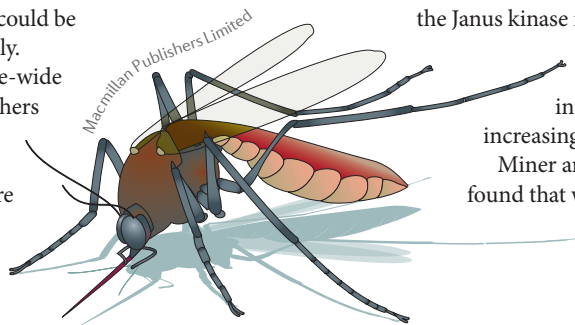
mice. Transfer of nsP1-specific or E2-specific CD4⁺ T cells into T cell receptor-deficient mice led to joint inflammation. To explore the effects of T cell modulation on disease pathogenesis, Teo *et al.* tested several clinically approved T-cell-suppressive drugs in CHIKV-infected mice. “Our research shows that fingolimod treatment blocks the movement of CD4⁺ T cells to the joint of infected hosts, resulting in a reduction of joint swelling,” explains Lisa Ng, co-corresponding author on the paper.

In a separate study, Miner *et al.* tested eight different DMARDs, which are commonly used to treat patients with RA, in CHIKV-infected mice. “[Previous findings] suggested that DMARD therapies that work in RA and target T cells might also work for CHIKV,” says co-corresponding author Deborah Lenschow. Miner *et al.* showed that abatacept (a drug that blocks T cell co-stimulation) and the Janus kinase inhibitor tofacitinib

human monoclonal antibody partially decreased arthritis severity in infected mice, the combination of these two therapies abrogated the disease phenotypes, as demonstrated by a reduction in joint swelling, chemokine and proinflammatory cytokines levels, and infiltrating leukocytes. “[Our findings] provide a new avenue for possible therapy against CHIKV by repurposing RA-based drugs and combining them with antiviral approaches,” states co-corresponding author Michael Diamond.

Together, the results of these two studies show two different T-cell-targeting approaches that ameliorate chikungunya arthritis severity in mice. These therapeutic strategies could also be relevant for the treatment of inflammatory arthritis associated with other infectious diseases. As mouse models do not fully recapitulate human diseases, both groups intend to take these drugs forward to the next stage in drug-testing.

Jessica McHugh



reduced joint swelling in CHIKV-infected mice without increasing viral burden. Miner and colleagues also found that whereas treatment with either abatacept or an anti-CHIKV

ORIGINAL ARTICLES Teo, T. H. *et al.* Fingolimod treatment abrogates chikungunya virus-induced arthralgia. *Sci. Transl. Med.* <http://dx.doi.org/10.1126/scitranslmed.aal1333> (2017) | Miner, J. J. *et al.* Therapy with CTLA4-Ig and an antiviral monoclonal antibody controls chikungunya virus arthritis. *Sci. Transl. Med.* <http://dx.doi.org/10.1126/scitranslmed.aah3438> (2017)

INFLAMMATION

Hit the DEK!

An aptamer targeting the nuclear chromatin protein DEK blocks neutrophil extracellular trap (NET) formation and the development of arthritis in a mouse model, according to new research published in *Nature Communications*. “As DEK has been implicated in the pathogenesis of juvenile idiopathic arthritis (JIA) and other autoimmune diseases, our findings suggest that targeting DEK with aptamers or other modalities might prove to be of therapeutic benefit in several forms of arthritis, but especially in JIA,” says corresponding author Nirrit Mor-Vaknin.

Although primarily associated with chromatin integrity in the nucleus, DEK also acts as a secreted chemotactic factor. Moreover, DEK protein and anti-DEK autoantibodies are abundant in the synovia of patients with JIA. In the current study Mor-Vaknin *et al.* determined that *Dek*-knockout mice are far less prone than their wild-type

“**DEK protein and anti-DEK autoantibodies are abundant in the synovia of patients with JIA**”



counterparts to develop arthritis induced by intra-articular injection of zymosan.

“We wished to confirm this finding and to move towards therapeutics, but anti-DEK antibodies have failed to neutralize DEK function and might actually contribute to the autoimmune response,” Mor-Vaknin explains. “Therefore, we went to great lengths to develop an anti-DEK DNA aptamer.” Injection of the DEK-targeting single-strand DNA aptamer into the knees of wild-type mice blocked zymosan-induced joint inflammation and neutrophil recruitment.

The aptamer also reduced NET formation in zymosan-injected mouse joints. Consistent with this observation, neutrophils isolated from *Dek*-knockout mice showed very little NET formation following stimulation with lipopolysaccharide (LPS), in comparison with cells from wild-type mice. This deficiency was abrogated by the addition of

recombinant DEK before LPS stimulation; notably, recombinant DEK did not enter the cell but associated with NETs in the extracellular space. “DEK is critical for NET formation, likely due to its role as a chromatin factor,” Mor-Vaknin reasons.

Demonstrating the relevance of these findings to human disease, Mor-Vaknin and colleagues showed that DEK is also released by activated human neutrophils into the extracellular space where it associates with NETs. Moreover, DEK-containing NETs were spontaneously formed by neutrophils isolated from the synovia of patients with JIA. In neutrophils from healthy donors stimulated with PMA, incubation with an anti-DEK aptamer (but not a control aptamer) led to reduced NET formation and localization of DEK in the cytoplasm.

The researchers plan to develop the anti-DEK aptamer for clinical use in humans. “We envision the aptamer as being used for local injections and ultimately systemically,” says Mor-Vaknin. “This latter approach will likely require further optimization of the aptamer and the method of delivery.”

Sarah Onuora

ORIGINAL ARTICLE Mor-Vaknin, N. *et al.* DEK-targeting DNA aptamers as therapeutics for inflammatory arthritis. *Nat. Commun.* **8**, 14252 (2017)

 EXPERIMENTAL ARTHRITIS

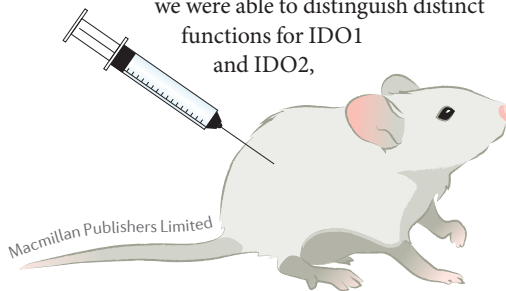
Do you want to treat arthritis? IDO2!

“
Treating mice with ... autoimmune arthritis with this anti-IDO2 antibody reduced the severity of disease”

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Therapeutically targeting indoleamine 2,3-dioxygenase 2 (IDO2) using a specific monoclonal antibody alleviates experimental arthritis, according to new findings published in *Clinical Immunology*. “Treatment with anti-IDO2 antibody inhibits autoreactive T and B cell responses and alleviates joint inflammation in the KRN preclinical model of autoimmune arthritis, fully recapitulating genetic IDO2 deficiency,” states Laura Mandik-Nayak, corresponding author of the study.

The indoleamine 2,3-dioxygenase enzymes (IDO1 and IDO2) catalyze the rate-limiting step in the catabolism of tryptophan. “Through a series of genetic knockout studies in mice, we were able to distinguish distinct functions for IDO1 and IDO2,



identifying IDO2, and not the better studied IDO1, as a proinflammatory mediator of autoimmune disease,” explains Lauren Merlo, lead author of the study. “However, small molecules that can be used to specifically target IDO2 *in vivo* have yet to be identified, so in the current study, we explored the use of a highly specific, monoclonal antibody therapy for IDO2,” she continues.

Treating mice with the KRN model of autoimmune arthritis with this anti-IDO2 antibody reduced the severity of disease compared with mice treated with a control antibody, regardless of whether the antibody was administered before or after the onset of disease. Merlo and colleagues reported similar findings in mice with collagen-induced arthritis. Using the KRN model to track autoreactive lymphocytes, the researchers pinpointed some of the mechanistic effects of anti-IDO2 antibody administration, including reduced T cell numbers in all subsets except regulatory T cells, and a decrease in IL-21 levels in mice

treated with the anti-IDO2 antibody as compared with those given a control antibody.

As an intracellular molecule, IDO2 would not traditionally be considered a candidate target for antibody therapy. “Mechanistic studies showed that anti-IDO2 is able to access its intracellular target to exert its anti-arthritic effect by internalization via the FcγRIIb receptor on B cells,” explains Merlo. “This work validates IDO2 as a therapeutic target for rheumatoid arthritis and adds to a growing literature demonstrating antibody treatments that can target intracellular antigens to offer feasible and disease-selective approaches to treat disease,” adds Mandik-Nayak.

Joanna Collison

ORIGINAL ARTICLE Merlo, L. M. F. et al. Therapeutic antibody targeting of indoleamine-2,3-dioxygenase (IDO2) inhibits autoimmune arthritis. *Clin. Immunol.* <http://dx.doi.org/10.1016/j.clim.2017.01.016> (2017)

FURTHER READING Merlo, L. M. F. et al. IDO2 is a critical mediator of autoantibody production and inflammatory pathogenesis in a mouse model of autoimmune arthritis. *J. Immunol.* **192**, 2082–2090 (2014)

IN BRIEF

RHEUMATOID ARTHRITIS**The lung as a site for anti-CCP generation?**

In a study of patients with rheumatoid arthritis (RA) and first degree relatives (FDRs) of patients with RA, 70% (14 of 20) and 25% (17 of 67), respectively, tested positive for anti-cyclic citrullinated peptide (anti-CCP) antibodies in their sputum, including some FDRs who were seronegative. In FDRs, elevated sputum levels of anti-CCP antibodies were associated with elevated numbers of macrophages and neutrophils and increased levels of neutrophil extracellular traps in the sputum.

ORIGINAL ARTICLE Demoruelle, M. K. *et al.* Anti-citrullinated protein antibodies are associated with neutrophil extracellular traps in the sputum in relatives of rheumatoid arthritis patients. *Arthritis Rheumatol.* <http://dx.doi.org/10.1002/art.40066> (2017)

VASCULITIS SYNDROMES**Shared genetic risk for Behçet disease and Crohn's disease**

Genotyping analysis of a Turkish cohort of 1,900 patients with Behçet disease and 1,779 controls, in addition to two replication cohorts, adds *ADO-EGR2*, *RIPK2*, *LACC1*, and *IRF8* to the list of known susceptibility loci shared by Behçet disease and Crohn's disease. A number of immune-related loci, such as *IL1A-IL1B* and *FUT2*, were also associated with Behçet disease, implicating the host response to microbial exposure in susceptibility to Behçet disease.

ORIGINAL ARTICLE Takeuchi, M. *et al.* Dense genotyping of immune-related loci implicates host responses to microbial exposure in Behçet's disease susceptibility. *Nat. Genet.* <http://dx.doi.org/10.1038/ng.3786> (2017)

RHEUMATOID ARTHRITIS**Sirukumab effective in patients refractory to anti-TNF therapy**

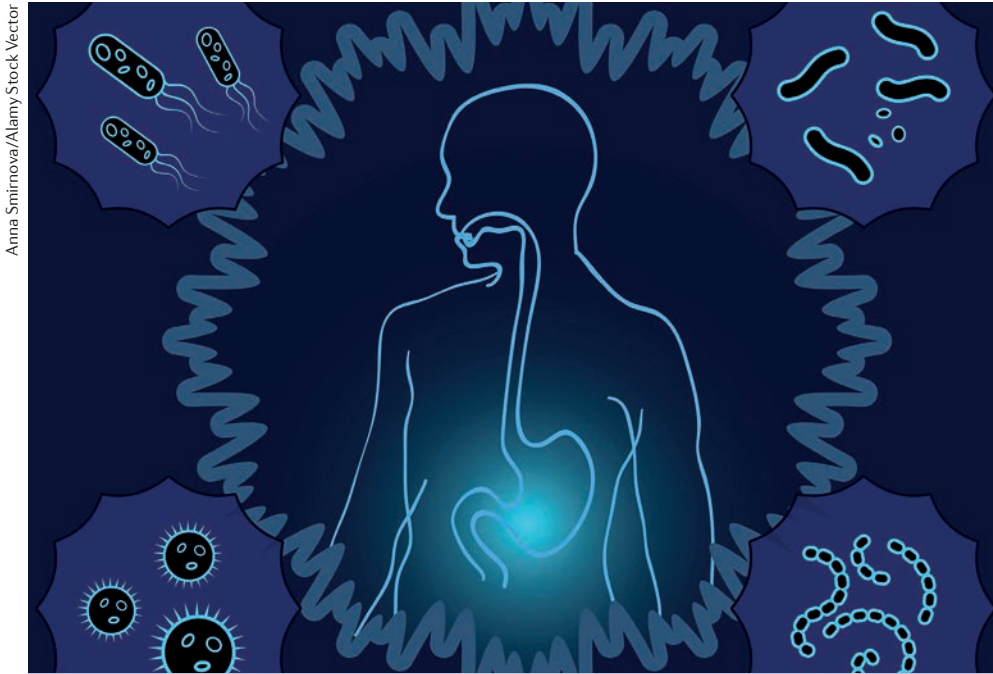
In the phase III SIRROUND-T study, treatment with the IL-6-specific antibody sirukumab was well tolerated and showed clinical efficacy in patients with RA refractory to anti-TNF therapy. At week 16, 40% (117 of 292) of patients treated with 50 mg sirukumab every 4 weeks and 45% (132 of 292) of patients treated with 100 mg sirukumab every 2 weeks achieved the primary outcome of $\geq 20\%$ improvement according to ACR criteria (ACR20 response), compared with 24% (71 of 294) of patients treated with placebo. Adverse event incidences were similar across groups, with the most common being injection-site erythema.

ORIGINAL ARTICLE Aletaha, D. *et al.* Efficacy and safety of sirukumab in patients with active rheumatoid arthritis refractory to anti-TNF therapy (SIRROUND-T): a randomised, double-blind, placebo-controlled, parallel-group, multinational, phase 3 study. *Lancet* [http://dx.doi.org/10.1016/S0140-6736\(17\)30401-4](http://dx.doi.org/10.1016/S0140-6736(17)30401-4) (2017)

RHEUMATOID ARTHRITIS**Baricitinib more effective than adalimumab**

Treatment with baricitinib, an orally administered inhibitor of Janus kinases 1 and 2, improved clinical features in patients with active RA and an inadequate response to methotrexate, and was more effective than adalimumab or placebo. 70% of patients treated with 4mg baricitinib daily achieved an ACR20 response by week 12, compared with 61% of those treated with 40mg adalimumab every other week and 40% of the placebo group. Baricitinib treatment also improved radiographic progression of joint damage, and was associated with reduced neutrophil counts and increased levels of creatinine and LDL cholesterol.

ORIGINAL ARTICLE Taylor, P. C. *et al.* Baricitinib versus placebo or adalimumab in rheumatoid arthritis. *N. Engl. J. Med.* <http://dx.doi.org/10.1056/NEJMoa1608345> (2017)



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SPONDYLOARTHROPATHIES

E. coli links IBD to spondyloarthritis

Spondyloarthritis (SpA) is a common extraintestinal manifestation in patients with inflammatory bowel diseases (IBD), but the mechanisms underlying this association have not yet been clarified. In a new study, Viladomiu and colleagues found that IgA-coated *Escherichia Coli* are enriched in patients with Crohn's disease-associated SpA (CD-SpA) as compared with patients with CD only. "Our microbial findings also correlate with patient-reported Bath ankylosing spondylitis disease activity index [scores]," says Randy Longman, corresponding author

“ IgA-coated *Escherichia Coli* are enriched in patients with Crohn's disease-associated spondyloarthritis **”**

of the study. "These findings may allow us to develop diagnostic tools to stratify patients with symptoms as well as identify patients at risk," he continues.

To investigate the role of specific microbial communities in the modulation of host immunity, Viladomiu and colleagues took advantage of a novel technique called IgA-seq, which couples the sorting of IgA-coated microbiota (bacteria recognized by the intestinal immune system) with ribosomal RNA gene sequencing. Using this approach, the researchers found

that the abundance of *E. coli* in the IgA⁺ fraction of faecal samples from patients with CD-SpA was increased compared with that from patients with CD only.

Further genetic analyses revealed that *E. coli* enriched in patients with CD-SpA were the adherent-invasive *E. coli* (AIEC) pathotype. Compared with non-AIEC control *E. coli* from patients with CD only, CD-SpA-derived AIEC were able to attach to the epithelium and increase the number of IL-17-producing CD4⁺ type 17 helper T (T_H17) cells when transferred into germ-free mice.

Viladomiu and colleagues also found that IL-17 production in both mucosal CD4⁺ T cells and serum from patients with CD-SpA was increased compared with that from patients with CD alone. Finally, the investigators demonstrated that in the K/BxN mouse model of inflammatory arthritis, CD-SpA-derived AIEC increased ankle thickness as compared with non-AIEC CD-derived control.

These findings suggest that AIEC mediates T_H17 systemic immunity, which in turn leads to CD-SpA. "While these data represent very exciting findings in a subset of patients with CD-associated peripheral SpA, further work is needed to evaluate these findings in axial, HLA-B27-associated disease as well as ulcerative colitis-associated SpA," concludes Longman.

Dario Umbarino

ORIGINAL ARTICLE Viladomiu, M. et al. IgA-coated *E. coli* enriched in Crohn's disease spondyloarthritis promote T_H17-dependent inflammation. *Sci. Transl. Med.* <http://dx.doi.org/10.1126/scitranslmed.aaf9655> (2017)

 SYSTEMIC SCLEROSIS

Antifibrotic effects of PDE4 blockade?

“
PDE4 inhibitors such as apremilast might ... have potential in the treatment of fibrosis in SSc”

New research published in *Annals of the Rheumatic Diseases* suggests inhibition of phosphodiesterase 4 (PDE4) could have disease-modifying antifibrotic effects in systemic sclerosis (SSc), particularly in those patients with inflammation-driven fibrosis. In preclinical models of SSc, PDE4 blockade prevented progression of chronic fibrosis and also reversed established fibrosis by reducing inflammatory cell activity and inhibiting the release of profibrotic cytokines from M2 macrophages.

Treatment with the PDE4 inhibitor rolipram hampered the development of skin fibrosis in bleomycin-challenged mice in a dose-dependent manner. Skin thickness, amount of fibrotic tissue and myofibroblast numbers were substantially lower in mice treated with rolipram in comparison with vehicle-treated mice. Notably, leukocyte

infiltration in lesional skin was markedly reduced by rolipram treatment, but fibroblasts were not directly affected.

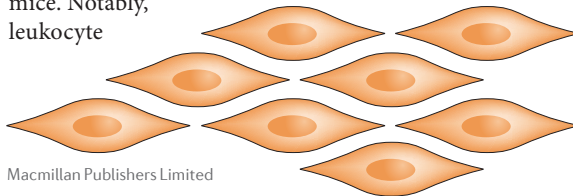
In peripheral blood monocytes isolated from healthy volunteers and patients with diffuse-cutaneous SSc, PDE4 blockade with rolipram inhibited the differentiation of monocytes into an alternatively activated M2 macrophage phenotype, but not into a classically activated M1 phenotype. Rolipram treatment also reduced mRNA expression of the profibrotic cytokines *IL6*, *IL13*, *TGFB1* and *TGFB2* in M2 macrophages (but not M1 macrophages), as well as secretion of IL-6. Consistent with these *in vitro* findings, numbers of M2 macrophages and tissue levels of IL-6 were reduced in skin sections from mice with bleomycin-induced fibrosis treated with rolipram.

PDE4 blockade was also shown to have antifibrotic effects in mice with established

bleomycin-induced fibrosis. In this model, treatment with the PDE4 inhibitor apremilast prevented progression of chronic fibrosis and also induced regression of established fibrosis. Additionally, leukocyte infiltration, M2 macrophage differentiation and tissue levels of IL-6 were reduced after apremilast treatment. Pharmacological PDE4 blockade also showed antifibrotic effects in the topoisomerase I mouse model of fibrosis and in murine sclerodermatous graft-versus-host disease, a model of diffuse-cutaneous SSc fibrosis.

Notably, apremilast is already clinically approved for the treatment of psoriasis and psoriatic arthritis. The findings of the current study suggest that PDE4 inhibitors such as apremilast might also have potential in the treatment of fibrosis in SSc, perhaps in early-stage SSc, which is characterized by inflammatory infiltrates, or in inflammatory subtypes of SSc in particular.

Sarah Onuora



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ORIGINAL ARTICLE Maier, C. *et al.* Inhibition of phosphodiesterase 4 (PDE4) reduces dermal fibrosis by interfering with the release of interleukin-6 from M2 macrophages. *Ann. Rheum. Dis.* <http://dx.doi.org/10.1136/annrheumdis-2016-210189> (2017)



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SPONDYLOARTHROPATHIES

Fine tuning the management of axial spondyloarthritis

Daniel Wendling and Clément Prati

Updated recommendations for the management of axial spondyloarthritis provide a useful framework for physicians treating this disease. However, the guidance on use of biologic therapies and treat-to-target strategies seems to raise more questions than it answers.

Refers to Van der Heijde, D. *et al.* 2016 update of the ASAS-EULAR management recommendations for axial spondyloarthritis. *Ann. Rheum. Dis.* <http://dx.doi.org/10.1136/annrheumdis-2016-210770> (2017)

to the management recommendations was produced by the task force, denoting the level of evidence for each recommendation, the grade of recommendation and the degree to which each member of the task force agreed with each recommendation. The results were then presented as five overarching principles and 13 recommendations³.

These new recommendations and principles³ highlight some interesting points for consideration. First, they encompass the whole spectrum of axial SpA, including non-radiographic forms. Although discussed in these recommendations as a concept, non-radiographic axial SpA is a reality in the clinic and should not be ignored. The second point to consider is how to integrate the recommendations for the initiation of a biological DMARD (bDMARD) into the general management of the disease, as has already been done in some national recommendations⁴. Finally, the third point to consider is the cost of managing and treating the disease (overarching principle number five in the 2016 recommendations³). This principle represents a new dimension in the treatment of axial SpA, as new treatments such as bDMARDs are particularly expensive.

Van der Heijde *et al.*³ provide a clear framework for the initiation and evaluation of bDMARD therapy for patients with axial SpA. This framework could help practitioners make informed decisions about the positioning of this treatment option within a disease management strategy. Van der Heijde *et al.*³ also emphasize the importance of objective signs of inflammation, such as elevated serum C-reactive protein level or clear signs of inflammation on MRI scans, which represent recognized markers of good response to bDMARD therapy.

The 2016 recommendations³ also include new therapeutic options from the growing range of biologic therapies, such as drugs targeting IL-17A. However, the proposed position of such therapies as a second choice after TNF inhibitors might be subject to discussion and could change as we gain experience using these drugs in the clinic. The arrival of biosimilars is also likely to have some economic implications for the prescription of bDMARDs, as discussed in overarching principle number five of the 2016 recommendations³. Additionally, these recommendations

Spondyloarthritis (SpA) is a common, chronic inflammatory disease with several types of phenotypic presentation (including axial, peripheral and enthesitic) that can have many possible extra-rheumatic manifestations (such as uveitis, psoriasis and inflammatory bowel disease)¹. Classification criteria published in 2009 have shed light on non-radiographic forms of axial SpA², and advances in our knowledge of the immunological mechanisms involved in the disease process have enabled the development of new therapeutic options. At the same time, new concepts and management strategies, such as treating to target, tight control and the therapeutic ‘window of opportunity’, have been proposed. These new

considerations prompted the Assessment of Spondyloarthritis International Society (ASAS) to produce a 2016 update to their management recommendations for axial SpA in collaboration with EULAR³.

These recommendations were produced using EULAR standardized operating procedures and were aimed at aggregating pre-existing recommendations for the management of ankylosing spondylitis and for the use of anti-TNF agents in axial SpA. The ASAS–EULAR steering committee defined the research questions for two systematic literature reviews that were discussed and presented to an international task force of 14 members. After a 1-day meeting, an update

recognize the possibility of tapering the dose or frequency of administration of anti-TNF agents for patients in sustained remission (strategies already employed by many in the clinic).

Although addressing several important points, the 2016 ASAS-EULAR recommendations highlight some unmet needs and unresolved questions, mainly regarding therapeutic strategies. Recommendation number three states that “treatment should be guided according to a predefined treatment target”³. Although this recommendation is in line with the preferred treat-to-target strategy⁵, the 2016 recommendations³ do not provide a clear definition of what such a treatment target should be. Even though there is no consensual definition of remission in axial SpA, validated definitions of low disease activity are available, which could serve as the basis for a treatment target in clinical practice. The ankylosing spondylitis disease activity score (ASDAS) is one such validated tool, and an ASDAS of <1.3 (defining inactive disease) could serve as a target for a therapeutic strategy, as could a reduction in ASDAS by at least 1.1 points during treatment^{3,4}. Another operational definition for a treatment target could be the maintenance of normal activities and capacities (domestic and professional).

Convincing medico-economic data are also lacking, particularly data evaluating the cost effectiveness of long-term use of biological treatments, which should be considered when interpreting these recommendations³. As the range of novel therapeutic possibilities (such as anti-IL-17 agents, anti-IL-23 agents and small-molecule inhibitors) is likely to increase in the near future⁶, head-to-head studies will be required to define a hierarchy of these options and clarify the position of each therapy within a disease management strategy. Studies should also address the feasibility and applicability of a treat-to-target strategy and confirm a potential window of opportunity for early

non-radiographic axial SpA is a reality in the clinic and should not be ignored

active treatment of this disease. Moreover, in light of available therapies, strategies that optimize the use of anti-TNF agents should be developed alongside recommendations for the biomonitoring of such drugs (evaluating circulating drug concentrations and levels of anti-drug antibodies)⁷. Studies that evaluate the evidence for a potential structural effect of NSAIDs and biologic treatments in axial SpA are also of interest⁸.

Finally, beyond any new treatments or management recommendations, the crucial point in clinical practice remains the early diagnosis of axial SpA and the identification of conditions that might interfere with evaluation of the disease, such as fibromyalgia⁹. Insufficient confidence in diagnosis might explain poor treatment outcomes. For example, treatment with a TNF inhibitor was not superior to placebo in a subgroup of patients classified as having non-radiographic axial SpA who had normal levels of C-reactive protein and an absence of inflammation of sacroiliac joints on MRI scans¹⁰. As emphasized in the new ASAS-EULAR recommendations³, simply fulfilling the disease classification criteria is not sufficient for diagnosis, and objective signs of inflammation are of value.

Overall, the new ASAS-EULAR recommendations³ are useful not only to provide a framework for physicians, but also to pave the way for a research agenda that looks for answers to the many questions that have arisen from our knowledge and practice to date.

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Competing interests statement

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Risk of premature death in gout unchanged for years

Chang-Fu Kuo and Shue-Fen Luo

The increased risk of mortality in patients with gout is increasingly recognized, and multiple guidelines call for better management of this disease and its comorbidities. A new study, however, has found that excess mortality in patients with gout has remained unchanged since 1999.

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(1.10 and 1.09, respectively). The mean follow-up time in both cohorts was slightly over 3 years, which covers only the relatively early clinical course of gout; whether a longer exposure to gout and associated comorbidities, along with poor management, would affect the premature mortality gap requires further study to confirm.

By contrast, a similar UK study examining premature mortality trends in patients with rheumatoid arthritis (RA) found that the absolute mortality rate as well as the magnitude of excess death (compared with matched controls) improved over the same period⁴. The improvement in mortality rates in both patients with gout and those with RA, as well as their matched controls, is suggestive of an improvement in general patient care. For example, statins are essential in cardiovascular disease treatment and prevention today, and have been shown to reduce the risk of premature mortality. The surge in the rate of statin prescriptions in UK primary care between 1995 and 2013 (REF. 5) could have exerted an effect on all patients, including those with gout and RA.

So why has the relative risk of death in patients with gout remained unchanged while excess death has reduced substantially in those with RA? Firstly, in the period studied by Fisher *et al.*³ the specific care of patients with RA improved greatly owing to the wider availability of biologic agents and an increased prescription of methotrexate in the UK. By contrast, the use of urate-lowering therapy (ULT), which has the potential to effectively cure gout, remained unchanged between 1997 and 2012, with only one-third of patients with gout receiving ULT⁶. Moreover, improved control of RA disease activity reduces the requirement for NSAIDs, which are known to increase the risk of cardiovascular, renal and gastrointestinal comorbidities, whereas the use of NSAIDs in patients with gout, as Fisher *et al.* show, reduced only marginally, from 34.4% in the early cohort to 30.7% in the late cohort³. Secondly, evidence for survival benefits attributable to effective control of RA disease activity is robust, whereas the effect of serum uric acid levels on mortality among patients with gout is controversial⁷. The optimal treatment target (serum uric acid level) and duration of ULT require more study to define. Thirdly, approximately one-third of patients with incident

Gout is the most common form of inflammatory arthritis, and its prevalence is rising globally. The manifestations of gout have been known in detail for centuries, and the mechanism of this disease — intense inflammation caused by monosodium urate (MSU) crystal deposition secondary to long-term uncontrolled hyperuricaemia — is well understood. In fact, among all forms of inflammatory arthritis, gout is probably the best understood. In addition, effective therapeutic agents that suppress inflammation (such as NSAIDs, colchicine, glucocorticoids and IL-1 inhibitors) and that reduce serum uric acid levels (xanthine oxidase inhibitors, uricosuric agents and pegloticase) are available for use in routine clinical practice. Yet the management of gout and the compliance of affected patients with definitive treatments are historically the worst among chronic illnesses¹.

Many barriers to effective gout treatment, from both the patient's and physician's perspective, have been discussed previously¹. Remedies to some of these barriers have been identified and included in current treatment recommendations and guidelines. Despite these efforts, however, the prognosis of gout, including mortality, is worse than in the general population. Studies from the UK, USA and Taiwan repeatedly consolidate the evidence for a higher risk of mortality, particularly for cardiovascular and metabolic causes of death, in patients with gout². A new

study from the UK³ further shows that the increased mortality risk in patients with gout has remained unchanged since 1999.

“the increased mortality risk in gout patients has remained unchanged since 1999”

Fisher *et al.*³ used The Health Improvement Network (THIN), an electronic primary care database representative of the UK general population, to compare the mortality of patients with incident gout in ‘early’ (diagnosed 1999–2006) and ‘late’ (diagnosed 2007–2014) cohorts, with that of their respectively matched controls. The crude death rate was higher in the early cohort (29.1 deaths per 1,000 person-years) than in the late cohort (23.0 deaths per 1,000 person-years), with a similar trend in the matched controls for the early and late cohorts (23.5 and 18.8 deaths per 1,000 person-years, respectively). Not surprisingly, the relative risk of death was higher in both gout cohorts as compared with their respective controls. However, the premature mortality gap, which denotes the excess risk of death imposed by gout, was similar between each of the early and late cohorts and their respective controls. Notably, the adjusted hazard ratio for death was similar for those diagnosed with gout in 1999–2006 and those diagnosed 2007–2014

gout already have a comorbidity at diagnosis, and half of those will have at least one major comorbidity within 5 years of diagnosis⁸. Many of these comorbidities, such as diabetes mellitus and chronic kidney disease, have been linked to chronic hyperuricaemia. Therefore, by the time they receive a diagnosis of gout, many patients have probably sustained substantial irreversible damage as a consequence of long-term hyperuricaemia and its associated comorbidities, which could translate to a relatively 'fixed' mortality gap compared with the general population. In this regard, initiating treatment for gout or hyperuricaemia only after the occurrence of arthritis seems inadequate. A staging system proposed in 2014 incorporates asymptomatic hyperuricaemia and MSU crystal deposition without signs or symptoms of gout in the spectrum of gout, which could serve as a basis for testing the potential benefit of screening for and treating asymptomatic disease⁹.

It is frustrating to find that the premature mortality gap of gout has not improved in a decade of substantial medical advances. Clinicians need to become more aware of optimized care for patients with gout, as reported in the 2016 EULAR recommendations for the management of gout¹⁰. Key principles include ample patient education and lifestyle advice, consideration and discussion of ULT early in the disease course, maintaining optimal

“ It is frustrating to find that the mortality of gout has not improved in a decade of substantial medical advances ”

serum uric acid levels (<6 mg/dl; <5 mg/dl in those with severe gout) and screening and management of associated comorbidities. Further research to define the cardiovascular, renal and metabolic benefits of ULT, to promote awareness of the disease among both physicians and patients, to test the role of screening for and treating asymptomatic hyperuricaemia and asymptomatic MSU deposition, and to investigate strategies to manage comorbidities associated with gout should help us to better understand the clinically relevant and manageable factors that would affect outcomes in patients with gout and, subsequently, improve clinical practice to close the premature mortality gap in gout.

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The authors declare no competing interests.

Sjögren syndrome genetics vary according to ancestry

Tove Ragna Reksten and Roland Jonsson

A pioneering new study scrutinising the genetic aetiology of Sjögren syndrome across different ancestries and clinical subtypes shows that the striking heterogeneity of associations observed in Sjögren syndrome cannot be explained by subphenotype differences alone.

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between populations, and difficulties in controlling for distributions of subphenotypes (such as autoantibody status, glandular involvement and disease severity) in patient cohorts. Evidence for the influence of ethnicity on Sjögren syndrome phenotype⁵ suggests that assessment of the influence of genes on disease frequencies and phenotype distributions is warranted, as opposed to only environmental factors such as diet and socioeconomic status that otherwise might affect study outcomes. This need was addressed by Taylor *et al.*⁶ in a multisite project analysing samples and clinical information collected from individuals of Native American, Asian, and European ancestry, which enabled analyses that accounted for both intercontinental and intracontinental genetic substructures of these populations.

In this study, data from a global GWAS corroborated established associations with regions in HLA genes, *STAT4* and *IRF5*, and identified novel suggestive associations in regions previously linked with other autoimmune diseases, including *SH2D2A* and *KLRG1* (REF. 6) (Supplementary information S1 (table)). Interestingly, *KLRG1* seemed to be associated with Sjögren syndrome solely in those of Asian ancestry, and the data suggest that the *GTF2I* association previously identified in Asians is also implicated in Europeans. Taylor *et al.*⁶ confirmed the prominent discrepancy in the locations and significance of HLA associations between ethnic groups that has been noted elsewhere⁷⁻⁹ (Supplementary information S1 (table)). Furthermore, their findings reassert the high genetic heterogeneity among Asians and Europeans with Sjögren syndrome, and identify associations in Asian populations not measurable in Europeans owing to low allele frequency. As allele frequencies did not fully explain the observed association differences, Taylor *et al.*⁶ investigated subphenotype effects by examining the correlation between global ancestry and the fulfilment of Sjögren syndrome criteria including labial salivary gland focus score ≥ 1 , ocular staining score (OSS) ≥ 3 and presence of anti-Ro/SSA and anti-La/SSB antibodies. The finding that involvement of all three criteria is higher in Asian individuals indicates that European ancestry is protective in Sjögren syndrome, in line with the reported results of the aforementioned epidemiologic study in a multiracial population⁵.

Sjögren syndrome is an autoimmune rheumatic disease characterized by focal lymphoid infiltration in lacrimal and salivary glands and the presence of anti-nuclear antibodies, anti-Ro/SSA antibodies and/or anti-La/SSB antibodies, as described in the latest classification criteria¹, but the aetiology of this disease is highly elusive. Sjögren syndrome manifests as severe dryness of the eyes, mouth, skin and mucosa, and is often accompanied by other symptoms such as fatigue, arthralgia, neuropathies and swelling of salivary glands and lymph nodes. The reported prevalence of Sjögren syndrome varies greatly, ranging from 0.05% in Norwegians² to 0.60% and 0.77% in Greek³ and Chinese populations⁴, respectively. A 2014 epidemiological study in a multiracial population demonstrated further differences between ethnic groups, including a twofold increase in disease prevalence, increased likelihood of autoantibody positivity and earlier age at onset of primary Sjögren syndrome in patients of non-European background as compared with those of European background⁵. Now, the first multi-ethnic genome-wide association study (GWAS) in Sjögren syndrome provides new insights into the links between genetic aetiology, ancestry and clinical subphenotypes⁶.

Genetic factors implicated in the pathogenesis of Sjögren syndrome include well-established associations with genes pivotal for antigen presentation, innate immune responses, and lymphocyte activation and

signalling. In the wake of data steadily emerging from GWASs in Sjögren syndrome^{7,8}, it has become evident that allele frequencies and representative single-nucleotide polymorphisms (SNPs) differ substantially within established associations. This heterogeneity is particularly noticeable in reported associations in the HLA system. Class II HLA-DR and HLA-DQ associations have been described in a range of white populations and in Chinese, Hispanic and Jewish Israeli populations (reviewed by Cruz-Tapias *et al.*⁹); however, disease susceptibility and protective alleles or haplotypes differ between ethnic groups. These observations are supported by large GWASs conducted in Han Chinese⁷ and white European populations⁸. In addition to HLA genes, overlapping associations include *STAT4* and *TNFAIP3*, whereas *IRF5*, *IL12A*, *BLK*, *CXCR5* and *TNIP1* were identified as risk loci in Europeans⁸ and an association with *GTF2I* was established in a Han Chinese population⁷.

Plausible explanations for these discrepancies in genetic associations include small sample sizes, differences in allele frequencies

their findings reassert the high genetic heterogeneity among Asians and Europeans with Sjögren syndrome

If subphenotypes indeed drive the observed European–Asian heterogeneity, analysis of only positive cases (that is, those that are positive for the subphenotype characterized by fulfilment of the three criteria outlined above) would render the confidence intervals in the two populations more similar. With the exception of HLA-DPB1, which was associated with focus score and antibody positivity in both populations, subphenotypes were not found to drive the association differences. Taylor *et al.*⁶ adjusted for possible confounding factors arising from the use of data from different recruitment sites and used standardised methods to determine diagnosis. Nonetheless, Sjögren syndrome is considered underdiagnosed in mildly affected individuals, particularly in medically underserved communities, and recruitment at the various sites could be skewed, possibly contributing to the differences in phenotype.

With the exception of HLA-DPB1 ... subphenotypes were not found to drive the association differences

Notably, the non-European control samples were analysed on a different genotyping platform, which provided an overlap set limited to approximately 300,000 SNPs for analysis of the Asian group. Despite these limitations, Taylor

*et al.*⁶ identified novel suggestive associations in regions implicated in other autoimmune diseases, warranting further investigation. Identifying population-specific risk factors and disease pathways is pivotal to increasing our understanding of Sjögren syndrome and to developing targeted treatments. Although genetic associations provide insights into the aetiology of the disease, understanding the contribution of epigenetic modifications, as demonstrated in DNA methylation studies in salivary glands and B cells¹⁰, along with that of environmental factors such as diet, smoking, infections and climate, is essential when attempting to evaluate genetic aetiology, ancestry and subphenotype heterogeneity.

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The authors declare no competing interests.

SUPPLEMENTARY INFORMATION

See online article: [S1 \(table\)](#)

ALL LINKS ARE ACTIVE IN THE ONLINE PDF

What rheumatologists need to know about CRISPR/Cas9

Gary J. Gibson and Maozhou Yang

Abstract | CRISPR/Cas9 genome editing technology has taken the research world by storm since its use in eukaryotes was first proposed in 2012. Publications describing advances in technology and new applications have continued at an unrelenting pace since that time. In this Review, we discuss the application of CRISPR/Cas9 for creating gene mutations — the application that initiated the current avalanche of interest — and new developments that have largely answered initial concerns about its specificity and ability to introduce new gene sequences. We discuss the new, diverse and rapidly growing adaptations of the CRISPR/Cas9 technique that enable activation, repression, multiplexing and gene screening. These developments have enabled researchers to create sophisticated tools for dissecting the function and inter-relatedness of genes, as well as noncoding regions of the genome, and to identify gene networks and noncoding regions that promote disease or confer disease susceptibility. These approaches are beginning to be used to interrogate complex and multilayered biological systems and to produce complex animal models of disease. CRISPR/Cas9 technology has enabled the application of new therapeutic approaches to treating disease in animal models, some of which are beginning to be seen in the first human clinical trials. We discuss the direct application of these techniques to rheumatic diseases, which are currently limited but are sure to increase rapidly in the near future.

CRISPR

Segments of prokaryotic DNA containing short repetitions of DNA sequences, which are interrupted by so-called spacer DNA derived from past invaders. CRISPR serves as the bacterial adaptive immune system that protects against invading genetic materials.

Rheumatic diseases represent a diverse family of complex diseases. Many are associated with disorders of the immune system and most have a strong genetic predisposition. Their causes are generally unknown. Advances in gene editing technologies offer the ability to define the underlying biology of these diseases and provide rational targets for new drug development. Initial gene editing techniques used zinc finger nucleases or transcriptional activator-like nucleases (TALEN) to provide the necessary double-strand DNA breaks. Both nucleases are modular proteins that can be engineered to fit a desired sequence and thus create double-strand breaks at a defined DNA sequence.

The gene editing technology, clustered regularly interspersed short palindromic repeats (CRISPR) associated protein 9 (CRISPR/Cas9), is the latest addition to this molecular tool box and has seen a spectacular level of research interest since the 2012 publication of data suggesting its potential as a gene editing tool for eukaryotes¹. The Cas9 nuclease uses short RNAs to target the desired DNA sequence, avoiding the laborious and expensive protein engineering necessary with the two previously described techniques. This landmark paper¹ was soon followed by a succession of papers establishing and adapting the technique for gene editing in eukaryotes^{2–8}.

The rapid pace of publication has continued to the present time, establishing CRISPR/Cas9 as a broadly versatile and technically simple technique for gene editing. This technique will undoubtedly transform the way we conduct basic biology research in the future.

In this Review, we briefly describe the developments in CRISPR/Cas9 technology that have improved its specificity, greatly simplified the creation of complex animal models of disease and enabled more efficient insertion of DNA sequences, the repression and activation of multiple genes in a single cell, the control of disease-carrying vectors and the identification of multiple genes, gene pathways and gene interactions essential for specific phenotypic changes and disease pathologies. Such applications could have a profound effect on our understanding of the biology underlying rheumatic diseases, and could lead to the identification of new therapeutic targets and the possibility of radical new treatment strategies.

Brief history of CRISPR gene editing

The characteristic CRISPR palindromic repeats were first recognized as an interesting feature in *Escherichia coli* in 1987 (REF. 9) but were not further investigated until much later. With an expanding number of sequenced

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Key points

- Advances in CRISPR technology have provided the capacity to precisely identify and define the function of genes and noncoding regulatory elements associated with disease development and susceptibility
- CRISPR technology has made the generation of mouse models of disease much quicker and less expensive than traditional approaches, and has facilitated the development of much-needed larger animal models of disease
- CRISPR technology has enabled the generation of gene drives, whereby genetic changes propagate rapidly through a species, providing the potential to eliminate disease vectors and thus vector-borne diseases such as malaria
- Successful treatment of mouse models of human diseases suggests that CRISPR technology can be applied to treat human diseases in the future
- CRISPR technology has the ability to facilitate a breakthrough in our understanding of the more common and complex human diseases, including rheumatic diseases
- The potential of CRISPR/Cas9 technology in the development of new treatment strategies is confidently expected to have a major effect on the practice of rheumatology

CRISPR/Cas9

RNA-guided gene editing platform based on the Cas9–gRNA ribonucleoprotein complex. The two-component complex can mediate gRNA-programmed recognition of specific DNA sequences and create a site-specific double-strand cleavage of the targeted DNA.

Cas9

An RNA-guided DNA endonuclease enzyme that is the universal component of the RNA-guided CRISPR/Cas9 gene editing machinery. Cas9 by itself is inactive; upon binding to the gRNA scaffold, Cas9 goes through conformational changes that initiate its target recognition, binding and cleavage activity.

Spacer

The spacer sequence refers to the 5' end, ~20 nucleotide variable sequence of the targeting gRNA construct. The spacer contains a targeting sequence that matches a region of DNA substrate and guides Cas9 nuclease activity.

Protospacer

The protospacer sequence refers to the targeted site on the DNA substrate. The nucleotide sequences of the spacer and the corresponding protospacer are identical.

genomes available, the prevalence of CRISPR palindromic sequences was recognized in a wide range of archaea and bacteria and the CRISPR name was coined¹⁰. CRISPR loci vary between bacterial and archaeal species but have the characteristic feature of a sequence of short, repetitive, partially palindromic sequences separated by equally short (30 to 40 base pairs) spacer sequences. The CRISPR loci are flanked by a variable number of CRISPR associated (Cas) genes¹¹. Recognition that a spacer sequence in *E. coli* was homologous with that of an *E. coli*-infecting virus (also known as a bacteriophage or phage) lead to the realization that CRISPR constituted an adaptive immune system for bacteria¹².

Unsurprisingly, bacteria and archaea have a variety of viral defence systems; they are outnumbered ten to one by phages¹³. The mechanisms of innate immunity in bacteria and archaea have been known for a long time, and include induction of cell death, expression of restriction enzymes and the presence of a sticky cell coat¹⁴. However, the cellular machinery and complex interactions that constitute an adaptive immune response had been considered a property of eukaryotes. The identification of CRISPR loci in nearly all species of archaea and most species of bacteria established that these organisms also have an adaptive immune system to defend against viral invasion (reviewed elsewhere^{11,13}). The CRISPR system performs two of the fundamental functions of an immune system. First, this system enables the acquisition of an immunological memory. Upon attack by a phage, some of an organism's Cas genes are activated to cut DNA sequences, termed protospacers, from the attacking phage and integrate them into their own CRISPR array, thus creating an immunological memory of their viral attackers^{11,13}. To avoid targeting its own DNA, a specific sequence termed the protospacer adjacent motif (PAM), which is rarely present in the host genome, is selected by the host organism. The PAM sequence varies according to the CRISPR/Cas type. The second immune system function provided by CRISPR is destruction of invading viruses. CRISPR RNA (crRNA) is generated from the stored CRISPR sequences in response to viral infection and then used to guide an

attack on the invader^{11,13}. The crRNAs are initially transcribed as long transcripts, which are then cleaved by endogenous RNase or specific Cas proteins to make smaller crRNAs, which direct Cas nucleases to cleave both DNA strands of the invader (FIG. 1).

The wide variety of CRISPR modules present in bacteria and archaea are divided into two classes, five types and 16 subtypes primarily on the basis of the number and type of Cas genes involved¹⁵. Type II CRISPR has been developed for gene editing in eukaryotes. Cas9, a large protein with two nuclease active sites, one that cleaves the target strand and another that cleaves the non-complementary strand¹⁸, is the nuclease used in type II CRISPR and requires an additional small RNA, *transactivating crRNA* (tracrRNA) for target recognition and cleavage.

Jinek *et al.*¹ first demonstrated that CRISPR/Cas9, in combination with crRNA and tracrRNA, could be used to specifically target DNA cleavage *in vitro*. In addition, they demonstrated that crRNA and tracrRNA could be combined to create a single guide RNA (gRNA) to direct sequence-specific Cas9 double-stranded DNA cleavage, and suggested that this technique might represent a simple, programmable RNA method that could be used for genome targeting and genome editing in eukaryotes (FIG. 2). This paper opened the flood gates for CRISPR/Cas9 directed genome editing. Within months, several papers were published that described the application of CRISPR/Cas9 gene editing in mammalian cells and made substantial improvements to the technique^{2–8}. The nucleotide sequence of *cas9* was reconstructed by codon optimization and inclusion of nuclear localization signals to optimize nuclear expression in mammalian cells³. The efficiency of gRNA was substantially improved by restoring the critical 3' hairpin structure, and the capacity of the system to edit several genomic sites using multiple gRNA sequences encoded in a single construct was demonstrated^{2–8}.

The CRISPR system in bacteria and archaea has limited application to rheumatology; however, it is the basis for understanding the mechanisms of action and components of CRISPR/Cas9 that have been adapted to genome editing in eukaryotes.

Improvements in CRISPR/Cas9 technology

Off-target mutations

DNA cleavage can occur with the CRISPR/Cas9 system even if there is imperfect complementarity between the gRNA and target DNA, particularly if the mismatches are in the 5' region of the target sequence. In the original report by Jinek *et al.*¹, gRNA was shown to tolerate up to five mismatches. This finding raised serious concerns for the use of CRISPR/Cas9 in genome editing, particularly for *in vivo* editing. Fortunately, methods to minimize and potentially eliminate off-target mutations have been developed over the past few years. Freely available software developed by measuring off-target cleavage of thousands of gRNAs has enhanced gRNA design, enabling the elimination of promiscuous gRNAs, minimizing off-target cleavage and maximizing effectiveness^{16,17}. Additional strategies that reduce off-target cleavage include reducing the size of the gRNA target site

Protospacer adjacent motif (PAM)

A three base pair DNA sequence immediately following the protospacer or the DNA sequence targeted by the Cas9/gRNA ribonuclease. The canonical PAM sequence for CRISPR/Cas9 gene editing machinery is 5'-NGG-3'.

Guide RNA (gRNA)

gRNA, also known as short guide RNA (sgRNA) is a short synthetic RNA sequence consisting of a scaffold structure and a programmable ~20 nucleotide spacer at the 5' end. The ~80 nucleotide RNA scaffold structure is essential for mediating both Cas9 protein binding and activation. The unique spacer sequence dictates the DNA target site to be recognized and cleaved by Cas9 protein.

Non-homologous end joining (NHEJ)

A cellular pathway that repairs double-strand breaks in DNA. NHEJ is active throughout the cell cycle and requires no repair template. NHEJ is frequently imprecise and the repair process can generate an open reading frame shift with insertions, deletions or mutations at the site of double-strand breaks. The inaccurate nature of the NHEJ repair process forms the basis of the CRISPR/Cas knockout strategy.

Homology-directed repair (HDR)

The HDR pathway (also known as homologous recombination), involving a homologous template (either a sister chromatid or an exogenous DNA template), repairs double-strand DNA breaks accurately according to the template. The template or donor DNA consists of left and right arms identical to sequences flanking the double-strand break. Between the arms, any DNA sequence or marker can be inserted and HDR will force the additional genetic material to be knocked in to the particular locus. HDR is usually believed to be active only during S and G2 phases of the cell cycle.

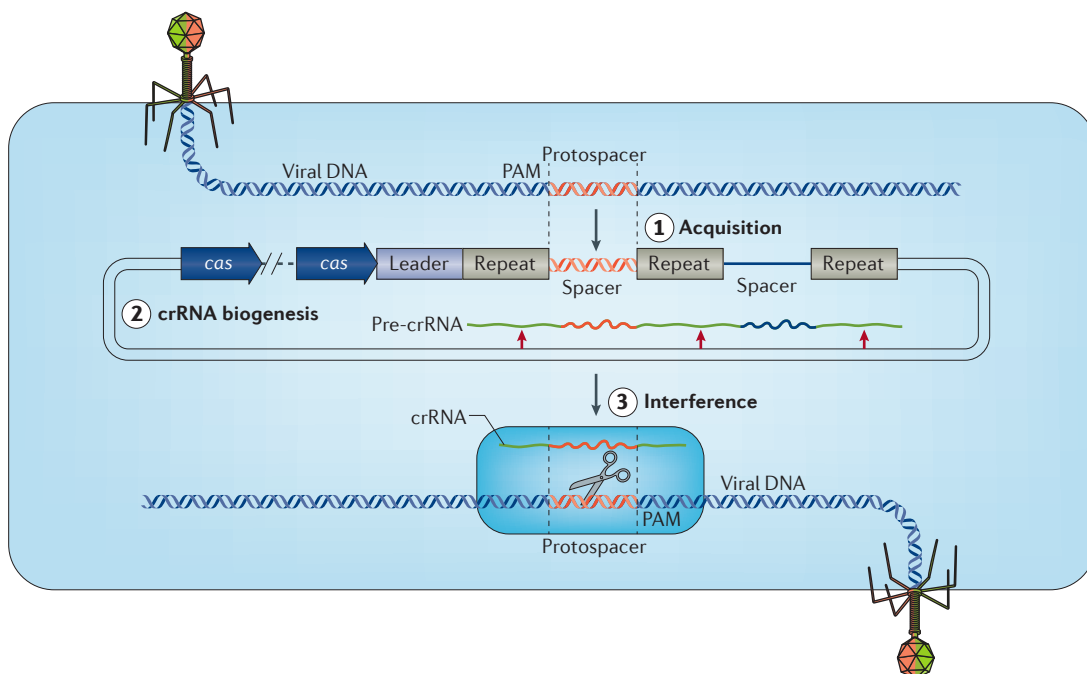


Figure 1 | **Adaptive immune system of bacteria and archaea.** CRISPR-associated (Cas) genes (*cas*) (blue arrows) encode proteins required for new spacer sequence acquisition, CRISPR RNA (crRNA) biogenesis and target interference. Step 1. Acquisition. Unique sequences (protospacers) are acquired from invading viruses and inserted into the host genome, separated by partially palindromic repeats (Repeat). Adjacent to protospacers are short sequences called protospacer adjacent motifs (PAMs). Step 2. crRNA biogenesis. In response to viral invasion, long CRISPR transcripts (pre-crRNA) are processed into short crRNAs that guide Cas proteins to invading DNA through complementary base-pairing. Step 3. Target interference. Cas nucleases initiate double-strand breaks in the DNA at the target site. Permission obtained from Annual Reviews © Sorek, R. *et al. Annu. Rev. Biochem.* 82, 237–266 (2013).

from 20 nucleotides to 17–18 nucleotides¹⁸. These shortened gRNAs seem to have the same efficiency as full-length gRNA in directing DNA targeting and cleavage, but show decreased off-target cleavage and increased sensitivity to gRNA:DNA mismatches¹⁸. One of several molecular modifications of Cas9 has been to mutate one of the nuclease active sites such that the enzyme cleaves only one of the DNA strands (termed a Cas9 nickase). Using Cas9 nickase and two paired and appropriately offset gRNAs, larger DNA double-strand breaks were created and the specificity improved in some sites by 50-fold to 1,000-fold¹⁹.

A promising new approach based on the detailed ultrastructure of the Cas9 protein–gRNA complex bound to target DNA has been described^{20,21}. Modifying regions of Cas9 to reduce the strength of interaction with DNA was reasoned to increase the reliance on the gRNA:DNA interaction and thus increase the sensitivity to gRNA:DNA mismatches. Kleinstiver *et al.*²⁰ engineered a version of Cas9 that targets sites of hydrogen bonding to the phosphate backbone of the target DNA strand. After systematically comparing multiple mutations and combinations of mutations, they demonstrated that one variant with four substitutions, in which alanine was substituted for charged amino acids, functioned as a high fidelity Cas9 (Cas9HF) with on-target activity similar to the wild-type, but with largely undetectable off-target mutations²⁰. Slaymaker *et al.*²¹ used a similar

rational engineering approach, but targeted the interaction between Cas9 and the complementary DNA strand by neutralizing three positively charged amino acid residues to generate a Cas9 mutant with similarly enhanced specificity (eCas9). Both studies used broadly specific, sensitive methods for detecting off-target mutations and examined multiple cell systems. Although these improvements go some way towards quashing concerns over off-target mutations, confidence in their use will only come with the continued application of high specificity Cas9 nucleases in a wide variety of systems, as off-target activity has been shown to be highly dependent on the cell type and culture system studied²².

Homology directed repair

The generation of knockout mutations using CRISPR/Cas9 is exceptionally efficient, primarily because of the high efficiency of DNA cleavage and high error rate of non-homologous end joining (NHEJ). Double-strand breaks in the host DNA are highly cytotoxic lesions that are efficiently repaired by NHEJ, the predominant repair mechanism in eukaryotes. This repair is usually inaccurate and frequent insertions and/or deletions (indels) occur²³. The consequent frameshift mutations generate premature stop codons, which result in loss of function of the target gene.

By contrast, homology directed repair (HDR) is typically highly inefficient²⁴. HDR makes use of homologous recombination to intentionally generate precise and

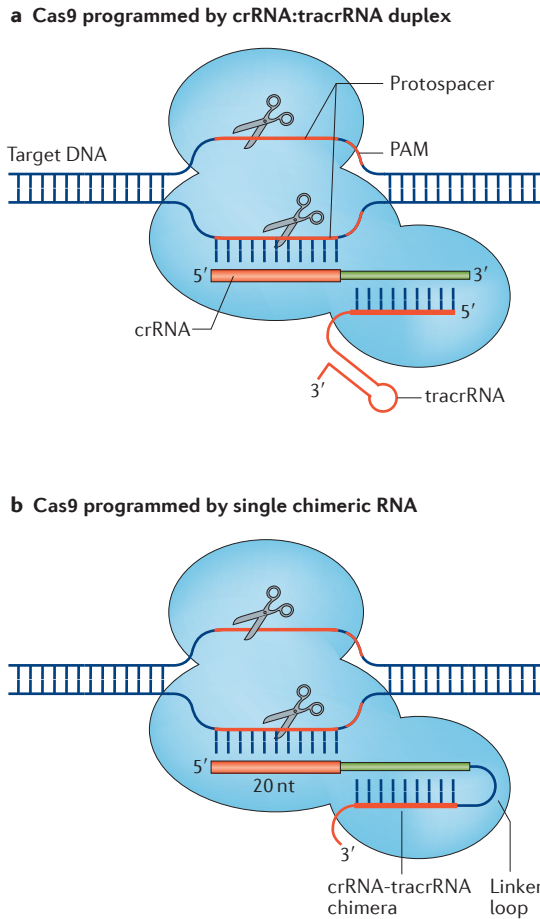


Figure 2 | Cas9 targeting using crRNA (CRISPR RNA)–tracrRNA (transactivating crRNA) or a single guide RNA chimera. a | In type II CRISPR/Cas9 systems, Cas9 is guided by a two-RNA structure formed by tracrRNA and crRNA to cleave targeted double-stranded DNA (dsDNA). **b** | A chimeric RNA generated by fusing crRNA to tracrRNA via a linker loop is able to target and cleave dsDNA. Permission obtained from Science © Jinek, M. *et al. Science* **337**, 816–821 (2012). Nt, nucleotide

specific alterations to the DNA sequence (FIG. 3). HDR is essential for many gene editing applications, particularly correction of genetic mutations. The double-strand DNA break generated by Cas9 can boost the HDR pathway by several orders of magnitude²⁵. However, efficiency still remains low, meaning that very large numbers of cells are required for successful insertion of the target sequence. Several approaches for enhancing HDR activity, including use of inhibitors of the NHEJ pathway²⁶ and use of longer homology arms for the donor DNA²⁷, have been described^{26,28,29}.

A promising new approach based on detailed investigation of the interaction of Cas9 with its target DNA substrate provides substantial improvements in the efficiency of HDR²⁹. The study demonstrated that Cas9 binds tightly to its DNA substrate for at least 5 hours after DNA cleavage²⁹, blocking access for donor DNA templates. However, one end of the cleaved DNA, the PAM-distal, non-target strand, was free of protein

interaction and could anneal exogenous DNA. By targeting this free DNA strand and optimizing donor DNA orientation, polarity and length, the researchers achieved a 60% frequency of HDR²⁹. This approach still needs to be corroborated in a variety of systems, but the increase in efficiency suggests that homologous recombination-based gene targeting should be amenable to routine laboratory manipulation. If similar widespread improvements in efficiency are achieved, this technique will substantially advance the prospects of CRISPR/Cas9-based therapeutic gene editing.

Gene repression and activation

The CRISPR/Cas9 system also offers the capability to selectively switch genes on or off without manipulating their sequence. Several groups have demonstrated that Cas9 can be mutated in both nuclease domains to generate a nuclease-deactivated Cas9 (dCas9)^{30,31}. dCas9 can be converted into a programmable gene activator or repressor via fusion with protein regulators while maintaining its ability to strongly bind specific DNA sites via target-directed gRNAs.

Systems that employ multiple activator proteins fused to dCas9 achieve consistently high levels of gene activation, ranging from 10-fold to 1,000-fold across multiple cell types and species^{32,33}. Examples of such fusion proteins include the following: VPR, which is a fusion of multiple synergistic activators, VP64 (an engineered tetramer of the herpes simplex VP16 transcriptional activator domain), transcription factor p65 and the Epstein–Barr virus replication and transcription activator (Rta); scaffolds, such as the SunTag array that binds multiple VP64 activator domains; and the synergistic activator mediator, a modified gRNA that contains binding sites for RNA-binding proteins fused with transcription activators^{33–35} (FIG. 4). A histone demethylase and a histone acetyltransferase have also been fused to dCas9 to specifically suppress or activate gene enhancers or promoters^{36,37}. These systems offer another approach to modifying gene expression, as well as helping to decipher site-specific epigenetic modifications and the role of histone methylation and acetylation in cellular function.

Fusion of dCas9 with transcription repressors (such as the Krüppel-associated box (KRAB) domain) has been effective in the generation of CRISPR-mediated gene interference (CRISPRi) (FIG. 4). When localized to DNA, KRAB recruits a protein complex that initiates chromatin remodelling, methylation and deacetylation^{30,38}. CRISPRi is similar to RNA interference (RNAi), a process whereby specific RNA molecules bind to mRNA, initiate its breakdown and thus inhibit gene expression. RNAi technology has been available for >15 years and is actively investigated as a tool for inhibiting specific gene expression both in the laboratory and as gene therapy. CRISPRi differs from and has some advantages over RNAi in that it primarily affects the process of transcription rather than affecting the levels of mature mRNA in a cell. CRISPRi is based on the Watson–Crick base-pairing model of gRNA binding to DNA and offers the same technical simplicity and broad versatility as CRISPR/Cas9.

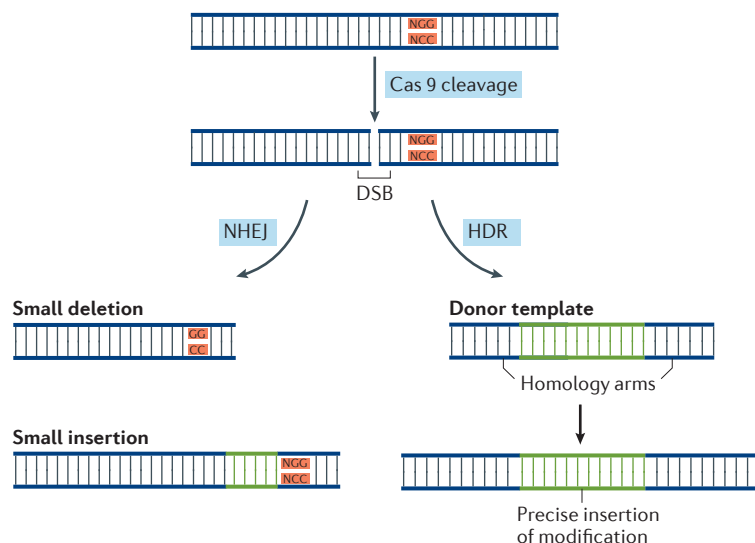


Figure 3 | Endogenous repair of double-strand DNA breaks by non-homologous end joining (NHEJ) or homology directed repair (HDR). Double-strand DNA breaks (DSB) generated by Cas9 in the absence of a donor template are repaired by NHEJ, which is frequently inaccurate, resulting in the insertion of small deletions or insertions. Inclusion of a donor template can result in HDR, leading to the introduction of desired mutations or modifications through homologous recombination.

CRISPRi can also target noncoding sequences, including noncoding RNAs. In addition, CRISPRi can efficiently knock down gene expression by 90–99%; however, the effects of CRISPRi are highly dependent on the target sites of gRNAs and differ across genes, suggesting that chromatin structure and the presence of regulatory elements can limit gene knockdown³¹.

Multiplexing

One of the exciting prospects for the use of CRISPR technology is the capacity to simultaneously activate, repress and knock out multiple specific genes in a single cell. This capacity has been made more accessible by the surprising observation that short gRNAs (14 nucleotides) bind strongly to their target sequence while simultaneously inhibiting the nuclease activity of Cas9 and preventing DNA cleavage³². These 14 nucleotide gRNAs activate genes to the same degree as 20 nucleotide gRNAs when combined with dCas9 fused to the gene activator VPR. When active Cas9 fused with the gene activator VPR (Cas9–VPR) was targeted with 20 nucleotide gRNA, the DNA cleavage activity (and associated gene mutation and, usually, gene deletion) observed was similar to that seen with wild-type Cas9, but when Cas9–VPR was targeted with 14 nucleotide gRNA, gene activation occurred. Kiani *et al.*³² demonstrated that gene knockout and activation of target genes could be performed simultaneously in single cells by transfection with Cas9–VPR and targeting with 14 nucleotide (activation) or 20 nucleotide (knockout) gRNAs³².

Our understanding of the cell-specific regulation of gene expression is rudimentary. The ability of CRISPR systems to activate, inhibit and knock out multiple genes in a single cell creates the capacity to decipher complex gene networks such as the multi-layered immune

systems associated with rheumatic diseases, the vast majority of which are multigenic. The capacity offered by dCas9 and multiplexed CRISPR systems to activate and repress multiple genes simultaneously should begin to break down barriers to understanding and, eventually, the manipulation of rheumatic diseases.

Applications of CRISPR/Cas9

Gene editing of mammalian cells

The simplest and, to date, most common application of the CRISPR/Cas system is in the generation of cell lines with complete and permanent loss of function of target genes. This application requires only transfection with plasmids containing *cas9* and the gRNA targeting the desired gene. For many cell types (particularly chondrocytes), transfection can be inefficient, partly due to the large size of *cas9* (4,104 bp)²². The creation of permanent cell lines expressing *cas9* in safe genomic loci overcomes the problems associated with transfection and creates a versatile and useful tool that can be used to create an almost unlimited range of cell lines with targeted mutations. A permanent chondrocyte cell line has been established that expresses Cas9 (rat chondrosarcoma Cas9, or RCS Cas9)³⁹. Subsequent editing of a target locus requires only transfection of this cell line with a specific gRNA. Transfection with gRNA complementary to the third exon of the aggrecan gene resulted in indel mutations in >80% of transfected cells³⁹. Most indels were predicted to generate premature stop codons and, because the target site was near the 5' end of the gene, resulted in loss of expression of the aggrecan core protein. Several genes have now been knocked out in RCS Cas9 cells by transfection with specific gRNAs, including *Has2* (REF 40), *Col6a1*, *Col6a2*, *Col6a3*, *Inpp1* and *Kank1* as well as miR-140 (all G.J.G., unpublished data). The associated loss of cell-surface hyaluronan in cells lacking hyaluronan synthase 2 (*Has2*) resulted in loss of the pericellular proteoglycan matrix and helped to define the role of hyaluronan in retention of pericellular matrix⁴⁰.

RCS Cas9 or similar cell lines are expected to facilitate the investigation of the complex interactions regulating chondrocyte function, differentiation, homeostasis and the role of disease-associated genetic traits in cartilage degeneration. One example is the a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) proteinase family, which has a central role in cartilage matrix turnover and cell–matrix interactions, although the complex structure and membrane association of ADAMTS proteinases has made identification of their precise role challenging. The generation of cell lines with targeted mutations using CRISPR/Cas9 from cells such as the RCS Cas9 cells could provide an excellent system to identify the specific, detailed function of ADAMTS proteinases and their role in cartilage pathology⁴¹. A wide variety of genes and noncoding regions that regulate cell function and pathologic changes associated with rheumatic diseases are likely to be identified in the near future using CRISPR/Cas9 technology (BOX 1). The availability of cell lines relevant to rheumatology research, like RCS Cas9, will be vital for verifying the role of these elements and more precisely defining their function.

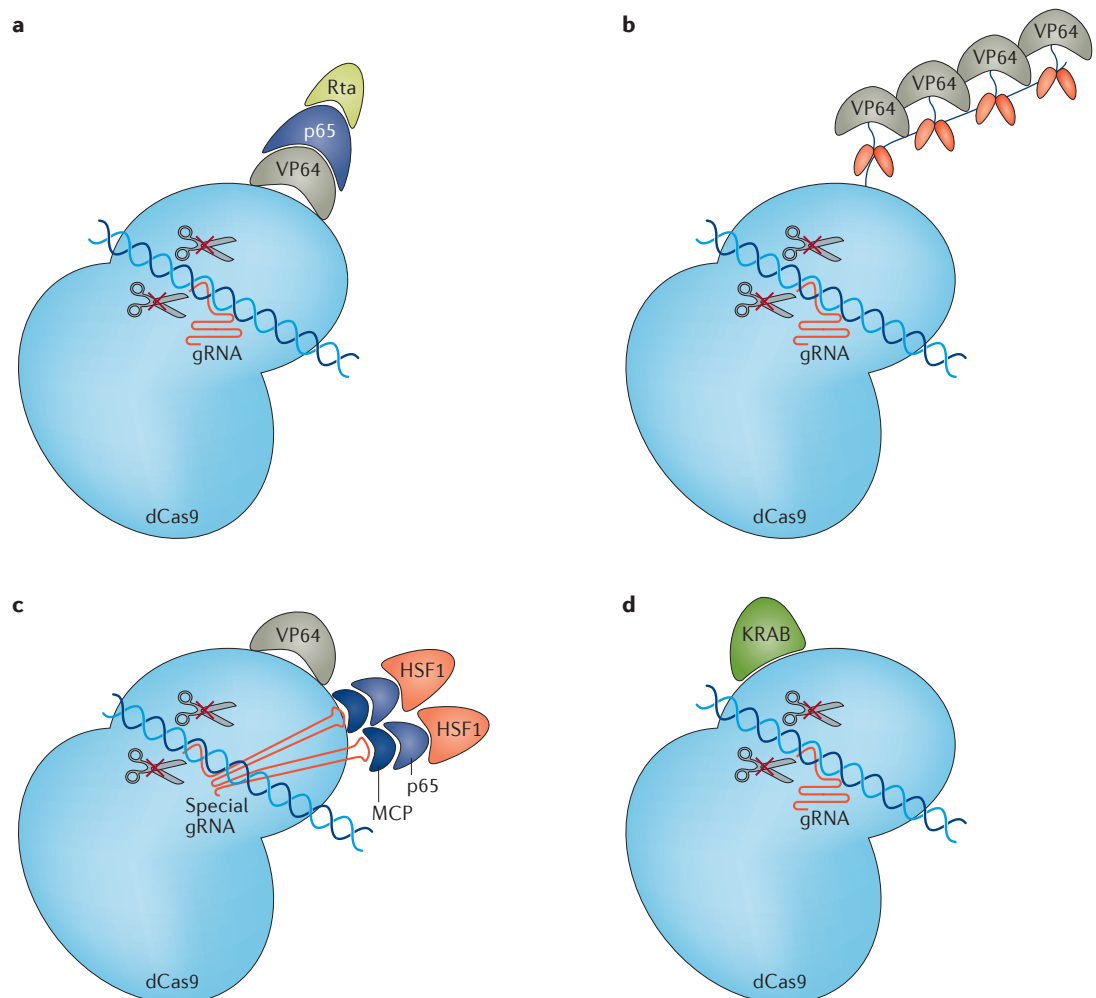


Figure 4 | Engineering nuclease-deactivated Cas9 (dCas9) for gene activation and repression. Several strategies can be used to generate gene activators: **a** | fusion of dCas9 with three activator domains, namely the herpes simplex activation domain (VP64), transcription factor p65 and the Epstein–Barr virus replication and transcription activator (Rta) (together known as VPR); **b** | an array of small peptide epitopes fused to dCas9 to recruit multiple copies of single chain variable fragment fused to VP64 (Sun Tag); or **c** | a modified guide RNA (gRNA) encoding extra loop structures that bind to the MS2 coat protein (MCP) that is in turn fused to p65 and heat shock factor 1 (HSF1). **d** | Similar strategies are employed for the generation of gene repressors and include fusion of dCas9 with the repressor domain Krüppel-associated box (KRAB). Modified with permission from NPG © Chavez, A. *et al. Nat. Methods* **13**, 563–567 (2016).

Analysis of gene regulatory elements

Noncoding *cis*-regulatory elements are estimated to make up as much as 10% of the human genome, and evidence suggests that 75% of polymorphisms associated with heritable diseases occur within these sequences⁴². The precise identification of such regulatory elements and our understanding of their role in regulating development-specific, cell-specific and disease-specific expression is very limited. New methods that analyse the entire genome, such as ChIP-seq and DNase-seq, provide extensive identification of distal regulatory elements within a specific cell type⁴³. The ability to delete large regions of the genome and prepare daughter cell lines with specific gene edits, multiple edits or gene deletions using CRISPR/Cas9 technology has provided a mechanism to precisely identify regulatory elements and decipher the function of individual enhancer regions and their interactions. For example,

researchers have identified a new class of regulatory elements with temporary enhancer activity that was lost after a few cell divisions and contributed to the complex temporal regulation of cell-specific gene expression⁴⁴. The role of distal enhancer elements that regulate expression of one of the critical matrix degrading enzymes, MMP13 is also beginning to be defined⁴⁵. This study⁴⁵ demonstrated the interplay between the transcription factors RUNX2 and C/EBPβ and the vitamin D receptor in regulation of osteoblastic differentiation. Similar studies in chondrocyte cell lines are expected to define the genome environment and interactions that regulate MMP13 expression during cartilage homeostasis, growth and degradation. More broadly, application of this approach will enable the identification of regulatory elements and the definition of their function and interaction in any system or cell type, including those most relevant to rheumatologists.

Box 1 | Application of CRISPR/Cas9 in rheumatology research

Examples of published applications of the CRISPR/Cas9 technology related to rheumatology research are relatively few, owing to the fact that the technology is only a few years old. A large proportion of the studies employing CRISPR/Cas9 are proof-of-principal for new modifications, developments and adaptations of the CRISPR toolbox.

A chondrocyte cell line expressing Cas9 has been developed that enables quick gene-editing by simple transfection with gRNAs targeting the gene of interest³⁹. These cells have been used to demonstrate the role of aggrecan in isolating chondrosarcoma cells from the host immune system³⁹ and further defining the role of cell-surface hyaluronan⁴⁰.

CRISPR/Cas9 technology has begun to define the interplay of genes and noncoding regions regulating the expression of the central matrix degrading proteinase MMP13 in bone cells⁴⁵.

The use of CRISPR/Cas9 gene screening has identified the genes present in mosquitos and humans that are necessary for the replication of families of viruses^{53,54}; together with the development of CRISPR/Cas9 gene drives^{82,84}, these findings offer the potential to eliminate insect vectors of diseases, including those with rheumatologic significance, such as Lyme disease.

Gene screening using CRISPR/Cas9 libraries has provided a new understanding of pathways of the innate immune system and identified several critical regulatory genes not previously recognized^{55,56,61}. These studies have the potential to provide new targets for the treatment of various rheumatic and associated diseases, including systemic lupus erythematosus⁵⁸, crystal-induced arthritides⁵⁹ rheumatoid arthritis and inflammatory bowel disease⁶⁰.

The potential and scope of CRISPR/Cas9 technology is enormous. These few examples of applications to rheumatic diseases will rapidly increase in the near future as new applications that are either underway or proposed in laboratories around the world are published.

Polymorphisms identified by genome-wide association studies (GWAS) are commonly in the form of single nucleotide polymorphisms (SNPs)⁴². The DNA regions typically contain multiple closely spaced SNPs that are co-inherited and thus unable to be distinguished by classical genetic association studies^{42,43}. However, CRISPR/Cas9 technology can specifically distinguish genetic variants associated with disease traits from bystander variants and enable researchers to start to decipher their causal role^{42,43}. An excellent example of the power of this technology was demonstrated in a study of the role of SNP variants in Parkinson disease⁴⁶. The accumulation of α -synuclein in the brains of patients with Parkinson disease has been associated with pathophysiology. Using CRISPR/Cas9 genetic modification of multiple SNPs, the authors of this study⁴⁶ showed that a specific SNP variant increased expression of α -synuclein by reducing the binding of transcription inhibitory factors.

The capacity to identify and modify SNPs has received a further major boost with publication of a method that the authors term 'RNA guide tuning'⁴⁷. This technique identifies gRNAs that are able to distinguish target sites differing by only a single base. The authors predict that CRISPR/Cas9 technology using these gRNAs will enable identification of the causal role of thousands of disease-associated SNPs, including those that have been reported for rheumatic diseases⁴⁸. The authors also speculate that in the future these tuned gRNAs could provide a means to disable disease alleles delineated by SNPs, in order to treat the associated disease⁴⁷.

CRISPR/Cas9 genomic screen

CRISPR/Cas9-mediated genome-wide knockout screen system. In this platform, a gRNA library targeting most genes in the genome with multiple sites per gene is cloned into lentiviral vectors and delivered as a pool into target cells that express Cas9. A low multiplicity of infection is used to ensure that each cell will receive no more than one gRNA or viral particle. By proper phenotype selection, gRNAs that are enriched or depleted in cells are determined and, correspondingly, genes that are required for that particular phenotype can be systematically identified.

Gene drives

Gene drives are genetic manipulations that enable a gene to force its inheritance to all, rather than half, of its offspring.

Gene Screening

The human genome project provided an almost complete catalogue of our genes⁴⁹. A CRISPR/Cas9 genomic screen now offers a high-throughput method of assigning functions to these genes. Several groups have synthesized genome-wide libraries of gRNAs that target almost the entire human and mouse genomes multiple times^{50–52}. Lentiviral libraries either contain gRNAs alone or gRNAs in addition to Cas9. Libraries with gRNAs alone require stable cell lines expressing Cas9, whereas libraries expressing gRNA and Cas9 can be used with almost any cell line (although these libraries require much higher cell numbers than for gRNAs alone owing to the size of the construct and consequent low viral titre). The technique for gene screening requires transduction of target cells with the gRNA library, growth of infected cells in culture and selection for transduced cells by either antibiotic resistance or fluorescence activated cell sorting^{50–52}. Cell populations selected for any target phenotype, for example survival, growth, differentiation or resistance to anticancer agents, are then isolated. The gRNA (identified by barcodes using deep sequencing) present or lost from the cell population is compared with the transfected library. Second generation gRNA libraries typically contain >100,000 gRNAs targeting 17,000–20,000 genes, each gene targeted with 5–10 gRNAs. The libraries have been carefully designed for efficient gene-knockout and minimal crossreactivity.

Genome-wide CRISPR/Cas9 screening has focused largely on cancer, but has also identified genes necessary for viral infection and innate immune system pathways. The host genes necessary for dengue and Zika virus infection^{53,54} include endoplasmic reticulum peptidases and oligosaccharide transferases that are expressed in both mosquito and human hosts. Most of the genes identified in a hepatitis C virus (another *Flaviviridae* virus) screen are distinct from those of dengue and Zika and include viral receptors, RNA binding proteins and enzymes associated with the conversion of riboflavin to flavin adenine dinucleotide⁵⁴. These studies emphasize the power of the CRISPR/Cas9 screening approach, and the findings represent much needed new pharmacologic targets for inhibition of *Flaviviridae*. In combination with targeted gene drives (described below), they present the exciting potential to control and possibly eliminate vector-borne viral diseases, including those most relevant to rheumatologists, such as Lyme disease and chikungunya.

Of direct interest to rheumatologists, several research groups have employed CRISPR/Cas9 gRNA libraries to identify innate immune system pathways, including a comprehensive unbiased CRISPR/Cas9 analysis to identify genes controlling the induction of TNF in response to dendritic cell stimulation⁵⁵. In this study, the authors used bone-marrow-derived dendritic cells isolated from transgenic mice expressing Cas9. The cells were infected with a library of gRNAs and monitored for gRNA abundance associated with high TNF expression in response to stimulation with lipopolysaccharide. The authors of this study identified and validated the role of many genes not previously associated with innate immune

circuits and described new pathways associated with endoplasmic reticulum stress and the polymerase associated complex, a regulator of transcription elongation, not previously implicated in inflammatory gene expression⁵⁵. In addition, two papers examining the innate immune pathways in macrophages have provided new understanding of pyroptosis and the inflammasome-mediated immune diseases^{56,57} implicated in systemic lupus erythematosus⁵⁸, crystal-induced arthritides⁵⁹ rheumatoid arthritis, inflammatory bowel disease and rare hereditary periodic fever syndromes⁶⁰. Although the involvement of caspase activation associated with the inflammasome protein complex and downstream activation of proinflammatory cytokines (including IL-1 β) had been recognized previously, the molecular pathways identified upstream of pyroptosis and the mechanisms inducing cell lysis and cytokine release were previously unknown. The two studies^{56,57} identified critical roles for Nek7 kinase upstream of inflammasome activation and caspase cleavage of gasdermin in driving cell lysis and the release of inflammatory cytokines. An additional study using the CRISPR/Cas9 system in human macrophages also revealed an alternative inflammasome pathway for secretion of IL-1 β that does not seem to be active in mouse macrophages⁶¹.

These studies, published since 2015, emphasize the utility of the CRISPR/Cas9 system for unbiased description of critical molecular pathways. This technique will provide rapid expansion of our understanding of complex molecular pathways across diverse biological systems, and might reveal new therapeutic targets for a wide variety of diseases, including rheumatic diseases.

Animal models and xenotransplantation

CRISPR/Cas9 technology has had an enormous effect on the ability to develop mouse models of disease. The technique makes the generation of genetically engineered mice quicker and cheaper than traditional techniques. Generation of transgenic mice using CRISPR/Cas9 takes ~11 weeks, in contrast to the traditional approach using embryonic stem cells that takes ~1 year⁶². In its simplest form the CRISPR/Cas9 approach involves injection of a single plasmid construct consisting of *cas9* and gRNA genes into fertilized mouse oocytes⁶³. Targeted sites in the genome are cleaved and mutation rates resulting from error prone NHEJ are high with ~50% of pups affected⁶³. CRISPR/Cas9 also enables the generation of complex models with large deletions, inversions and duplications⁶⁴. The technique enables the generation of mice carrying mutations in multiple genes⁶⁵ and the disruption of large topological domains⁶⁴ that would be very difficult and time consuming to generate by traditional methods. Many hundreds of genetically engineered mice have been generated using this technique⁶⁶.

As with all areas of CRISPR/Cas9 technology, improvements in generating mouse models of human disease are advancing rapidly. The development of an adult-onset and tissue-specific model of heart disease⁶⁷ has opened the door for simple and efficient development of temporally and tissue-specific models of other human diseases. Carroll *et al.*⁶⁷ described the generation

of a transgenic mouse line expressing Cas9 exclusively in cardiomyocytes with no overt effects. Delivery of gRNAs targeting the gene encoding cardiac myosin heavy chain 6, *Myh6* using adeno-associated virus (AAV) in adult mice demonstrated high levels of cardiac-specific mutation and cardiac failure⁶⁷. The use of mice with tissue-specific expression of Cas9 overcomes the difficulty of delivering components of the CRISPR/Cas9 complex that are at the packaging limit of many viral delivery systems. In addition, this method enables analysis of the function of any cardiac gene, including those that are embryonic lethal or widely expressed in other tissues, by the simple delivery of specific gRNAs. Mice with expression of Cas9 specifically in other tissues are expected to be developed and will enable analysis of gene function in a wide variety of adult-onset diseases. Mice with cartilage-specific or joint-specific expression of Cas9 would be invaluable in the description of the gene function associated with a wide variety of rheumatic diseases. This idea is made more tantalizing by the flexibility of the CRISPR/Cas9 system to mutate multiple coding and noncoding sites in a genome.

CRISPR/Cas9 technology might overcome our reliance on mouse models of disease, and several models of disease and disease resistance have been generated in other species including goats, cattle, ferrets, fish, monkeys and even elephants⁶⁸. A surprisingly large number of studies have reported the use of CRISPR/Cas9 to engineer mutations in domestic species, particularly in pigs, sheep, cattle and goats^{69–73}. Most studies employ somatic cell nuclear transfer. The technique involves editing the desired gene in a somatic cell, usually fibroblasts, and replacing the nucleus in isolated oocytes with the nucleus of the gene-edited somatic cell. Gene editing can also be performed in embryos by direct injection of CRISPR components into the pronucleus or cytoplasm (reviewed elsewhere^{74,75}).

One application that has received a lot of research and commercial interest addresses the growing demand for human tissue for transplantation and the chronic shortage of organ donors. For many years scientists proposed the use of pig organs for transplantation with such enthusiasm that several companies were established with this goal in mind. However, this work ran into two major obstacles: namely, endogenous viruses and immune incompatibility. The ability of the CRISPR/Cas9 system to delete multiple copies of a gene in a single cell system has enabled eradication of 60 copies of the family of porcine retroviruses from the pig genome and enabled the deletion or mutation of 20 genes known to trigger a human immune response⁶⁹. These advances suggest porcine cartilage and bone for human transplantation might provide a radically new approach for treatment of end-stage arthritides.

A study examining the role of the Mohawk (Mkx) transcription factor has demonstrated the value in moving from transgenic mice to larger animals, in this case transgenic rats. Studies in mice suggested an important role in tendon development, with the *Mkx* mouse knockout resulting in tendon hypoplasia^{76,77}. The rat *Mkx*^{-/-} (generated by direct injection of *cas9* and gRNA

into fertilized oocytes) had similar hypoplasia to the mice, but onset was earlier and more severe⁷⁸. Unlike the *Mkx*^{-/-} mice, the *Mkx*^{-/-} rats developed chondral lesions and heterotopic ossification of the Achilles tendon. As well as providing access to more tissue and the capacity to conduct more cell-based and biochemical experiments, the larger size of the animal, the authors suggested, increased the mechanical stimulation to the tendons which resulted in chondrogenic differentiation and a more severe phenotype⁷⁸. For diseases with skeletal pathology, the transition to larger animal models made feasible by CRISPR/Cas9 technology is expected to provide a quantum leap in our understanding and ability to model human diseases.

Gene drives

During normal sexual reproduction the copy of a gene inherited from one parent will not spread through a wild population because in each generation there is only a 50% chance of passing it on. However, if the gene is modified so that it causes the gene from the other parent to be modified in the same way, the offspring will always receive the modified gene and the gene will rapidly spread through a wild population, potentially reaching 100% of the population within a few generations. These gene drives (as they have been termed) have been proposed as a way of eliminating disease vectors, controlling invasive species, immunizing threatened species and generating crops with resistance to herbicides^{79,80}. Although proposed many years ago little progress had been made in the development of gene drives, primarily because of the difficulty in precisely engineering genomes. The development of the CRISPR/Cas9 system has caused a rapid reversal of that inactivity. Several publications have described model gene drives confined to the laboratory in fruitflies and mosquitoes^{81–84}. The systems employed in these studies were similar and comprised a construct with three components: a *cas9* gene, a gRNA targeting the sequence of interest and homology arms enabling Cas9 cleavage of the second allele and expression of the Cas9-gRNA via HDR. Three genes were targeted in *Anopheles gambiae*⁸⁴ and *Anopheles stephensi*⁸² (the main malaria vectors) with transmission rates of 90–99%. The studies suggest that this approach, in combination with the identification of the genes that are essential for viral replication, could be developed to eliminate malaria from affected regions^{82,84}. Similar approaches might be used to target other vector transmitted diseases including Lyme disease, hepatitis C, dengue and Zika virus.

The capacity to wipe out or at least drastically alter entire wild populations clearly has serious ecological concerns. These concerns have received wide attention and have been the subject of a National Academy of Sciences Report^{85,86}. The use of gene drives remains controversial and studies to date are not sufficient to allow the release of gene-drive-modified organisms into the environment.

Treating human disease

A cancer study in which one gene was inserted and another deleted in T cells was the first using CRISPR/Cas9 technology to pass the first committee (NIH Recombinant

DNA Advisory Committee) on its way to clinical trials⁸⁷. An additional similar trial is about to begin in China⁸⁸. These researchers plan to use CRISPR technology to insert a receptor for a protein often expressed in tumours, but not in healthy cells, into patients' T cells, and to delete PD-1, a T cell surface protein that has been shown to dampen cell activity after an immune response. These trials are based on very promising studies in mice⁸⁹ that demonstrated tumour regression using this approach.

Several other promising studies in animal models suggest that CRISPR/Cas9 technology will soon be applied to treat diseases that affect humans (TABLE 1). Three concurrent publications provide proof of principle that CRISPR/Cas9 technology can be used to correct Duchenne muscular dystrophy (DMD)^{90–92}. DMD is a devastating progressive muscle disease that is typically caused by small mutations in the dystrophin gene, resulting in the generation of a premature stop codon and thus the loss of protein expression. Dystrophin is a very large muscle protein composed of many domains, some of which are dispensable for protein function. Because most mutations affecting patients with DMD occur in these non-essential protein regions it has been proposed that exon skipping strategies would provide effective treatment in the majority of patients. The authors of these three studies investigated a mouse model of the human disease with a nonsense mutation in exon 23 of the dystrophin gene^{90–92}. AAV delivery of *cas9* and two gRNAs targeting the 3' and 5' ends of exon 23 caused skipping of this exon and expression of a functional dystrophin protein. Postnatal systemic delivery of AAV vectors also restored dystrophin expression and enhanced muscle function. Dystrophin expression in muscle progenitors was consistent and continued improvement in muscle function was maintained for at least 6 months post-treatment. With continued development to enhance safety and efficacy it is hoped that this technology will realize its promise in treating patients with DMD.

Delivery of the CRISPR components remains one of the challenges for treatment of human disease (CRISPR delivery is reviewed in detail elsewhere^{22,93,94}). AAV delivery shows great promise; however, the relatively small packing capacity of AAV presents a problem. The most widely used *cas9* comes from *Streptococcus pyogenes* and is at the limits of AAV's packing capacity. A smaller *cas9* isolated from *Staphylococcus aureus* should overcome these problems, but has not been widely used to date⁹⁵. In addition, the long-term persistent expression of the CRISPR system that results from viral delivery exaggerates the problems of off-target effects. Even very low levels of off-target cleavage could become problematic if expression is maintained for the extended periods that occur with viral delivery. Transient expression can be achieved by use of lipid nanoparticles for delivery (reviewed elsewhere²²). Nanoparticle delivery of Cas9 with AAV delivery of gRNAs and a repair template has shown success in the treatment of a mouse model of tyrosinaemia, achieving therapeutic correction in 6% of hepatocytes after one treatment⁹⁶. Furthermore, a very encouraging chondroprotective effect has been obtained using intra-articular nanoparticle delivery

Table 1 | Preclinical research using CRISPR/Cas9

Disease	Protein (gene)	Platform	Delivery	Refs
Metabolic liver disease	Ornithine carbamoyltransferase (<i>Otc</i>)	Mouse model	Intravenous delivery of two AAVs; HDR	102
Retinitis pigmentosa	X-Linked retinitis pigmentosa GTPase regulator (<i>RPGR</i>)	Patient iPSCs	Transfection; HDR	103
Dominant dystrophic epidermolysis bullosa	Collagen alpha-1(VII) chain (<i>COL7A1</i>)	Patient iPSCs	Transfection; NHEJ	104
Hereditary tyrosinemia	Fumarylacetoacetase (<i>Fah</i>)	Mouse model	Lipid nanoparticle delivery of Cas9; AAV delivery of gRNA and repair template; systemic injection.	96
Fanconi anaemia	Fanconi anemia group I protein (<i>FANCI</i>)	Patient iPSCs	Cas9 nickase; repair template transfection; hematopoietic differentiation	105
Duchenne muscular dystrophy	Dystrophin (<i>Dmd</i>)	Mouse model	Exon deletion using paired gRNAs; AAV systemic and muscle delivery	90–92
Hepatitis B virus	Cell surface antigen	Cell culture, Mice	Transfection; NHEJ	106
Sickle-cell anaemia	Haemoglobin (<i>Hbb</i>)	Mouse model	<i>Ex vivo</i> repair of haematopoietic stem cells and transplantation back into mice.	107
β-Thalassemia	Haemoglobin (<i>HBB</i>)	Patient iPSCs	iPSCs derived from patient fibroblasts; HDR; excisable antibiotic selection	108
Cystic fibrosis	Cystic fibrosis transmembrane conductance regulator (<i>CFTR</i>)	Patient iPSCs	Skin iPSCs; HDR; excisable antibiotic selection; differentiation to lung epithelial cells	109
Latent HIV infection	C–C chemokine receptor type 5 (<i>CCR5</i>)	Haematopoietic stem cells	CCR5 ablation in haematopoietic stem cells	110

Abbreviations: AAV, adeno-associated virus; gRNA, guide RNA; HDR, homology-directed repair; iPSC, induced pluripotent stem cell; NHEJ, non-homologous end joining.

of NF-κB siRNAs for treatment of a mouse model of post-traumatic osteoarthritis⁹⁷. Importantly, this peptide nanoparticle complex delivered RNA deep within the cartilage, providing a unique vehicle for the treatment of cartilage diseases⁹⁷. Nanoparticle constructs containing Cas9 protein and gRNA (ribonucleoprotein complexes) have been shown to provide very efficient gene manipulation in cells⁹⁸. Although untested to date, the intra-articular delivery of CRISPR ribonucleoprotein complexes using similar peptide nanoparticles offers a unique opportunity to treat arthritic joint diseases.

Genetic diseases, such as DMD, will probably comprise the first focus of the therapeutic use of CRISPR technology. With the rapid rate of improvements and diversification in CRISPR technology and delivery, it is expected that many of the promising prospects for treatment of human genetic disease will reach clinical trials. We optimistically anticipate that as CRISPR/Cas9 technology expands our understanding of more complex diseases and reveals new treatment strategies, our understanding and capacity to treat the more common human diseases, including rheumatic diseases, will also expand.

Conclusions

CRISPR/Cas9 technology is transforming molecular biology in a way similar to how PCR transformed it >30 years ago. The application of PCR for the analysis of gene expression and function, diagnosis of disease, DNA cloning, phylogeny associations, molecular fingerprinting and much more has made a critical contribution to our capacity to perform analyses and test gene function. Similarly, the ability to quickly and simply edit the genome using rapidly expanding CRISPR/Cas9

technologies across a wide range of systems from cell culture to animal models has already transformed our capacity to address fundamental previously intractable questions of normal gene function and the molecular pathogenesis of diseases. CRISPR/Cas9 technologies are showing enormous promise in untangling the interactions of gene networks responsible for the regulation of specific biologic and disease functions. GWAS have identified many hundreds of variants associated with diseases, including rheumatic diseases^{48,99}. Large population studies have identified common variants associated with immune traits¹⁰⁰ and provided detailed analysis of common variants among autoimmune diseases^{100,101}. CRISPR/Cas9 technology provides genome-wide high-throughput screening and fine mapping of these regions, including the ability to distinguish causal and bystander SNPs. As the application of these analyses to research questions continues, and advances in CRISPR/Cas9 and other CRISPR technologies are developed, their effect will no doubt be enormous. These advances are anticipated to offer a new and exciting conceptual understanding of the complex regulation of cell function and the molecular pathogenesis of human diseases. Like PCR, the CRISPR technologies will rapidly become accepted as just another critical component of the molecular biology tool kit.

The capacity of CRISPR/Cas9 to repair endogenous genes while preserving the physiological regulation of gene expression offers the potential for human gene therapy. Although the efficiency and accuracy of CRISPR-based gene editing is bound to continue to advance, the primary challenges of gene therapy (probably the greatest of which are the development of safe and efficient delivery systems) remain to be

overcome before extensive application of gene therapy using this technology can be widely employed. The initial PCR technique was very cumbersome and time consuming until heat stable polymerases transformed the technique to the quick, simple, routine procedure

that is used in every laboratory. Although CRISPR technology is already transformative, additional new technologies, such as new gene delivery techniques, might be expected to have a similar additive effects on its applications.

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G.J.G. contributed to researching data for the article, writing, and reviewing and editing the manuscript before submission. M.Y. contributed to discussion of content and writing the article.

Competing interests statement

The authors declare no competing interests.

Beyond TNF: TNF superfamily cytokines as targets for the treatment of rheumatic diseases

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Abstract | TNF blockers are highly efficacious at dampening inflammation and reducing symptoms in rheumatic diseases such as rheumatoid arthritis, psoriatic arthritis and ankylosing spondylitis, and also in nonrheumatic syndromes such as inflammatory bowel disease. As TNF belongs to a superfamily of 19 structurally related proteins that have both proinflammatory and anti-inflammatory activity, reagents that disrupt the interaction between proinflammatory TNF family cytokines and their receptors, or agonize the anti-inflammatory receptors, are being considered for the treatment of rheumatic diseases. Biologic agents that block B cell activating factor (BAFF) and receptor activator of nuclear factor- κ B ligand (RANKL) have been approved for the treatment of systemic lupus erythematosus and osteoporosis, respectively. In this Review, we focus on additional members of the TNF superfamily that could be relevant for the pathogenesis of rheumatic disease, including those that can strongly promote activity of immune cells or increase activity of tissue cells, as well as those that promote death pathways and might limit inflammation. We examine preclinical mouse and human data linking these molecules to the control of damage in the joints, muscle, bone or other tissues, and discuss their potential as targets for future therapy of rheumatic diseases.

Over 30 years have passed since the molecular identification of TNF as a mediator of fever and cachexia¹, and approximately 20 years since the first introduction of TNF inhibitors into clinical practice for the treatment of rheumatoid arthritis (RA)². During this time, much has been learned about the basic biology of the 19 structurally related cytokines of the TNF superfamily (TNFSF), their receptors (TNF receptor superfamily, TNFRSF), the intracellular signalling pathways activated by these receptors, as well as the unique and overlapping roles of TNFSF cytokines in a number of inflammatory and autoimmune diseases. TNFSF proteins organize lymphoid tissue development, co-stimulate lymphocyte activation and can either increase lymphocyte survival and function or induce cell death^{3–6}. Outside the immune system, TNFSF cytokines can promote the development and survival of osteoclasts, as well as cells in the mammary glands, hair follicles and sweat glands. TNFSF cytokines can also regulate neuronal activity and drive inflammatory responses in a range of tissue structural cells, including epithelial cells and fibroblasts. These insights have led to intensive efforts to treat other inflammatory diseases through TNF neutralization, and multiple TNF-blocking agents (such as adalimumab,

certolizumab pegol, etanercept, golimumab and infliximab) are now approved for diseases such as juvenile idiopathic arthritis, psoriasis, psoriatic arthritis, spondyloarthropathies, inflammatory bowel disease and uveitis^{7,8} (TABLE 1). Investigations into the targeting of other TNFSF members have led to a number of clinical trials in different diseases and resulted in the successful development of belimumab, an antibody against B cell activating factor (BAFF, also known as TNFSF13B), and denosumab, an antibody targeting receptor activator of nuclear factor- κ B (NF- κ B) ligand (RANKL, also known as TNFSF11), for the treatment of systemic lupus erythematosus (SLE) and osteoporosis, respectively^{9–11}.

Clinical targeting of TNF, BAFF and RANKL has been reviewed elsewhere^{7–10,12–17}, as has the targeting of all the TNF and TNFRSF members in both immune and nonimmune disorders¹¹. In this Review, we focus on TNF family proteins that are produced by the immune system but are not yet targets of approved drugs. These molecules might be crucial to the immune response underlying rheumatic diseases and are promising future targets for intervention and therapy in diseases such as RA and SLE (FIG. 1). Although blocking nerve growth factor binding to its receptor TNFRSF16 (also known

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Key points

- TNF inhibitors are among the most effective protein-based drugs for reducing inflammation associated with several rheumatic diseases
- In addition to TNF, the TNF superfamily (TNFSF) comprises other ligand–receptor combinations that might participate in the pathogenesis of rheumatic disease
- TNFSF members initiate several processes, including immune activation, tissue inflammatory responses and cell death or suppression
- Many TNFSF proteins other than TNF are being evaluated in preclinical mouse or human studies as possible therapeutic targets in rheumatic diseases
- TNFSF members can be targeted to either restore tolerance in rheumatic diseases or to regulate tissue cell responses

as nerve growth factor receptor) is of primary interest for the treatment of pain associated with osteoarthritis, TNFRSF16 is not an immune-system-related molecule and so we do not present a discussion here but refer readers to several other published articles^{11,18–21}.

TNF superfamily

Multiple functional polymorphisms in the genes encoding TNFSF cytokines, their receptors and their signalling proteins are associated with susceptibility to autoimmune diseases^{11,22}. Yet, many functions of TNFSF proteins remain poorly understood. TNFSF and TNFRSF proteins have many structural and biological similarities (FIG. 1). TNFSF molecules are trimeric type II transmembrane proteins characterized by C-terminal TNF homology domains that can be cleaved from cells to form soluble ‘cytokine-like’ molecules²³. Their receptors are type I transmembrane proteins that have varying numbers of extracellular ligand-binding cysteine-rich domains²³. The extracellular domains of the TNFRSF can also be cleaved to form soluble molecules, which might be useful as biomarkers for inflammation, although their exact function is not clear. Engagement of receptors by their cognate ligands is thought to primarily lead to trimerization of the receptors, which can further form higher-order oligomers on a cell's surface. Although overall sequence similarity between TNFRSF molecules is low (20–30%), once engaged by their cognate ligands they can drive common or overlapping signalling pathways^{24–26} (FIG. 2). Moreover, membrane-bound TNF family ligands can also signal through themselves when engaged to their cognate receptors (a process known as reverse signalling), which might contribute to their function. When crosslinked on the surface of cells, various consequences of reverse signalling have been described, such as proinflammatory cytokine production (for example IL-1 and IL-6) and cell maturation, which depend on the cell type that receives the TNFSF ligand signal²⁷.

The expression of TNF family proteins is quite broad and dynamically regulated (FIG. 1). Many ligand–receptor pairs are constitutive or inducible on lymphocytes, including antigen presenting cells (APCs; such as dendritic cells, macrophages and B cells) and T cells, and normally participate in promoting T and B cell responses, which are central to most autoimmune and rheumatic diseases. Similarly, death-inducing molecules

can also be expressed by lymphocytes, and participate in maintaining self-tolerance and limiting adaptive immune responses. Additionally, a number of TNF family ligands and/or receptors are constitutive or inducible in non-lymphoid cells including epithelial cells, fibroblasts, smooth muscle cells, and endothelial cells. These molecules participate in the proinflammatory and anti-inflammatory crosstalk that occurs between tissue structural cells and the immune system, which might either contribute to autoimmune tissue pathology or limit damage.

Below, we discuss the biological activities of TNFSF members and their potential involvement in rheumatic diseases. For simplicity, we have grouped TNFSF proteins, as described above, into immune cell activators, tissue inflammatory proteins and molecules that induce cell death or immune suppression. This classification is not absolute and the reader should be aware that molecules such as TNF, CD40 ligand (CD40L, also known as TNFSF5), LIGHT (also known as TNFSF14), TNF-like ligand 1A (TL1A, also known as TNFSF15), and TNF-related apoptosis inducing ligand (TRAIL, also known as TNFSF10) can exert functions on both immune cells and tissue cells (FIG. 1). Moreover, a number of proteins, including TNF and Fas ligand (FasL, also known as TNFSF6), are able to promote cell death as well as being proinflammatory, depending on the target cell type and the context in which they are active.

Immune cell activation**CD40L**

CD40 (also known as TNFRSF5) is a stimulatory receptor expressed on dendritic cells, macrophages and B cells, whose signals drive activation, maturation, survival and inflammatory cytokine production^{28,29} (FIG. 3). CD40 is crucial in both inducing IgG autoantibodies and driving immunoglobulin class switching^{30,31}, and is also a primary driver of T cell immunity. Its ligand, CD40L, is induced in T cells shortly after activation and, via ligation of CD40 on professional APCs, can lead to an increase in antigen presentation and activation of T cells by upregulating MHC molecules and inducing expression of stimulatory ligands such as CD86 and those belonging to the TNF superfamily, which are described below (for example OX40 ligand (OX40L))^{32,33}.

Studies have long linked the interaction between CD40L and CD40 to rheumatic disease pathogenesis. In the early 1990s, studies of multiple autoimmune models, including collagen-induced arthritis and lupus-like disease in NZB/SWR or NZB/NZW F1 mice^{34–36}, demonstrated markedly reduced signs of inflammation in mice lacking either CD40 or CD40L, or in wildtype mice treated with CD40L blocking reagents. Similar to other molecules discussed below, the idea that the CD40L–CD40 axis is also active in human disease largely derives from expression studies in patients. The caveat with expression studies is that detection of the molecules in serum or tissues does not automatically imply they are functional or important, but could simply reflect the presence of activated immune cells. However, such data,

Table 1 | Clinical trials of TNF and TNF receptor superfamilies

TNF family ligand	TNF family receptor	Biologic agent targeting receptor or ligand	Name of biologic agent	Stage of drug development for targeted disease(s)
TNF	TNFR1, TNFR2	Chimeric anti-TNF mAb	Infliximab	Approved: AS, CD, PsA, psoriasis, RA, UC
		Human TNFR2-Fc fusion protein	Etanercept	Approved: AS, JIA, PsA, psoriasis, RA
		Human anti-TNF mAb	Adalimumab	<ul style="list-style-type: none"> Approved: AS, Crohn disease, JIA, PsA, psoriasis, RA, UV Phase III (recruiting): UC Phase III (recruiting): Behçet disease
		Human PEGylated Fab anti-TNF mAb	Certolizumab pegol	<ul style="list-style-type: none"> Approved: CD, RA Phase III (completed): AS, PsA Phase III (ongoing): psoriasis Phase II (recruiting): UC
		Human anti-TNF mAb	Golimumab	<ul style="list-style-type: none"> Approved: AS, PsA, RA Phase IV: UC Phase II (completed): asthma
		Recombinant human TNF conjugated to KLH	TNF-Kinoid	Phase II (completed): CD, RA
LT α 3	TNFR1, TNFR2	Human TNFR2-Fc fusion protein	Etanercept	Approved: AS, JIA, PsA, psoriasis, RA
		Human anti-LT α mAb	Pateclizumab (MLTA3698A)	Phase II (completed): RA
LT α 1 β 2	LT β R	Human LT β R-Ig fusion protein	Baminercept (BG9924)	Phase II (terminated due to lack of activity): RA, Sjögren syndrome
OX40 ligand	OX40	Human anti-OX40L mAb	Oxelumab	Phase II (discontinued owing to lack of activity): asthma
		Human anti-OX40 mAb [†]	KHK4083	Phase II (recruiting): UC
		Human anti-OX40 mAb	GBR830	Phase II, (recruiting): AD
CD40L	CD40	Humanized anti-CD40L mAb	Ruplizumab (BG9588)	Phase II (discontinued owing to safety issues): lupus nephritis
		Humanized anti-CD40L mAb	Toralizumab (IDEC-131)	Phase II (discontinued owing to safety issues): CD, MS
		Anti-CD40L-Tn3 fusion protein	MEDI4920	Phase I (recruiting): RA
		Chimeric anti-CD40 mAb	FFP104 (PG102)	Phase I (recruiting): CD, primary biliary cirrhosis
		Human anti-CD40 Fc-silent mAb	CFZ533	Phase I-II (recruiting): Grave disease, MG, RA, SS, transplantation
		Human anti-CD40 mAb	ASKP1240 (4D11)	<ul style="list-style-type: none"> Phase II (completed): psoriasis Phase II (ongoing): transplantation
RANKL	RANK	Human anti-RANKL mAb	Denosumab	<ul style="list-style-type: none"> Approved: osteoporosis Phase III (ongoing): RA Phase II (recruiting): OA Phase I-II (recruiting): CD
TWEAK	Fn14	Humanized anti-TWEAK mAb	BIIB023	<ul style="list-style-type: none"> Phase II (terminated due to lack of activity): lupus nephritis Phase I (completed): RA
APRIL	TACI, BCMA	Human TACI-Ig fusion protein	Atacicept	<ul style="list-style-type: none"> Phase II (completed): RA Phase II (ongoing): SLE Phase II (terminated due to safety issues): lupus nephritis Phase II (terminated due to increased disease): MS
BAFF	BAFFR, BCMA, TACI	Human anti-BAFF mAb	Belimumab	<ul style="list-style-type: none"> Approved: SLE Phase II (completed): MG, RA, Sjögren syndrome Phase II (ongoing): SSc
		Human anti-BAFF mAb	Tabalumab (LY2127399)	<ul style="list-style-type: none"> Phase III (completed): RA, SLE Phase II (completed): MS
		Human TACI-Ig fusion protein	Atacicept	<ul style="list-style-type: none"> Phase II (completed): RA Phase II (ongoing): SLE Phase II (terminated due to safety issues): lupus nephritis Phase II (terminated due to increased disease): MS
		Human BAFF-binding peptibody	Blisibimod (AMG623)	Phase III (ongoing or recruiting): SLE

Table 1 (cont.) | Clinical trials of TNF and TNF receptor superfamilies

TNF family ligand	TNF family receptor	Biologic agent targeting receptor or ligand	Name of biologic agent	Stage of drug development for targeted disease(s)
LIGHT	HVEM, LT β R	Human LT β R-Ig fusion protein	Baminercept (BG9924)	<ul style="list-style-type: none"> Phase II (completed and terminated due to lack of activity): RA Phase II (terminated due to unavailability of biologic): Sjögren syndrome
		Human anti-LIGHT mAb	KHK252067	Phase I (completed): CD, UC
NGF*	NGFR	Humanized anti-NGF mAb	Tanezumab (RN624)	Phase III (recruiting): chronic back pain, osteoarthritis
		Human anti-NGF mAb	Fulranumab (AMG-403)	Phase III (ongoing): osteoarthritis
		Human anti-NGF mAb	Fasinumab (REGN475)	Phase III (ongoing): osteoarthritis Phase III (recruiting): chronic back pain

AD, atopic dermatitis; APRIL, A proliferation-inducing ligand; AS, ankylosing spondylitis; BAFF, B-cell-activating factor; BAFFR, BAFF receptor; BCMA, B-cell maturation antigen; CD, Crohn's disease; Fn14, Fibroblast growth factor-inducible protein 14; HVEM, Herpes virus entry mediator; JIA, juvenile idiopathic arthritis; LT, lymphotoxin; LT β R, LT β receptor; NGF, nerve growth factor; NGFR, NGF receptor; mAb, monoclonal antibody; MG, myasthenia gravis; PsA, psoriatic arthritis; RA, rheumatoid arthritis; RANK, receptor activator of nuclear factor kappa-B (NF- κ B); RANKL, RANK ligand; SSc, systemic sclerosis; TAC1, transmembrane activator and CAML interactor; TNFR, TNF receptor; TWEAK, TNF-related weak inducer of apoptosis; UC, ulcerative colitis. *NGF is not a canonical TNF family ligand on the basis of structure, although NGFR is part of the TNFR superfamily. †Depleting and/or antagonist biologics (all other biologic agents displayed are antagonists).

particularly for conventional cytokines such as IL-5, IL-13, and IL-17, has aided their clinical targeting and enabled patient stratification into those most likely to respond to biologic agents. Therefore, with TNFSF molecules the expression data are highly useful regardless of the caveats, especially if linked to either other disease markers or the magnitude of clinical symptoms. Soluble CD40L in serum, or CD40L expression in inflamed tissue, epithelial cells, endothelium or T cells, is upregulated in patients with RA, psoriatic arthritis, ankylosing spondylitis, SLE, Sjögren syndrome and systemic sclerosis (SSc), often correlating with disease severity or levels of autoantibodies^{28,29}. Additionally, polymorphisms near the genes encoding CD40L or CD40, which are thought to lead to elevated or prolonged expression, have been associated with susceptibility to SLE, RA and other rheumatic disorders (such as Behçet disease)^{37–44}.

Animal studies have shown that the neutralization of CD40L has a strong suppressive effect on pathogenic T cell development and antibody responses. These results, together with data from human expression and association studies, made CD40L an attractive therapeutic target for rheumatic diseases, particularly SLE and RA. As reviewed elsewhere^{11,28,29}, phase I–II trials in several patient groups, including patients with lupus nephritis, demonstrated some beneficial activity of antibodies against CD40L (such as ruplizumab, ab1793 and toralizumab)^{45–47}. Unfortunately, the thromboembolic activity of these antibodies, linked to crosslinking of CD40L expressed by platelets, led to discontinuation of their further development (TABLE 1). To circumvent the thromboembolic effect, preclinical studies in mice or nonhuman primates are assessing new biologic agents that block CD40L without causing aggregation of the molecule; these biologic agents either lack an Fc region or are mutated to prevent their binding to Fc receptors. Results suggest that they can be as efficacious as the parent (Fc intact) antibody — without the thromboembolic effect — in scenarios such as animal models of lupus^{48–50}. However, in certain settings Fc effector function might be necessary for therapeutic activity, as shown by the lack

of activity of an aglycosylated anti-CD40L antibody in nonhuman primate transplantation studies⁴⁸. MEDI4920, a Tn3-fusion protein with reactivity to CD40L, is currently in phase I safety trials. Additionally, antagonist and/or depleting antibodies against CD40 have been produced (ch5D12, chi220–BMS-224819, ASKP1240, FFP104, CFZ533), with encouraging preclinical results⁵¹, and some of them are being tested in phase I–II trials in Sjögren syndrome⁵², RA⁵³ and other autoimmune conditions (TABLE 1). If these strategies can overcome the adverse effects associated with agents that block CD40–C40L interactions, such agents are an attractive avenue, and the possibility for clinical benefit in rheumatic diseases is high.

OX40L

OX40L (also known as TNFSF4) is an inducible molecule expressed on several cell types, although arguably most importantly, on APCs. OX40 (also known as TNFRSF4) is largely found on activated T cells as well as natural killer T cells and innate lymphoid cells such as natural killer cells^{5,54} (FIG. 3). OX40L can trigger signalling through its receptor OX40, resulting in a range of activities including expansion and accumulation of effector T cells (such as type 1 T helper cells (T_H1), type 2 T helper cells (T_H2), type 17 T helper cells (T_H17) and cytotoxic T lymphocytes) and their cytokine production^{5,6,11,54}. Additionally, reverse signalling through OX40L can promote expression of inflammatory cytokines (such as IL-12 or TNF) in APCs^{5,54}, although the importance of this activity as compared with that driven by OX40 is not clear at present.

Data from human and mouse studies suggest that the OX40–OX40L axis has an important role in rheumatic diseases. Blockade of OX40L reduces bone and cartilage destruction in mouse models of collagen-induced arthritis^{55,56} or autoimmune arthritis⁵⁷, with results from the former model being attributed to reduced numbers of collagen-specific T cells. Synovial fluid samples of patients with RA contain elevated numbers OX40-expressing T cells, suggesting OX40 signalling

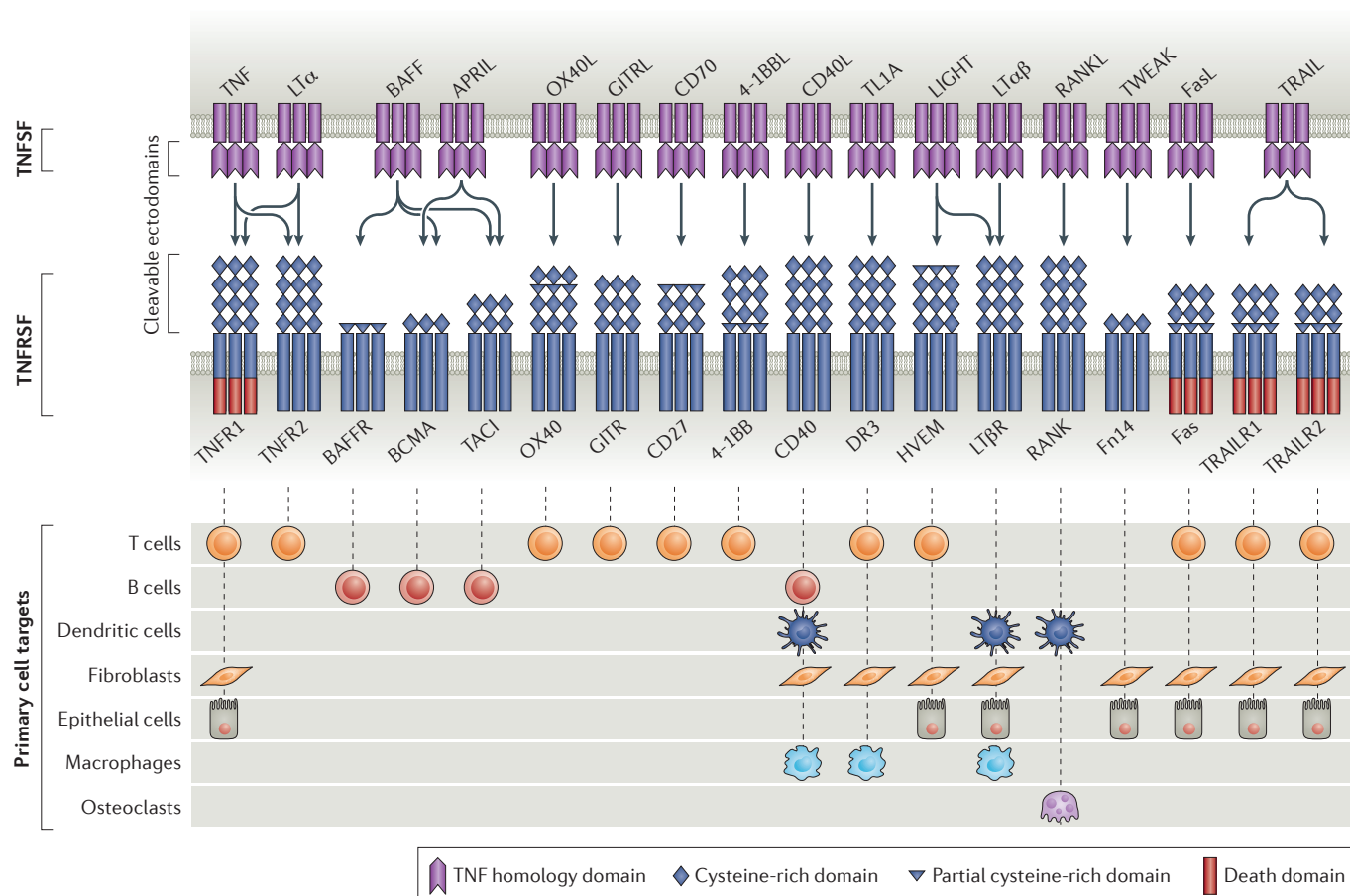


Figure 1 | Select members of the TNF and TNFR superfamily implicated in rheumatic diseases. TNF superfamily ligands (TNFSF; top) are active primarily as non-covalently associated homotrimers and can be soluble or membrane-expressed. TNF superfamily receptors (TNFRSF; bottom) contain variable numbers of cysteine-rich domains in their ligand-binding extracellular regions. TNFRSF are mainly membrane-expressed, but can form soluble receptors via enzymatic cleavage of the ectodomains. Also depicted are the primary cell targets that respond to TNFSF through TNFRSF signalling, although this list is not comprehensive in terms of the expression characteristics of each molecule. TNFRSF molecules whose main function is to promote apoptotic cell death (TNFR1, Fas, TNF-related apoptosis-inducing ligands 1 (TRAIL1) and TRAIL2) can recruit a death-inducing signalling complex to their cytoplasmic domains via a death domain. 4-1BBL, 4-1BB ligand; APRIL, a proliferation-inducing ligand; BAFF, B-cell-activating factor; BAFFR, BAFF receptor; BCMA, B-cell maturation antigen; CD40L, CD40 Ligand; DR3, death receptor 3; FasL, Fas ligand; Fn14, fibroblast growth factor-inducible immediate-early response protein 14; GITRL, glucocorticoid-induced TNF receptor-related (GITR) ligand; HVEM, herpes virus entry mediator; LT, lymphotoxin; OX40L, OX40 ligand; RANKL, receptor activator of nuclear factor- κ B (RANK) ligand; TAC1, transmembrane activator and CAML interactor; TL1A, TNF-like ligand 1; TWEAK, TNF-related weak inducer of apoptosis.

controls T cell numbers in human RA^{56,58}. Targeting of OX40 with cytotoxic drugs to deplete T cells has also shown some therapeutic benefit in an animal model of adjuvant arthritis⁵⁹. Surprisingly, signalling via OX40L antagonizes the activity of RANK in promoting osteoclast development from macrophage progenitors. OX40L-deficient mice are accordingly osteopenic⁵⁶, although the implication of this finding in the context of therapeutic inhibition of OX40–OX40L interactions in arthritis is not clear.

In patients with SLE who have proliferative glomerulonephritis, OX40L is upregulated in glomeruli, most likely on endothelial cells⁶⁰ and/or dendritic cells⁶¹. Similarly, studies have shown that in peripheral blood

and renal biopsy samples from patients with lupus nephritis, OX40 expression by CD4⁺ T cells correlates with disease activity, urine proteinuria and serum creatinine^{62–65}. Furthermore, on the basis of an initial report⁶⁶, many studies have confirmed an association between susceptibility to developing SLE and polymorphisms upstream of the *OX40L* gene (also known as *TNFSF4*), which probably leads to its increased expression. The OX40–OX40L axis is also involved in kidney disease, as patients with Henoch–Schönlein purpura with nephritis have elevated levels of serum OX40L and OX40⁺ T cell numbers compared with patients without nephritis⁶⁷. Surprisingly, no reports have yet demonstrated a functional role for these molecules in

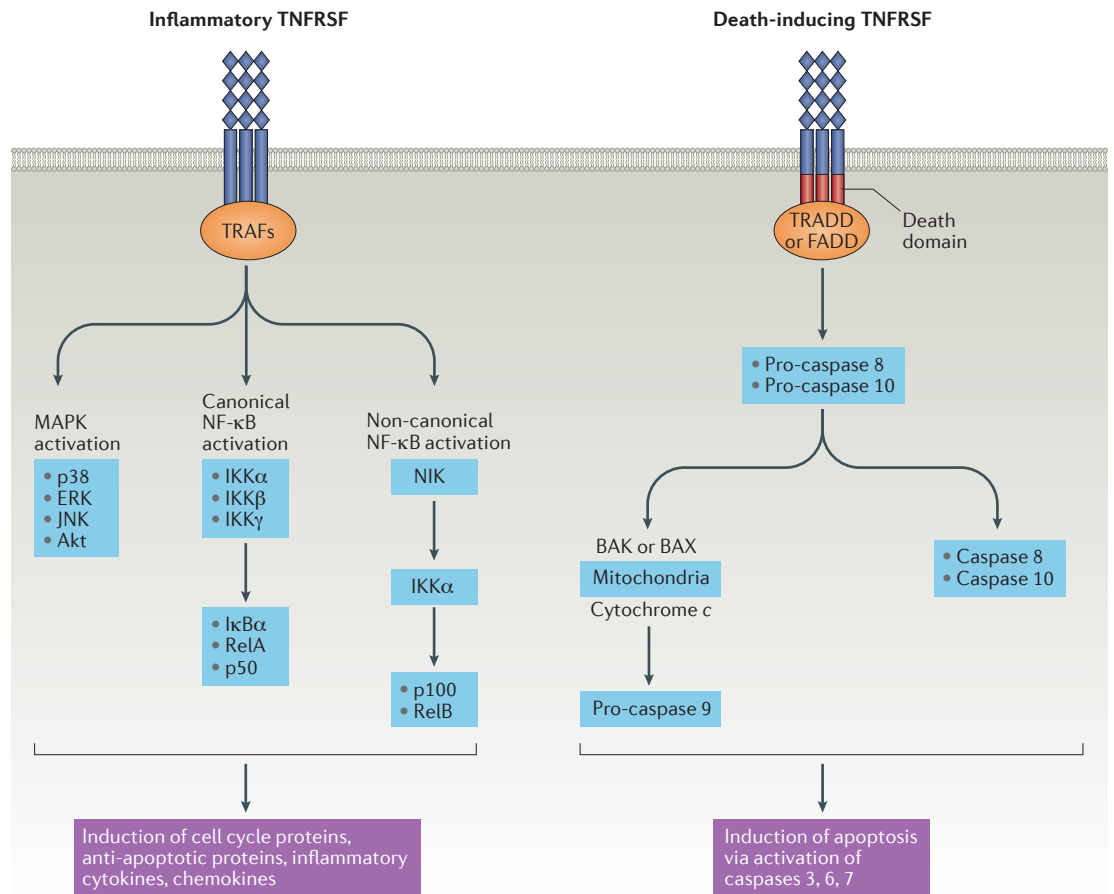


Figure 2 | General TNFRSF receptor signalling. TNF receptor superfamily (TNFRSF) proteins recruit one or several adaptor proteins (TNFR associated factors 1 to 6 (TRAFs), TNFR associated death domain protein (TRADD) and Fas associated death domain protein (FADD)) after ligand binding. As a generalization, TNFRSF proteins that utilize TRAFs (left) can be regarded as proinflammatory and induce proliferation (cell cycle proteins), survival (anti-apoptotic proteins), differentiation and production of inflammatory mediators such as cytokines and chemokines, according to the responding cell type. These processes can be induced via activation of one or both nuclear factor κ B (NF- κ B) signalling pathways (canonical and non-canonical) as well as via MAP kinase cascades. The canonical NF- κ B signalling pathway is IKK β -dependent and involves phosphorylation of inhibitor of κ B (I κ B α) and nuclear translocation of NF- κ B subunit p50 and transcription factor p65 (RelA); the non-canonical NF- κ B signalling pathway is IKK α -dependent and involves activation of NF- κ B-inducing kinase (NIK), processing of p100 to p52, and nuclear translocation of p52 and RelB. The MAP kinase cascades involve c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), p38 or other kinases such as serine/threonine-protein kinase (AKT). TNFRSF members that contain a 'death domain' (right) and recruit the death domain-containing adaptor protein Fas-associated death domain protein (FADD), such as Fas, TNF-related apoptosis-inducing ligands 1 (TRAIL1) and TRAIL2, are often regarded as anti-inflammatory as they generally lead to cell death (apoptosis or necroptosis) through activation of cysteine-aspartic proteases (caspases) and receptor-interacting serine/threonine-protein (RIP) kinases (not shown). TNFR1 and death receptor 3 (DR3), the receptors that recruit the TRADD adaptor proteins, can activate inflammatory responses as TRADD can recruit TRAF proteins, but additionally activate death pathways through secondary complexes containing TRADD, Fas-associated death domain protein (FADD) and caspase 8. Adapted from *Nat. Rev. Drug Discov.* **12**, 147–68 (2013) © Macmillan Publishers Limited¹¹.

mouse models of nephritis, even though human studies imply that OX40–OX40L crosstalk between T cells and endothelial or dendritic cells might contribute to disease.

As well as controlling the accumulation and/or activity of pathogenic effector T cells, OX40–OX40L interactions have been associated with production of pathogenic antibodies. Transgenic mice overexpressing OX40L display elevated levels of anti-DNA antibodies⁶⁸. Furthermore, soluble OX40 and/or OX40L are increased in the plasma of patients with early-stage RA compared with healthy individuals, and correlate with levels of anti-citrullinated

protein antibodies and IgM rheumatoid factor⁶⁹. Similarly, an association between OX40L expression on myeloid APCs (dendritic cells and monocytes), SLE disease activity and anti-ribonucleoprotein (RNP) antibodies has been described⁶¹. As activated B cells express OX40L, this ligand could directly signal and contribute to autoantibody production. However, the primary rationale for the association with anti-RNP antibodies is that OX40L on dendritic cells can signal via OX40 expressed by T cells and might aid formation of follicular T helper cells that drive B cell differentiation⁶¹. An association between polymorphisms

in the OX40L locus and Sjögren syndrome or SSc has also been confirmed in multiple studies^{70,71}, with levels of soluble serum OX40 being elevated in patients with early-stage SSc⁷². Lastly, biopsy samples from patients with Wegener granulomatosis, another rheumatic disease that is associated with elevated levels of anti-neutrophil cytoplasmic antibodies (ANCA) and glomerulonephritis⁷³,

contain OX40-expressing T cells⁷³. Although clinical grade drugs that neutralize OX40L (such as oxelumab and KY1005) or OX40 (such as KHK4083) exist, at present no trials have attempted to target the OX40–OX40L interaction in rheumatic diseases (TABLE 1). However, such interventions have strong therapeutic potential and might be beneficial, particularly in RA and SLE.

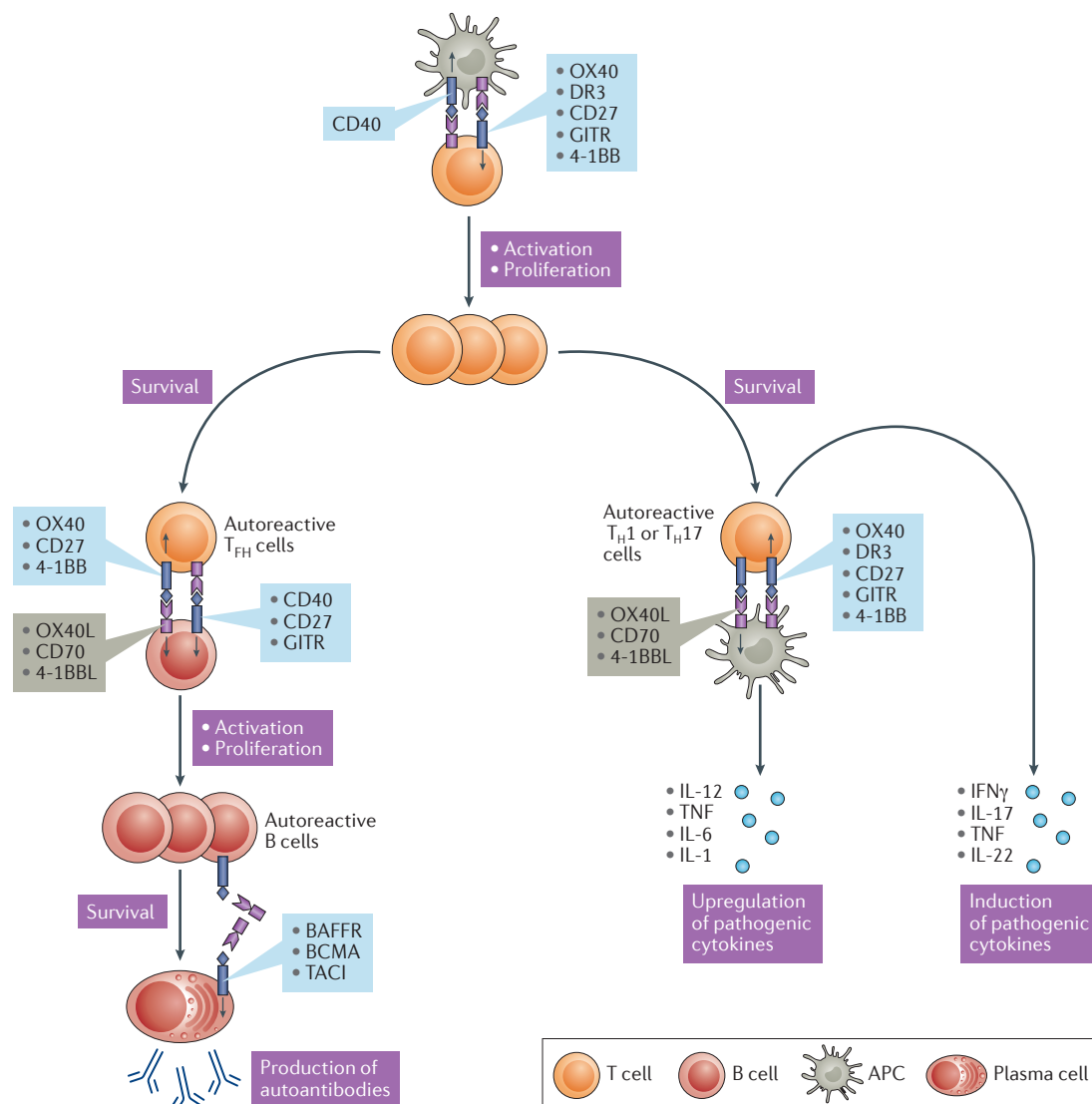


Figure 3 | TNFSF activities enhancing immune cell activation. The simplified diagram highlights the possible interactions between TNF superfamily (TNFSF) ligands and TNF receptor superfamily (TNFRSF) proteins expressed on several cells in the immune system (antigen-presenting cells (APCs), B cells, and T cells). Driven by the appropriate antigen, T cells can receive TNFRSF signals through OX40, glucocorticoid-induced TNF receptor-related protein (GITR), death receptor 3 (DR3), CD27, and 4-1BB. These signals enhance their activation, promote division and survival to augment the size of the autoreactive pool, induce differentiation of follicular helper T (T_{HF}) cells that control antibody responses and induce the expression of cytokines that drive tissue pathology. APCs (dendritic cells and macrophages), via CD40, can upregulate MHC molecules, co-stimulatory ligands (including TNFSF molecules) and inflammatory cytokines, which aid the T-cell response. B cells can receive signals from CD40, CD27, GITR, B-cell-activating factor (BAFF) receptor (BAFFR), B-cell maturation antigen (BCMA) and transmembrane activator and CAML interactor (TACI). These signals drive activation, division and survival, class switching, and plasma cell differentiation, resulting in production of pathogenic autoantibodies. Reverse signalling through membrane-expressed TNFSF ligands such as OX40 ligand (OX40L), CD70 and 4-1BBL, expressed on dendritic cells, macrophages and B cells, can also augment production of inflammatory cytokines and help B cell differentiation. Other reported activities of TNFRSF signalling on immune cells such as mast cells, eosinophils, neutrophils, basophils, Natural killer T cells and innate lymphoid cells are not shown, but these can further result in production of inflammatory mediators that contribute to tissue pathology and amplify the T-cell and B-cell response.

TL1A

Death receptor 3 (DR3, also known as TNFRSF25) is another stimulatory receptor expressed by T cells (FIG. 3) that can regulate effector cell accumulation and/or reactivity regardless of T helper phenotype^{74–76}. Its ligand, TNF-like ligand 1A (TL1A, also known as TNFSF15), can be induced in APCs such as dendritic cells and macrophages, as well as in endothelial cells^{5,6,11,77,78}. TL1A–DR3 interactions might drive many inflammatory responses, especially mucosal inflammation^{77,79}, and increasing evidence suggests a role in rheumatic disease.

Levels of TL1A are elevated in the synovial fluid and serum of patients with RA, and are associated with both autoantibody levels and atherosclerotic lesion development^{80–86}. Interestingly, human synovial fibroblasts are capable of expressing TL1A after stimulation with TNF or IL-1 β , suggesting a potential local source of TL1A in addition to professional APCs⁸². In line with the notion that soluble TL1A could be pathogenic in RA, injection of recombinant TL1A into mice with already-developed collagen-induced or bovine serum albumin (BSA)-induced arthritis leads to an increase in the severity of disease, including increased cartilage damage, bone destruction and increased levels of autoantibodies^{82,87}. In these arthritis models, DR3 and TL1A deficiency, or TL1A inhibition in wild-type mice, resulted in reduced swelling and bone erosions, and/or increased kinetics of disease resolution, demonstrating the therapeutic potential of targeting TL1A^{87–89}. The reason for reduced disease activity is not clear but could be due to a combination of lower T-cell activity and reduced infiltration of destructive cells such as neutrophils, which is possibly linked to defective chemokine expression. An alternative explanation is that TL1A could have a role in enhancing RANKL-triggered osteoclast differentiation in macrophage precursors that express DR3 (REF. 87). This process could cooperate with the immune-mediated inflammatory effects of TL1A and contribute to bone dysregulation. Although genome-wide association studies have not identified TL1A or DR3 as susceptibility loci for inflammatory arthritides, a duplication in the gene encoding DR3 (*TNFRSF25*) has been linked to RA⁹⁰; in addition, an association study indicated that several SNPs downstream of the gene encoding TL1A (*TNFSF15*) were linked to the development of spondyloarthritis (SpA)⁹¹. SpA, a disease closely related to RA, can be characterized by gut inflammatory phenotypes and T_H17 cells are thought to be involved in SpA pathogenesis; both of these features are known to be connected with TL1A activity^{77–79}. Lastly, DR3 and/or TL1A were found to be upregulated in lesional skin plaques and serum from patients with psoriasis, another disease with a T_H17 component that can be directly associated with arthritis^{92,93}. Although the implications of these observations regarding the pathogenesis of SpA and psoriasis are not clear, these data suggest that DR3 and TL1A are involved in bone and joint disorders and manifestations that arise from these inflammatory diseases.

Data directly implicating TL1A involvement in SLE pathogenesis are currently lacking, except for one report describing a weak correlation between elevated TL1A

levels in serum and SLE disease activity⁹⁴. However, during acute kidney allograft rejection, renal tubular epithelial cells express DR3 (REF. 95), and renal vascular endothelial cells express TL1A⁹⁶. DR3 activity might be protective against nephrotoxicity in some settings^{96,97}, but whether these molecules contribute to nephritis as seen in SLE is an open question. Overall, the data presented above indicate that inhibition of TL1A–DR3 activity might be beneficial for patients with arthritis, and possibly for those with other autoimmune conditions such as SLE.

GITRL

Glucocorticoid-induced TNF receptor-related ligand (GITRL, also known as TNFSF18) is an inducible molecule expressed in professional APCs, and other cell types such as endothelial cells. Its receptor, glucocorticoid-induced TNF receptor-related protein (GITR, also known as TNFRSF18), can stimulate T cell, dendritic cell and B cell activation (FIG. 3). Studies have implicated these molecules in controlling many immune-inflammatory responses, although functional data relating them to rheumatic disease are largely restricted to arthritis at present^{6,11,98,99}. GITR-deficient mice display reduced joint inflammation in collagen-induced arthritis compared with wild-type mice, including decreased T-cell reactivity and lower levels of inflammatory mediators such as TNF¹⁰⁰. Serum from patients with RA have increased levels of GITRL compared with healthy controls, a finding associated with increased IL-17 levels¹⁰¹. Furthermore, GITR and GITRL have been detected in synovial tissue sections from patients with RA (primarily in T cells and macrophages); synovial fluid from these patients has also been found to contain both GITR and GITRL as soluble molecules^{102,103}. In line with the idea that soluble GITRL is pathogenic, injection of recombinant GITRL into mice with collagen-induced arthritis increases disease kinetics and clinical symptoms¹⁰¹, as does treatment with an agonistic GITR antibody¹⁰⁴; this treatment also increases production of the T-cell-derived inflammatory cytokines such as IL-17, TNF and IFN γ ^{101,104}. Furthermore, stimulation of GITR on synovial fluid macrophages leads to upregulation of several inflammatory proteins including TNF, IL-6 and MMP-9 (REF. 102). Lastly, soluble GITRL and/or GITR might represent useful biomarkers for other rheumatic diseases, as in patients with SLE or Sjögren syndrome the levels of these molecules are increased^{105,106}. Given that their expression correlate with disease severity^{105,106}, GITR–GITRL activity might also contribute to the pathogenesis of these diseases

CD70

CD27 (also known as TNFRSF7) is constitutively expressed on most T cells, and the interaction with its ligand CD70 (also known as TNFSF7) can provide signals to T cells to control their accumulation and reactivity, similarly to that seen with OX40, GITR and DR3 (REFS 3–5) (FIG. 3). In addition to T cells, CD70 is inducible on dendritic cells and B cells, and can induce reverse signals within these APCs to increase their activation

status²⁷, therefore participating in the crosstalk between T cells and B cells and antibody production. Genetic deletion or neutralization of either CD27 or CD70 in mice has revealed a pathogenic role for the CD27–CD70 axis in many inflammatory settings^{4–6,107}. For example, in mice with collagen-induced arthritis, blocking CD27–CD70 interactions with anti-CD70 antibody reduces bone and cartilage erosion and inflammatory infiltrates in the joints, and decreases collagen-specific antibody production, even when the treatment is initiated after disease onset¹⁰⁸. In the synovial fluid of patients with RA, soluble CD27 levels and CD27⁺ T cell numbers are elevated and correlates with the levels of rheumatoid factor, supporting a role for CD27 in human RA¹⁰⁹. Furthermore, in patients with RA, CD70 expression is increased in CD4⁺ T cells that produce the effector cytokines IFN γ and IL-17 (REFS 110,111). Although the implication of this upregulation is not clear, these CD4⁺ T cells are probably highly pathogenic, given that ligation of CD27 on B cells by CD70 can promote B cell differentiation. Synovial fluid samples from patients with juvenile idiopathic arthritis are also characterized by increased expression of soluble CD27 (REF. 112).

A correlation between CD27 or CD70 expression and disease activity is also observed in other rheumatic diseases, although functional data are in general lacking at present. Soluble CD27 levels correlate with disease activity in patients with SLE^{113,114}, and the proportion of plasma cells expressing high levels of CD27 additionally correlates with SLE disease indices¹¹⁵. Furthermore, several studies showed that T cells derived from patients with SLE express high levels of CD70 and are capable of driving B cell antibody production via CD27 (REF. 116). Similarly, T cells from MRL/lpr mice with lupus-like disease overexpress CD70 (REF. 117), although no studies to date have shown if CD70 expression is required for disease onset in these mice. Interestingly, plasmacytoid dendritic cells (pDCs), which are thought to be central to SLE pathogenesis via their type I interferon production, can strongly express CD70 (REF. 118). These pDCs can drive antibody secretion by B cells via CD27 without the participation of T cells, implicating pDCs as another important source of CD70. CD4⁺ T cells from patients with SSc and Sjögren syndrome have also been found to express high levels of CD70 (REFS 119,120). Thus, neutralizing the interaction between CD27 and CD70 could potentially dampen disease activity in RA and/or other diseases such as SLE. A clinical-grade antibody to CD70 (SGN-75) has been developed and conjugated to a toxin for targeting CD70⁺ B cell cancers¹¹. This reagent could be used, with or without toxin, for treatment of rheumatic disease, although no trials have so far been initiated.

4-1BBL

4-1BB (also known as TNFRSF9) is an inducible stimulatory receptor expressed on T cells and innate lymphoid cells that can promote their accumulation and/or activity; expression of its ligand, 4-1BBL (also known as TNFSF9), is also inducible on professional APCs^{5,6,121}. 4-1BB is similar to the molecules described above in terms of intrinsic activity (FIG. 3); As with OX40, GITR

and CD27, 4-1BB is currently being targeted with receptor agonists to promote antitumour T-cell responses in the context of clinical cancer immunotherapy¹²². However, only a few studies have shown 4-1BB and 4-1BBL involvement in inflammatory disease pathogenesis^{5,6,121}. As such, little data has been generated with regard to rheumatic disease. Serum samples of patients with RA contain elevated levels of soluble 4-1BB and 4-1BBL, which correlate with disease severity^{123,124}. Nevertheless, in collagen-induced arthritis in mice, a reagent that blocks the interaction between these two molecules had only a moderate effect in suppressing disease symptoms such as T-cell reactivity and inflammatory cytokines¹²⁵. Although this finding does not exclude a role for 4-1BBL–4-1BB interactions in promoting RA in humans, it is in contrast to the much more robust data obtained when other TNF family molecules (such as OX40L, CD70, GITRL and TL1A) were targeted in the same arthritis model. On the other hand, stimulation of 4-1BB with receptor agonists results in strong suppression of joint inflammation and bone destruction in mouse models of RA^{125,126}. This finding is not consistent with the idea that endogenous 4-1BB–4-1BBL interactions promote development or activity of pathogenic T cells in RA. A similar conclusion might be true for SLE; indeed, 4-1BB-deficiency in lupus-prone MRL/lpr mice exacerbates rather than ameliorates disease¹²⁷, in line with a regulatory rather than pathogenic role. Similar to mouse models of arthritis, 4-1BB agonists also fully inhibit lupus-like disease in MRL/lpr and NZB/NZW F₁ mice, including reduction of skin lesions, lymphadenopathy, autoantibody production and nephritis^{128–130}. These results suggest that the neutralization of 4-1BB or 4-1BBL might have little effect in rheumatic disease, whereas stimulation of 4-1BB could dampen inflammation.

Increasing tissue inflammation

Lymphotoxin and LIGHT

Lymphotoxin and LIGHT (also known as CD258 and TNFSF14, respectively) are TNFSF cytokines with interrelated functions that are similar to those of TNF. They can control T cell and APC responsiveness, and importantly, have marked effects on both development and homeostasis of lymphoid tissue and structural cell responses of non-haematopoietic tissue^{131–134} (FIG. 4). Soluble lymphotoxin (also known as LT α or TNFSF11) is a homotrimer that binds TNF receptors (TNFR1 and TNFR2), but might often be redundant with TNF. In RA, anti-TNF antibodies have been found to be as clinically effective as etanercept, a TNFR2-Fc fusion protein that blocks both LT α and TNF¹³⁵, and in a clinical trial of RA, pateclizumab, a specific blocker of LT α , showed much reduced efficacy compared with the TNF blocker adalimumab¹³⁶. These findings do not rule out an important role for LT α in some inflammatory diseases, but suggest that its role is secondary to that of TNF when TNF is present in abundance. By contrast, the other version of lymphotoxin, LT $\alpha\beta$ might exert distinct and unique functions compared with TNF and LT α . LT $\alpha\beta$ is membrane-bound heterotrimer composed of LT α and

a distinct β subunit, and exclusively binds to the $LT\beta$ receptor ($LT\beta R$, also known as $TNFRSF3$)¹³⁷. $LT\alpha\beta$ is constitutively expressed on resting B cells and can also be induced in activated T cells. $LT\beta R$ is expressed on some haematopoietic cells, such as dendritic cells and macrophages, but importantly, is expressed on tissue stromal cells such as fibroblasts, adipocytes, hepatocytes, endothelial cells, fibroblastic reticular cells, smooth muscle cells and epithelial cells¹³⁷. Studies of gene-knockout mice have shown a non-redundant role for $LT\alpha\beta$ - $LT\beta R$ interactions in controlling the development of lymph nodes and Peyer patch structures, which is due to the absence of $LT\beta$ -dependent RANKL production¹³⁷. RANKL acts on stromal cells to induce chemokine expression, which is critical for recruitment and proper positioning of lymphocytes within these structures¹³⁷. In mature lymphoid tissue, $LT\alpha\beta$ signals through $LT\beta R$ in follicular dendritic cells, controlling the expression of adhesion molecules (vascular cell adhesion protein 1 (VCAM1) and mucosal addressin cell adhesion molecule 1 (MADCAM1)), as well as chemokines, which maintain B cell organization in follicles¹³⁷. These mechanisms have also been implicated in controlling the arrangement of immune cells in tertiary lymphoid structures, which occur in tissues undergoing chronic inflammatory responses¹³⁷.

LIGHT binds to $LT\beta R$ and also to a receptor termed herpes virus entry mediator (HVEM, also known as $TNFRSF14$). LIGHT can be expressed by activated T cells and other lymphoid cells, and HVEM is expressed on many haematopoietic cells in addition to the same structural cells that express $LT\beta R$ (such as fibroblasts, epithelial cells and smooth muscle cells)^{132–134}. Whereas LIGHT does not participate in controlling lymphoid organogenesis, growing evidence suggests that its activity in tissue cells, via both $LT\beta R$ and HVEM, might be a strong component of the remodelling processes characteristic of many chronic inflammatory and autoimmune diseases, including epithelial–mesenchymal transition and myofibroblast differentiation¹³⁴ (FIG. 4). The physiological role of $LT\beta R$ and HVEM might be to protect the epithelium and other tissues against injury or infection^{138,139}. However, their reported activities in epithelial cells, fibroblasts, osteoclasts, adipocytes and hepatocytes suggest that if LIGHT or $LT\alpha\beta$ are produced in excess these receptors directly or indirectly induce the production of inflammatory cytokines, chemokines, extracellular matrix proteins and proteinases. These effects are similar to that seen with TNF - $TNFR1$ activity, implying that these molecules cooperate in orchestrating tissue inflammation^{134,140–144}.

An Fc fusion protein of $LT\beta R$, which can neutralize both $LT\alpha\beta$ and LIGHT, can block disease symptoms in many mouse models of rheumatic disease, including collagen-induced and adjuvant arthritis, several models of SLE and the Sjögren-syndrome-like salivary gland inflammation of non-obese diabetic mice^{131–133,145–148}. Additionally, genetic deletion of LIGHT protects mice from lung and skin inflammation and tissue remodelling in models of SSC^{144,149}. Despite these results, targeting the $LT\alpha\beta$ -LIGHT axis with baminercept,

a soluble $LT\beta R$ -Fc fusion protein, did not demonstrate clinical efficacy in RA and Sjögren's syndrome (TABLE 1), although some modulation of immune reactivity was noted¹⁵⁰. A caveat of these trials was the recruitment of difficult-to-treat patient populations that had previously shown inadequate responses to TNF inhibitors or other DMARDs. More specific reagents targeting LIGHT, $LT\beta$ or their receptors still have potential for the treatment of rheumatic diseases that involve tissue remodelling and inflammation, although they are more likely to be efficacious in patients who are also responsive to TNF -directed therapy. A fully human LIGHT blocking antibody has been generated and has successfully completed phase I safety trials (TABLE 1); this antibody is currently entering phase II studies of paediatric inflammatory bowel disease but hasn't yet entered any trials for rheumatic disease.

TWEAK

TNF -related weak inducer of apoptosis (TWEAK, also known as $TNFSF15$) has high degree of homology with TNF and is thought to primarily act on tissue cells^{151,152}. TWEAK is produced by a large range of myeloid and immune cells, but its receptor, fibroblast growth factor-inducible 14 (Fn14, also known as $TNFRSF12A$), is more highly expressed on non-haematopoietic cells than on lymphoid cells. Fn14 is upregulated by fibroblast-like growth factor^{153,154}, as well as by other factors associated with injury and inflammation¹⁵¹. TWEAK has pleiotropic effects in stromal cell types, including regenerative-like activities in hepatocytes, endothelial cells, myocytes and epithelial cells¹⁵² (FIG. 4). Arguably, the physiological role of the TWEAK-Fn14 axis is to protect against tissue injury, but like the LIGHT- $LT\alpha\beta$ axis, if TWEAK or Fn14 are excessively produced they could drive and orchestrate inflammation, fibrosis and tissue remodelling.

TWEAK and Fn14 are elevated in the synovium and serum of patients with RA and/or psoriatic arthritis, with levels correlating with disease severity in some instances, although their levels in joints are not affected by TNF inhibitor treatment^{155–158}. In normal fibroblasts or fibroblast-like synoviocytes, TWEAK can induce proliferation and upregulate the production of inflammatory cytokines such as IL-6, chemokines, adhesion molecules and proteinases^{159–162}. As such, blocking TWEAK reduces disease severity in collagen-induced arthritis in mice without affecting titres of anti-collagen antibodies^{163,164}, suggesting that TWEAK largely contributes to inflammation and bone destruction locally in the joint. Osteoclasts express Fn14, and consequently TWEAK can promote osteoclastogenesis, which is relevant to RA pathogenesis¹⁶⁵. These data suggest that neutralizing TWEAK has the potential to dampen disease activity in RA. Phase I trials of a blocking antibody against TWEAK (BIIB023) have been conducted in patients with RA¹⁶⁶, but further trials in RA have not yet been pursued (TABLE 1).

TWEAK has also been implicated in kidney disease. Fn14 deficiency or TWEAK blockade reduces a variety of renal pathologies in several mouse models of disease, including fibrosis after ureteral obstruction¹⁶²,

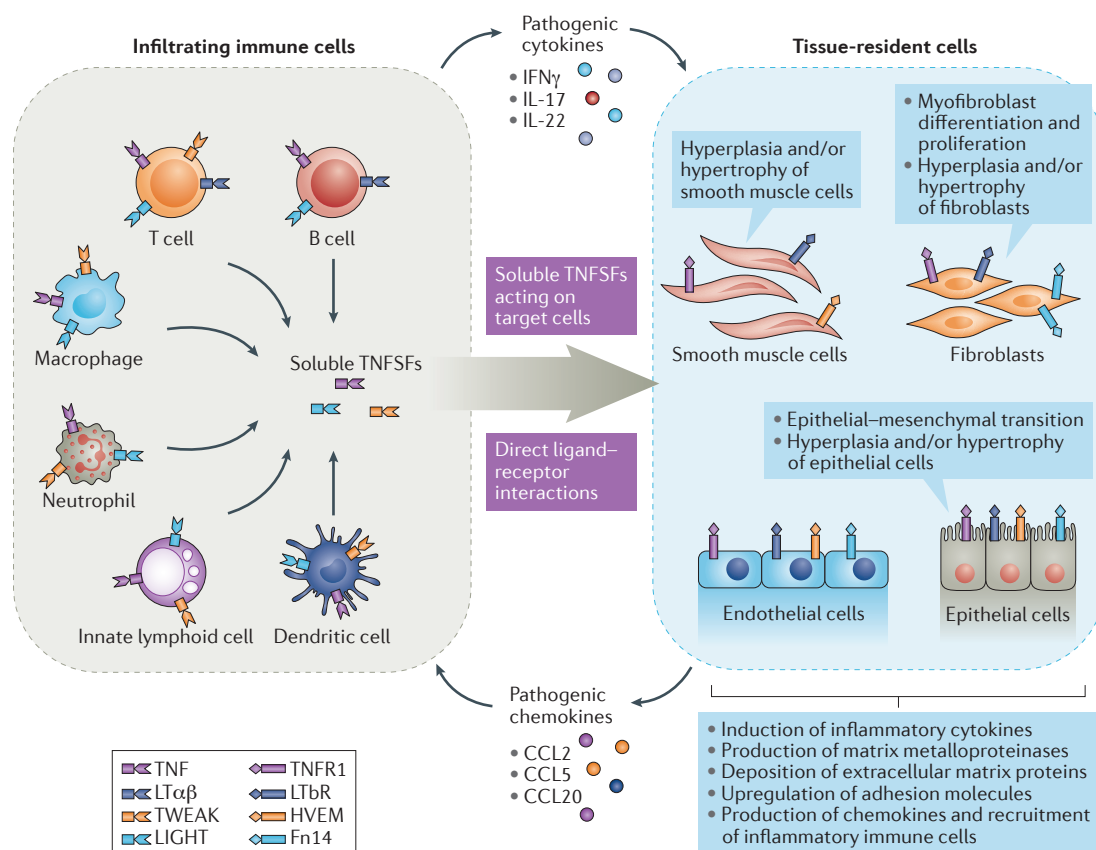


Figure 4 | TNFSF inflammatory activities in tissue cells. The simplified diagram shows the possible interactions between TNF superfamily (TNFSF) ligands and their receptors expressed on tissue cells (epithelium, endothelium, fibroblasts and smooth muscle cells) that can affect tissue homeostasis and inflammatory activity. The TNFSF molecules lymphotoxin (LT) $\alpha\beta$, LIGHT and TNF-related weak inducer of apoptosis (TWEAK), together with TNF, are likely to be produced primarily by cells of the immune system, including T cells, B cells, dendritic cells, macrophages, as well as neutrophils, mast cells and innate lymphoid cells. Amplification loops from tissue structural cells, including endothelial and epithelial cells, might further induce production of these molecules. Signals from TNFR1, lymphotoxin- β receptor (LT β R), herpes virus entry mediator (HVEM) and fibroblast growth factor-inducible protein 14 (Fn14) can directly promote tissue pathology through multiple processes, including differentiation events such as epithelial-mesenchymal transition and myofibroblast transformation, hyperplasia and hypertrophy of epithelial cells, fibroblasts, and smooth muscle cells, expression of extracellular matrix proteins and proteinases that contribute to tissue remodelling, production of chemokines and adhesion molecules that attract and maintain inflammatory immune cells within the inflamed tissue. CD40 and death receptor 3 (DR3) are also expressed on some tissue cells such as fibroblasts and could further amplify their inflammatory activity (not shown). Furthermore, receptor activator of nuclear factor- κ B ligand (RANKL) and TWEAK are regulators of osteoclast activation and differentiation (also not shown). TNFSF might additionally synergize with proinflammatory T-cell-derived cytokines such as IFN γ , IL-17 and IL-22, which also have receptors on tissue structural cells.

folate-induced interstitial nephritis¹⁶⁷, nephrotoxic serum-induced immune complex glomerulonephritis¹⁶⁸ and nephritis associated with chronic graft-versus-host disease¹⁶⁹. Additionally, in Fn14-deficient mice, renal, neuropsychiatric and dermatological manifestations were considerably reduced in the MRL/lpr model of spontaneous lupus-like autoimmunity^{170–172}. As with collagen-induced arthritis, titres of systemic auto-antibodies were not affected in these studies, further suggesting that Fn14 mediates local effects in target tissues. Which cell types receive Fn14 signals in the context of lupus nephritis or other kidney disease is an unresolved question. However, TWEAK can stimulate inflammatory mediator production (cytokines and/or chemokines) *in vitro* by a variety of different kidney cell

types, including renal tubular epithelial cells, podocytes and mesangial cells^{167,173,174}. In human SLE, TWEAK can serve as a urinary biomarker for nephritis¹⁷⁵. Despite these promising results, a trial investigating the efficacy of anti-TWEAK antibodies in SLE was terminated following failure to increase rates of renal remission in patients with nephritis already being treated with mycophenolate¹⁷⁶ (TABLE 1).

Cell death and immunosuppression

FasL and TRAIL

FasL and TRAIL have a potent ability to induce apoptosis. FasL can promote apoptosis in activated primary B cells, T cells and dendritic cells through Fas (also known as TNFRSF6)^{174,175}, and TRAIL has been shown to induce

apoptosis in activated mouse CD8⁺ T cells via TRAIL receptor 1 (TRAILR1, also known as TNFRSF10A) or TRAILR2 (also known as TNFRSF10B)^{177–179}. Defective activity of the FasL–Fas or TRAIL–TRAILR axis might increase the susceptibility to autoimmune disease. Genetic defects in FasL, or more commonly in Fas, result in spontaneous autoimmunity in mice and in autoimmune lymphoproliferative syndrome in humans^{180–184}. TRAIL-deficient mice are hypersensitive to diseases such as collagen-induced arthritis¹⁸⁵. Less is known about the role of TRAIL and its receptors in human cells. Although activated human T cells express TRAILR1 and TRAILR2, unlike FasL, TRAIL does not generally induce apoptosis in these cells¹⁸⁶. Dendritic cells might be more relevant targets for TRAIL in the human immune system as a deficiency in caspase 10, which is activated by TRAIL, underlies a variant of autoimmune lymphoproliferative syndrome, which is marked by accumulation of these cells¹⁸⁷. For these reasons, the function of FasL and TRAIL is mainly to restrain persistent immune responses to curb autoimmunity. Fas and TRAILRs can also be expressed outside the immune system; crosslinking of these molecules on cells such as synovial fibroblasts or dermal fibroblasts, which are associated with RA and SSc, respectively, might induce apoptosis^{188,189}. However, either an elevated activation state or increased proliferative activity of such cells might make them more resistant to the effects of the naturally produced death-inducing ligands^{189,190}, which could be another contributing factor to diseases such as RA.

The apoptotic potential of FasL and TRAIL, either to dampen activity of autoreactive T cells or to kill highly proliferative tissue cells, has led to the hypothesis that recombinant FasL or TRAIL, or biologic agents acting as receptor agonists, could be candidate therapeutics for rheumatic diseases¹⁹¹. Results from experimental studies on the injection of various forms of FasL or TRAIL into rodents have reinforced this idea^{192–196}. However, several factors might hinder this therapeutic strategy. Fas engagement has the potential to cause off-target effects, as exemplified by induction of hepatocyte cell death and acute liver failure in mice injected with Fas agonists¹⁹⁷. Although all activated T cells express Fas, stimulation with this molecule fails to induce efficient apoptosis of memory T cells or T cells in the early stages of activation, which are the likely T cells that would be active in rheumatic diseases^{178,198}. Rather, Fas might stimulate T-cell activation in some scenarios^{199–201}. Some data suggest that fibroblast-like synoviocytes can be induced to proliferate when treated with soluble FasL or with low doses of agonist Fas antibody, whereas only oligomeric FasL or high doses of anti-Fas agonists induce apoptosis^{202,203}. These factors further complicate the development of biologic agents to stimulate Fas or TRAILRs that might be therapeutically useful in rheumatic diseases.

Challenges and limitations

Is targeting one TNFSF member enough?

Several potential challenges exist when looking at modulating the activity of TNF family members other than TNF in rheumatic diseases. Blockade of TNF is

highly efficacious in treating patients with a wide range of inflammatory arthritides including RA, psoriatic arthritis, ankylosing spondylitis and juvenile idiopathic arthritis, and also in other inflammatory diseases such as plaque psoriasis, Crohn's disease and ulcerative colitis^{11,204}. However, whether neutralizing another TNFSF protein in isolation will produce the same strong and broad benefit is not clear. Blocking TNF might have a potent therapeutic effect for two main reasons. Firstly, TNF is a primary end-stage inflammatory mediator in tissues, as it is produced at high levels by multiple cell types (both immune and non-immune) and induced by many different stimuli. Secondly, TNF has two receptors that are both expressed on immune cells as well as stromal non-haematopoietic cells, broadening its activity from tissues to the immune system. In comparison, the majority of other TNFSF molecules are produced at lower levels, triggered by fewer stimuli, act on a smaller number of cell types and primarily control immune cells and not tissue cells. Hence, a number of TNFSF proteins, particularly the immune modulators, might have a narrower range of action compared with TNF, limiting the therapeutic effects of biologic agents that target them.

A possible example in this regard is BAFF, a molecule that primarily, although not exclusively, controls B cell activity. Preclinical data, particularly in mouse models, suggest that BAFF and B cells are central to lupus-like autoimmunity^{10,17}. However, belimumab, a BAFF inhibitor, although approved for SLE treatment and having considerable effects on human B cells, has been found to be only moderately efficacious in a small number of patients with SLE^{205,206}. This unexpected outcome could reflect differences between human SLE and the disease that manifests in animal models. As suggested above, the fact that BAFF primarily controls only one immune cell type and does not play an important role within the affected tissues of patients with SLE might also explain this outcome. Belimumab has only a moderate effect in patients with RA, although slightly more promising results have been observed in Sjögren's syndrome^{207,208}. Again, suppression of the B cell arm of the immune response might not be sufficient for a notable disease modification, given the activity of other immune cell types in these diseases and the strong tissue component, which is dependent on crosstalk between multiple immune cells and tissue structural cells. Thus, when considering other molecules such as CD40L, OX40L, GITRL, TL1A and CD70, which arguably exert the majority of their activity on T cells, B cells, dendritic cells and macrophages, and are possibly not functional within the affected tissues during the active phase of disease, we have to consider whether neutralizing only one of their interactions will produce a pronounced therapeutic effect.

Another obstacle for successfully targeting TNFSF proteins, particularly those that primarily control immune cell activation, is that an alteration in activity of cells such as T cells and B cells might take a long time to manifest in terms of disease symptoms. As current trials are typically short term and largely designed to compare to an already approved drug (such as a TNF

inhibitor) whose target or mechanism of action could be different, future success in this area might require careful trial designs and end points based around modulation of the perceived primary target cell or cells.

Towards immunological tolerance

Despite the caveats of targeting some TNFSF members discussed above, inhibiting molecules such as CD40L, OX40L, GITRL, CD70 and TL1A, which control the accumulation and activity of pathogenic T cells and B cells, might be a good strategy to re-establish immunological tolerance. Such targeting could prevent the formation of these disease-causing T cell and B cell populations, lead to their deletion and/or reset immune homeostasis in favour of regulatory T cells and B cells; such regulatory cells are now acknowledged to be critical for limiting autoimmunity.

Abatacept (a CTLA4–Ig fusion protein) is a drug already approved for RA therapy and used either as first-line treatment or in patients not responding to conventional therapy. This reagent is primarily thought to act by disrupting CD28 stimulatory signals in T cells. As CD28 can cooperate with TNFRSF proteins in driving T-cell activation^{4,209}, blocking one or more of these TNF family members might have therapeutic effects similar to those of abatacept in RA and possibly other rheumatic diseases. However, given the apparent overlap in the activities of several TNFRSF molecules on T cells and B cells, and the idea that TNFRSF and CD28 cooperate in driving T cell and B cell responses^{4,5,210}, we still have to consider that combination therapies that neutralize two or more interactions might be required to see marked and broad-reaching activity in many patients, regardless of the disease. Furthermore, as discussed above, therapeutic effects might take time to manifest in terms of disease control. Mouse transplantation models using fully MHC-mismatched allografts have shown that neutralization of CD40L with OX40L, or CD40L with CD70, with or without concomitant inhibition of CD28, can help to establish immune tolerance in situations where targeting the individual interactions is ineffective⁵. However, the best combined therapy for any given rheumatic disease is not obvious at present. Information regarding the timing of action of TNF family molecules during disease development will also be critical to any therapeutic success. Immune monitoring of levels of TNFSF ligands and receptors in fluids or tissues of patients with rheumatic disease will probably help, although this approach still assumes that their presence signifies their activity. Immune monitoring might also lead to an improved understanding of molecules that can be targeted simultaneously. Furthermore, translational studies in animal models that more realistically mimic the active phases of human rheumatic disease should aid the formulation of effective combination therapies.

Blocking tissue inflammation

Although the TNFSF members that primarily control T cells and APCs (FIG. 3) are probably good targets for restoring tolerance in rheumatic diseases, the molecules that regulate tissue cell responses (FIG. 4) similarly to TNF

might be more attractive targets for therapy. For example, several structural cell types express LT β R, HVEM and Fn14. A few reports have shown that molecules such as CD40 and DR3 are expressed and active in mouse and human fibroblasts in disease settings as diverse as RA, SSc and inflammatory bowel disease^{211–213}. What is not clear is how much synergy or overlap occurs between these receptors on structural cells in terms of function, and again whether blocking a single molecule in humans is likely to have a profound effect on any given disease phenotype. The failure of TWEAK–Fn14 blockade to achieve its end point in lupus nephritis might reflect the challenges inherent in nephritis trials²¹⁴, and trials in other diseases will be necessary to assess its full potential. However, TWEAK blockade could be an example of where combining treatment with a biologic agent targeting another protein is necessary, as TWEAK has a functional activity similar to that of other TNFSF molecules such as TNF and LIGHT.

Related to this discussion is the observation that anti-TNF treatment is ineffective in about one-third of patients with RA²⁰⁴. The reasons for this lack of response are not clear, but an open question is whether some patients do not respond to anti-TNF monotherapy because several other TNFSF molecules, such as TWEAK and LIGHT, are also active. Would these anti-TNF nonresponders (in any rheumatic disease) be the preferred population to treat with biologic agents targeting other tissue-acting TNFSF members? To test this theory, a clinical trial investigated the use of baminercept, which inhibits LIGHT and LT $\alpha\beta$, in patients with RA who were unresponsive to TNF blockers. Although some effect on biological activity was noted¹⁵⁰, this monotherapy was abandoned as it did not achieve the therapeutic end point. However, in this case redundancy or cooperative action between multiple TNFSF members, including LIGHT, TNF and TWEAK, could explain this lack of activity. Combination therapy might then be more efficacious than targeting molecules separately. This might apply to patients that do respond to TNF inhibitors as well as those who do not respond to anti-TNF therapy alone.

Conclusions

At present, our knowledge of the TNF family members is quite advanced and, at least in some cases, has translated well into the clinic. However, there have been notable failures despite preclinical data suggesting important roles for many of these molecules in rheumatic or other inflammatory diseases. As discussed above, the potential overlap in expression and activities of TNFRSF might hinder therapeutic approaches that only neutralize a single interaction. However, these setbacks should not discourage the enthusiasm for attempting to modulate these molecules alone or in combination. Historically, combination treatment of TNF inhibitors with other biological drugs (such as abatacept and anakinra) has not improved efficacy in the treatment of rheumatic disease and only increased adverse events such as infections^{215,216}. These findings might be specific to TNF, as the evolutionary role of this cytokine is arguably to limit replication of infectious pathogens. Therefore, neutralizing two

TNFSF members other than TNF might not result in a similar increase in such deleterious effects. Regardless, any combination will probably require extensive safety data before being introduced in the clinic. Advances in technology might enable two or more proteins to be targeted with a single biologic agent (such as with a bi-specific antibody), potentially making the path to inhibiting multiple interactions more feasible.

An alternative therapeutic strategy is to stimulate the death receptors Fas and TRAILR1/2, and attempt to induce apoptosis of immune or structural cells that contribute to disease pathology. The difficulty in this approach is being able to effectively induce death in the relevant cell types without having off-target effects; given the broad expression of death receptors, more direct approaches (such as bi-specific molecules) that focus the activity of an agonist reagent on individual cell types are probably needed. Another strategy might be to activate stimulatory receptors. In SLE (and multiple sclerosis), TNF inhibitors have not performed well^{217,218}, and in some cases promote lupus-like disease²¹⁹. Although the reason for this outcome is unclear, studies suggest that inhibition of TNF binding to TNFR2 can impair the expansion of suppressive CD4⁺ Foxp3⁺ T_{reg} cells, which maintain immune tolerance in some

settings^{220–222}. In this regard, similar functional observations have also been drawn for OX40, 4-1BB, CD27, DR3 and GITR. In particular, studies in mouse models of RA and SLE, as well as asthma, graft-versus-host disease and multiple sclerosis, have revealed that 4-1BB agonists are strongly suppressive, as they selectively expand both CD8⁺ T_{reg} cells that can inhibit effector CD4 T cells and/or CD4⁺ Foxp3⁺ T_{reg} cells^{5,125,223}. Similarly, stimulation of DR3, GITR or OX40 in some settings can expand T_{reg} cells, and in several mouse models results in suppression of asthma symptoms, allograft rejection, diabetes and multiple sclerosis-like disease^{224–230}. However, owing to the possibility of expanding pathogenic self-reactive T cells, agonist targeting might not be a first-line strategy; neutralization of these molecules is instead the logical choice for therapy. If clinical trials reveal contraindications for certain inhibitory reagents, drugs that stimulate TNFSF receptors might represent an alternative treatment option. Agonist antibodies to 4-1BB, OX40, CD27 and GITR are currently in clinical trials for the treatment of cancer to expand tumour-reactive T cells¹¹, and apart from some hepatotoxicity observed with anti-4-1BB at high doses, they have shown a relatively good safety profile, and could be tested in patients with rheumatic disease.

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Competing interests statement

M.C. has licensed patents on several TNF superfamily molecules. R.S. has issued patents on antibodies against the TNF superfamily molecule TL1A.

The emerging safety profile of JAK inhibitors in rheumatic disease

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Abstract | Tofacitinib is the first Janus kinase (JAK) inhibitor commercially approved for the treatment of rheumatoid arthritis. This compound and a number of other JAK inhibitors are currently being tested in phase II and III trials for the treatment of a variety of autoimmune inflammatory diseases. Whereas a characteristic safety profile is emerging for some JAK inhibitors, differences between individual agents might emerge on the basis of distinct potency against their molecular targets. Similarly to biological therapy, JAK inhibition can lead to serious and opportunistic infections, and viral infections seem to be particularly frequent. Although no malignancy signals have been identified to date, long-term follow-up and further research are needed to understand the risk of malignancy associated with these compounds. As is the case for biologic agents, vaccination is important to mitigate the risks of these emerging therapies.

Small-molecule therapies offer an important alternative to biological therapies for the treatment of inflammatory diseases. In the past 5 years, a number of small-molecule compounds targeting Janus kinases (JAKs) have been developed. Interest in these compounds initially stemmed from the observation that defects in JAKs caused severe immunosuppression in humans, and thus they could be targets for immunosuppressive therapy¹. This observation was coupled with the understanding that JAK-mediated signalling is involved in the pathogenesis of rheumatoid arthritis (RA), psoriasis, inflammatory bowel disease and other autoimmune conditions, and that inhibition of this pathway seems to be effective in these diseases². Currently, there are more JAK inhibitors in development than there are JAKs, and their differential ability with regard to JAK blockade could potentially distinguish their individual efficacy and safety profile. With one JAK inhibitor currently used in the clinic for the treatment of RA, and several others in phase III clinical trials, the safety of these compounds is just beginning to be understood. This Review provides a discussion of the current understanding of JAK inhibitor safety in the setting of inflammatory autoimmune disease, including an overview of changes in laboratory parameters, infection and malignancy risks associated with each compound.

Cytokine receptors and JAK signalling

Four JAKs exist in humans: JAK1, JAK2, JAK3 and non-receptor tyrosine-protein kinase TYK2. These kinases bind to type I and II cytokine receptors and transmit extracellular cytokine signals to activate various

signal transducers and activators of transcription (STATs), which drive the proinflammatory machinery of the cellular immune response. Various JAK complexes are known to mediate distinct cytokine signalling pathways. For example, the JAK1–JAK3 complex, which is essential for lymphocyte proliferation and homeostasis^{3–5}, is induced by interleukins such as IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, whereas IL-6 signalling is mediated by JAK1, JAK2 and TYK2. As a homodimer, JAK2 is essential in facilitating the signalling mediated by erythropoietin, granulocyte-macrophage colony stimulating factor and other factors essential to erythropoiesis, myelopoiesis and platelet production^{2,6}. Important signalling pathways in host defense include innate antiviral responses via type I interferon mediated by JAK1–TYK2 complexes, and IFN γ signalling mediated by JAK1–JAK2 complexes². Furthermore, these kinases also mediate interferon responses against non-viral pathogens such as *Mycobacterium tuberculosis*⁷. The immunomodulatory signals mediated by JAKs are summarized in FIG. 1.

Over the past decade, a number of JAK inhibitors have been developed, some of which have greater specificity for one or more JAKs than others. On the basis of the known functions of various JAKs and their interaction with cytokine receptors, it is tempting to speculate regarding the potential safety signals produced by JAK inhibitors according to their selectivity. To date, these correlations have proved challenging to establish given the overlap in activity of many of these compounds and the difficulty in understanding the functions of these kinase–receptor complexes. Despite these caveats, a characteristic safety profile, which includes infections and

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Key points

- Despite differences in selectivity between Janus kinase (JAK) inhibitors, a large overlap exists in their safety profiles
- All JAK inhibitors have been associated with a decrease in neutrophil number, although changes in numbers of lymphocytes and natural killer cells vary between compounds
- An increased risk of viral infections (particularly herpes zoster) seems to distinguish the safety profile of tofacitinib from that of biologic DMARDs
- Similarly to tofacitinib, other JAK inhibitors also seem to increase the risk of herpes zoster infection despite differences in JAK selectivity
- To date, no increased risk of malignancy has been reported with tofacitinib in rheumatoid arthritis; however, experience is limited and this risk must be evaluated in the long term with all JAK inhibitors
- The prevention of herpes zoster and other opportunistic infections is both feasible and important in the setting of JAK inhibition for the treatment of autoimmune inflammatory diseases

changes in laboratory parameters, seems to be emerging as data from various JAK inhibitor development programs accumulate.

JAK inhibitors approved and in development

Tofacitinib is approved for the treatment of RA in the USA as well as other countries including Australia, Japan, Russia, Argentina and Canada, but not yet in the European Union. Other JAK inhibitors in development, including compounds that will be soon assessed by regulatory agencies, are listed in TABLE 1. Most knowledge on the safety of JAK inhibitors comes from the relatively extensive experience with tofacitinib in the development programs for RA and other conditions, as well as from post-marketing reporting in countries where these drugs are approved.

Tofacitinib preferentially inhibits JAK1 and JAK3, but, like most JAK inhibitors, it also has activity against other JAKs (notably JAK2), albeit to a lesser extent^{8,9}. Studies have explored the inhibition of various JAKs by different compounds *in vitro*. Inhibition of JAKs seems to be dose-dependent and JAK inhibitors can have off-target activity, becoming 'pan-JAK' inhibitors at high doses^{8,9} (TABLE 2). These pharmacological studies are difficult to interpret and can produce conflicting results owing to differences in the methodology used for *in vitro* assays⁸.

Some of the changes in laboratory parameters associated with treatment with tofacitinib and other JAK inhibitors are similar to those observed with the use of biologic agents such as tocilizumab, and reflect the inhibition of IL-6. The changes include, for example, increased levels of liver transaminases and lipids¹⁰⁻¹². Data on many JAK inhibitors are still preliminary (and reported to date primarily as conference abstracts), and their relative specificities for different JAKs do not always explain the differences in laboratory parameters reported for each of these compounds (TABLE 3).

Tofacitinib. Changes in laboratory parameters, which are well characterized for tofacitinib, include an initial decrease in the number of lymphocytes, neutrophils,

natural killer (NK) cells and platelets, increased levels of liver transaminases and lipids (such as LDL cholesterol and HDL cholesterol); a small increase in serum creatinine level and a small reduction in creatinine clearance have also been observed¹³⁻¹⁶. From a clinical standpoint, these laboratory parameters were monitored during the development program, and only a small percentage of patients developed serious adverse events attributable to such changes. Among over 4,000 patients with RA in long-term extension studies, few patients developed severe declines in neutrophils (0% of patients with <500 cells per microlitre) or lymphocyte levels (1.3% of patients with <500 cells per microlitre), and these parameters reversed when tofacitinib treatment was stopped¹³. Furthermore, the percentage of patients developing grade 2 or 3 changes for neutrophils, lymphocytes and creatinine levels was similar in patients receiving 5 mg or 10 mg of tofacitinib twice a day¹³. The clinical effect of the observed decrease in NK cells remains unclear. In one study, baseline and nadir levels of NK cells were not associated with serious infections, herpes zoster infection or malignancy, although the analysis was limited to around 1,000 patients, and data were mainly collected in the first 6 months after therapy was started¹⁵. Whether a decrease in the number of NK cells predisposes some patients with RA to infection or malignancy needs to be further investigated. Lastly, although increases in creatine phosphokinase levels have also been observed, these changes have generally been graded as mild and have not been associated with rhabdomyolysis, renal failure or other serious adverse events¹³. Whereas the cause of the slight rise in creatinine levels in patients with RA treated with tofacitinib was initially unclear, further work has identified decreased creatinine clearance to be responsible. This phenomenon was examined in a small substudy of 148 patients with RA who were randomly allocated to receive either tofacitinib or placebo. The adjusted geometric mean measured glomerular filtration rate (GFR) decreased 8% from baseline over 6 weeks of treatment with tofacitinib 10 mg twice daily, although these changes were ameliorated to some degree after discontinuation of the drug, and no difference in measured GFR was observed between the placebo and tofacitinib groups at the end of the study¹⁶. To date, during the long-term use of tofacitinib within the RA development program, no increased risk of renal insufficiency or failure has been observed¹³.

Other JAK inhibitors. Treatment with baricitinib, which inhibits JAK1 and JAK2, has been found to lead to cellular and laboratory changes similar to those described above for tofacitinib treatment, but differences seem to exist in regard to lymphocyte and platelet counts, which decrease minimally or not at all, and levels of haemoglobin, for which a reduction was observed¹⁷⁻¹⁸. In phase III clinical trials, almost 1% of patients developed grade 3 reductions in lymphocyte levels within the first 24 weeks of baricitinib exposure¹⁷⁻²⁰. NK cell number transiently increased in the first 4 weeks after start of therapy, before decreasing below baseline levels afterwards. One phase III trial showed that a decrease in NK cell number occurred similarly with baricitinib and

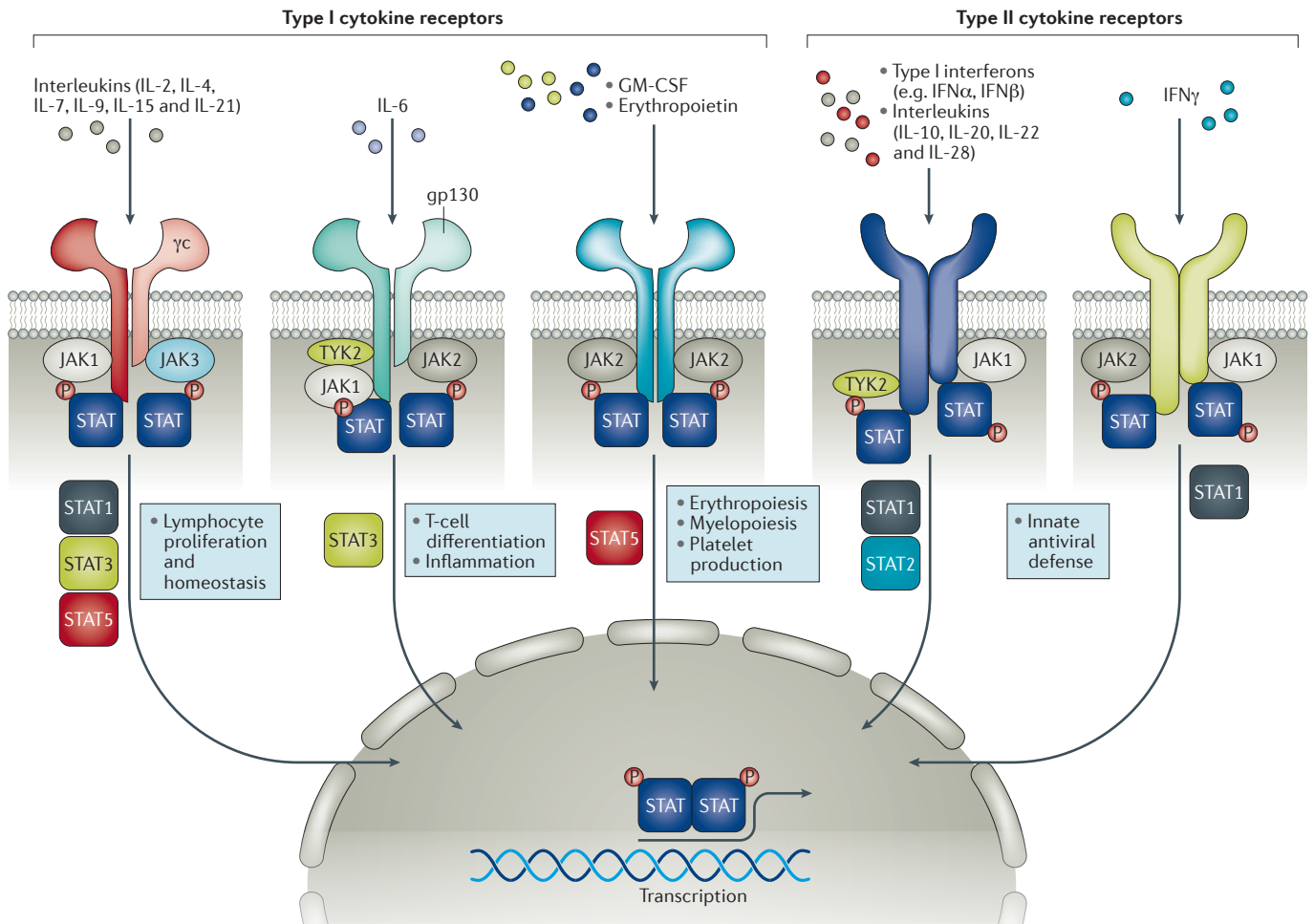


Figure 1 | Overview of JAK–STAT signalling in host defense and cellular homeostasis. Type I and II cytokines bind their receptors with subsequent intracellular signalling via the Janus kinase and signal transducer and activator of transcription (JAK–STAT) pathway. The cytoplasmic domain of cytokine receptors associates with various JAKs (JAK1, JAK 2, JAK 3 and non-receptor tyrosine-protein kinase 2 (TYK2)). These kinases act via autophosphorylation as well as STAT phosphorylation. Key host inflammatory responses are mediated through these interactions, including those that lead to autoimmune inflammatory diseases such as rheumatoid arthritis. Shown here are the signalling pathways of a select group of cytokines, as well as cytokine receptor dimerization and their association with JAKs. Of note, the figure depicts important interactions in the host defense against infection including the signalling of both type I interferons and interferon- γ (IFN γ). Type I interferons signal via type II cytokine receptors associated with JAK1 and TYK2, whereas IFN γ signals via type II cytokine receptors associated with JAK1 and JAK2. These signalling pathways are particularly important to host antiviral responses. Other signalling pathways mediated by JAK–STAT are important for cellular homeostasis, including lymphocyte production and erythropoiesis. GM-CSF, granulocyte-macrophage colony-stimulating factor.

placebo^{17,20}, but two other phase III studies reported that this decrease was more common in patients treated with baricitinib than in those treated with placebo in the first 24 weeks of therapy (19% versus 10% (REF. 19) and 22% versus 8% (REF. 20)). Patients with low levels of NK cells were not found to be at higher risk of infection (including herpes zoster), although these analyses were limited by the small number of patients included^{19,20}.

The differences in laboratory parameters observed between tofacitinib and baricitinib are incompletely explained by the differential activity of these drugs against JAK2 or JAK3. Changes in laboratory parameters were reported in a small number of patients with RA ($n=82$) treated with the JAK3-selective compound decernotinib²². Whereas levels of liver transaminases and

LDL cholesterol were shown to increase with decernotinib treatment (similarly to tofacitinib), no increase in HDL cholesterol levels were observed. A reduction in neutrophil levels was also observed, although lymphocytes and haemoglobin levels remained stable. Furthermore, two patients (4.9%) treated with decernotinib developed grade 3 lymphopenia²². It is difficult to compare these findings with those obtained with tofacitinib and baricitinib, as the experience with the latter compounds is much more robust. Whereas decernotinib has some activity against the JAK2 axis, and presumably against other JAKs depending on the dose, the similarities and differences between this JAK3 inhibitor and the two aforementioned compounds are difficult to explain owing to their different selectivity.

Table 1 | JAK inhibitors approved and in development

Compound	Target	Indication	Stage of development
ABT-494	JAK1	RA	Phase I
		Crohn's disease	Phase II
		Ulcerative colitis	Phase II
		Atopic dermatitis	Phase I
Baricitinib	JAK1, JAK2	RA	Phase III
		Psoriasis	Phase II
		Diabetic nephropathy	Phase II
		SLE	Phase II
		Atopic dermatitis	Phase II
Decernotinib	JAK3	RA	Phase II (development currently on hold)
CYT387	JAK1, JAK2	Myelofibrosis	Phase I-II
Filgotinib	JAK1	RA	Phase III
		Crohn's disease	Phase III
		Ulcerative colitis	Phase III
INCB018424	JAK1, JAK2	Psoriasis (topical treatment)	Phase II
Pacritinib	JAK2	Myelofibrosis	Phase II
Peficitinib	JAK1, JAK3	RA	Phase III
		Psoriasis	Phase II
Ruxolitinib	JAK1, JAK2	Myelofibrosis	Approved by FDA
Tofacitinib	JAK1, JAK3	RA	Approved by FDA
		Psoriasis	Phase III
		Ulcerative colitis	Phase III ongoing
		JIA	Phase I

JAK, Janus kinase; JIA, juvenile idiopathic arthritis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

Peficitinib also selectively inhibits JAK3. In a phase II study involving Japanese patients with RA, treatment with this drug resulted in decreased numbers of platelets and neutrophils, and a small increase in the levels of creatine phosphokinase, liver transaminases, lipids and creatinine over a 12-week period, although no consistent trends regarding changes in lymphocyte number were observed between peficitinib dose groups²³. Very limited data are available for the JAK1-selective compounds ABT-494 and filgotinib. In two phase II clinical trials investigating ABT-494 in patients with RA, laboratory changes were shown to be very similar to those observed with tofacitinib. Indeed, levels of liver transaminases, creatine phosphokinase, creatinine, LDL cholesterol and HDL cholesterol slightly increased, whereas the numbers of NK cells, lymphocytes and neutrophils decreased. Of note, these trials involved relatively small numbers of patients (220 and 249 patients), and treatment duration was only 12 weeks^{24,25}. In phase II studies involving approximately 900 patients with RA^{26,27}, treatment with filgotinib slightly increased creatinine levels and reduced the levels of neutrophils and platelets; however, no changes in the levels of lymphocytes and NK cells or LFT values were observed. Interestingly, HDL cholesterol levels rose to a greater extent than LDL cholesterol levels, therefore increasing the HDL:LDL ratio^{26,27}. By contrast, the HDL:LDL ratio observed with other JAK inhibitors remained stable.

Taken together, the findings described above suggest that various JAK inhibitors are associated with slightly different cellular and laboratory changes over time. However, their individual profiles with regard to JAK selectivity do not necessarily allow for the easy prediction of these differences. One factor that complicates the comparison between JAK inhibitors is the existence of different dose groups in phase II studies, as higher doses in some cases could diminish a compound's selectivity. For those compounds in the early stages of development, more long-term data are needed to further understand their safety profile.

Adverse effects of JAK inhibitors

The adverse events associated with use of JAK inhibitors are best known for tofacitinib. Approximately 15,000 person-years of exposure from long-term extension studies has been reported, and preliminary real-world data (that is, post-marketing reports) are now available. For other compounds with differential JAK activity, it remains to be seen if their mechanisms of action result in distinct adverse effects. For some adverse events such as malignancy, many more years of exposure and time are needed to characterize the risk associated with these compounds. For other events, such as serious infections, reasonable risk estimates exist that derive from trials investigating tofacitinib and

Table 2 | Comparison of enzymatic and whole-cell activity for selected JAK inhibitors

Compound	Enzyme assay IC ₅₀ (nM)*				Human whole blood IC ₅₀ (nM)					
	JAK1	JAK2	JAK3	TYK2	IL-15 [†] pSTAT5	IL-6 [§] pSTAT1	IL-12 pSTAT4	IFNα [¶] pSTAT3	IL-23 pSTAT3	CD34 ⁺ cells [‡] EPO ^{**} pSTAT5
Baricitinib	4.0	6.6	787.0	61.0	259	21.1	149	28.7	81.9	87.8
Decernotinib	112	619	74.4	>10,000	932	1,870	16,400	1,290	11,200	>20,000
Filgotinib	363	2,400	>10,000	2,600	2,140	918	13,362	1,500	10,123	13,200
Ruxolitinib	6.4	8.8	487.0	30.1	1,850	298	1,090	194	818	677
Tofacitinib	15.1	77.4	55.0	489	55.8	75.4	409	35.0	229	302

EPO, erythropoietin; IC₅₀, half maximal inhibitory concentration; IFNα, interferon-α; JAK, Janus kinase; pSTAT, phosphorylated signal transducer and activator of transcription; TYK, non-receptor tyrosine-protein kinase. *Run in the presence of 1 mM ATP. [†]IL-15 signals through JAK1–JAK3. [§]IL-6 signals through JAK1–JAK2 or TYK2. ^{||}IL-12 and IL-23 signal through JAK2–TYK2. [¶]IFNα signals through JAK1–TYK2. [‡]CD34⁺ cells spiked into human whole blood. ^{**}EPO signals through JAK2–JAK2. Adapted with permission from Clark, J. D., Flanagan, M. E. & Telliez, J. B. Discovery and development of Janus kinase (JAK) inhibitors for inflammatory diseases. *J. Med. Chem.* 57, 5023–5038 (2014). Copyright 2013 American Chemical Society.

baricitinib. Real-world data collected over the course of 5–10 years will advance our understanding of JAK inhibitor safety.

Malignancy. Similarly to TNF blockers and other biologic agents before they were approved for clinical use, JAK inhibitors have been hypothesized to promote malignancy. Exhaustive population-based studies suggest that TNF blockers do not increase the risk of solid or lymphoproliferative malignancy in patients with RA²⁸. ‘Cancer immunoediting’, the process whereby the human immune system destroys cancer cells within the body, is thought to rely upon a variety of cytokines (for example, IFNγ) and cell types (such as NK cells) that could be affected by JAK inhibition²⁹. Limited long-term data exist on the malignancy risk associated with use of JAK inhibitors. However, to date, the risk of cancer in patients with RA treated with tofacitinib seems to be similar to that observed with biological therapies³⁰.

In a 2015 study, among more than 5,600 patients with over 12,000 patient-years of exposure to tofacitinib, 107 patients were found to develop malignancy (excluding non-melanomatous skin cancer (NMSC)). The most common malignancy was lung cancer (*n* = 24), followed by breast cancer (*n* = 19) and lymphoma (*n* = 10). Overall, the rate of malignancy in these patients was 0.85 per 100 patient-years. The investigators also evaluated incidence rates within open-label extension data in 6-month intervals after the start of tofacitinib treatment. The 6-month interval rates were 0.66–1.04 per 100 patient-years, and remained stable over time. Standardized incidence ratios (SIRs) were calculated for tofacitinib-treated patients using information about malignancies in the USA general population in the Surveillance, Epidemiology and End Results (SEER) database. Both the overall SIR for malignancy and the SIRs for individual malignancy types were similar to those reported previously in RA³⁰. Furthermore, data from long-term extension studies, which included nearly 15,000 person-years of exposure, showed a malignancy rate (excluding NMSC) of 1.0 (0.8–1.1) per 100 patient-years¹⁴. To date, the malignancy rates associated with the use of tofacitinib seem to be similar to those reported in long-term extension trials investigating biologic agents

in patients with RA^{31–37}. Although the current picture is reassuring, long-term experience is limited with this molecule. The comprehensive evaluation of cancer risk associated with anti-TNF therapies took more than a decade. Therefore, long-term follow-up studies are necessary to assess whether tofacitinib is associated with an increased risk of cancer.

Among 3,400 patients with RA treated with baricitinib, the reported malignancy rate (excluding NMSC) was 0.720 per 100 patient-years¹⁸. For other JAK inhibitors currently in development, little long-term data is available and only a small number of malignancies has been reported^{17,38}.

Infections. In general, the emerging safety profile of JAK inhibitors seems to be similar to that of TNF blockers and other biologic agents, but there are several notable exceptions. The incidence rate of serious infection events (SIEs) in trials investigating the use of tofacitinib in patients with RA was similar to that in long-term extension studies and randomized controlled trials (RCTs) evaluating TNF blockers and other biologic agents³⁹.

A crude SIE incidence rate of 3.1 per 100 patient-years has been reported in RCTs and long-term extension studies investigating tofacitinib³⁹. The most frequent SIEs resulting from tofacitinib treatment are those that occur in patients with RA, including those treated with biologics. These SIEs include community-acquired pneumonia, urinary tract infections and skin or soft-tissue infections^{39,40}. In phase I–III trials and long-term extension studies of baricitinib, a similar incidence of SIEs (3.20 per 100 person-years) was reported among 4,229 treated patients¹⁸.

Whereas most infections associated with JAK inhibition are thought to be bacterial and their risk seems to be similar to that associated with biological therapy, a very different risk profile has emerged in regard to viral infections. Perhaps the most recognized infectious complication to date has been the reactivation of varicella zoster virus (VZV; that is, herpes zoster). Whereas VZV exposure is nearly ubiquitous and the lifetime risk of herpes zoster is approximately one in three, the risk of herpes zoster is strongly correlated to a decline in cell-mediated immunity⁴¹. Accordingly, age, RA, use of prednisone and

Table 3 | Mean changes in laboratory parameters associated with individual JAK inhibitors

	Tofacitinib	Peficitinib	Baricitinib	Decernotinib	Filgotinib	ABT-494
Selectivity	JAK1, JAK3	JAK1, JAK3	JAK1, JAK2	JAK3	JAK1	JAK1
Lymphocyte number	↓	No change	No change	↓	No change	↓
NK cell number	↓	NA	↓*	NA	No change	↓
Neutrophil number	↓	↓	↓	↓	↓	↓
Haemoglobin level	↑	↑	↓	No change	↑	↓
Platelet count	↓	↓	No change	NA	↓	NA
Liver transaminase level	↑	NA	↑	↑	No change	↑
Creatine phosphokinase level	↑	↑	↑	NA	NA	↑
HDL level	↑	↑	↑	No change	↑	↑
LDL level	↑	↑	↑	↑	No change	↑
Creatinine level	↑	↑	↑	↑	↑	↑

Shown are general trends reported in the development program of each compound. The magnitude of change varies by compound, and within each compound by dose. In some cases, changes were seen at only certain doses. Notably, grade changes (for example, grade 3) for laboratory parameters can occur in the opposite direction of mean trends for a given parameter. For some drugs (for example, decernotinib and peficitinib), the number of patients receiving the drug is limited and the estimations of laboratory change are less robust. JAK, Janus kinase; NK cell, natural killer cell; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NA, not available. *Initial rise followed by a decrease.

other factors associated with decreased cell-mediated immunity influence the risk of herpes zoster⁴². Whereas the risk of herpes zoster is approximately 1.5–2-fold higher in patients with RA than in the general population, certain therapies for RA seem to further increase this risk, most notably tofacitinib therapy^{43–45}. Incidence rates of herpes zoster infection reported in the tofacitinib development program for RA (4.4 per 100 patient-years) were 1.5–2-fold higher than those usually observed in patients with RA⁴⁶. Despite this increased risk, very few cases of multidermatomal or disseminated herpes zoster were reported, and no cases resulted in visceral disease or death. Interestingly, the rates of herpes zoster infection varied substantially by region, with the highest rates reported from certain regions of Asia. In Japan and Korea, incidence rates were 9.2 per 100 patient-years, nearly 2–3-fold higher than those observed in North America or Europe⁴⁶. This disparity suggests that genetic factors, differences in diagnosis or case-ascertainment or other factors might explain the differential risk observed between regions. A 2015 analysis revealed other important factors associated with herpes zoster risk among patients with RA treated with tofacitinib. Interestingly, the concomitant use of steroids or methotrexate considerably influenced the risk of herpes zoster infection in patients treated with tofacitinib, and patients treated with tofacitinib alone were at substantially lower risk than those treated with concomitant methotrexate or prednisone⁴⁷. In this study, patients received <10 mg per day of prednisone, and the risk modification by this drug is likely to be dose-dependent. Furthermore, herpes zoster risk with tofacitinib use is also dose-dependent, with higher risk observed at a dose of 10 mg twice a day⁴⁶. Lastly, a population-based study from the post-marketing period evaluated the risk of

herpes zoster with tofacitinib as compared with biologic agents (abatacept, rituximab, TNF blockers and tocilizumab) in patients with RA⁴⁸. This ‘real-world’ analysis showed a risk of herpes zoster with tofacitinib twofold higher than that associated with biological therapies.

Other viral opportunistic infections caused by tofacitinib treatment have also been reported. Latent viruses that are generally present in humans include cytomegalovirus (CMV), Epstein–Barr virus (EBV) and John Cunningham (JC) virus. To date, a small number of CMV infections have been reported from long-term extension trials in patients treated with tofacitinib, including at least one case of CMV retinitis⁴⁹. Reactivation of JC virus (that is, progressive multifocal leukoencephalopathy (PML)) has not been reported, nor have cases of EBV infection.

Currently, the ‘herpes zoster signal’ seems to be a ‘class effect’ as most JAK inhibitors show an elevated risk. With baricitinib, the incidence rate of herpes zoster is similar to that with tofacitinib. A study published in 2016 showed that herpes zoster incidence rates in patients treated with 4 mg of baricitinib daily were 4.3 per 100 patient-years within the first 24 weeks, whereas in patients treated with placebo incidence rates were 1.0 per 100 patient-years¹⁸. When considering the cumulative experience of phase I–III trials and long-term extension studies with baricitinib (4,421 patient-years of exposure), the reported herpes zoster incidence was 3.4 cases per 100 person-years, and none of these cases involved dissemination or death^{17,18}. Decernotinib was also reported to increase the risk of herpes zoster^{50,51}, whereas few data on the risk or incidence rates of infection are available for filgotinib. In phase II studies, six cases of herpes zoster were reported in patients with RA receiving filgotinib, whereas one case of herpes zoster

was reported among patients with RA receiving placebo. In a phase II study, four cases of herpes zoster infection were found in 225 Japanese patients with RA treated with peficitinib over just 12 weeks, an incidence of 6.3 cases per 100 person-years²³. In a phase II study investigating the effect of ABT-494 in 469 patients with RA, six cases of herpes zoster infection were found during 16 weeks of follow-up (12 weeks of exposure). Whereas incidence was not reported in this study, a 'back of the envelope' calculation suggests an incidence rate of approximately 5% per year, similar to that reported in tofacitinib or baricitinib trials. Of note, the ABT-494 study did not enroll within Asia, where higher rates of herpes zoster were noted with other JAK inhibitors^{24,25}.

Outside the rheumatology field, ruxolitinib, a JAK inhibitor that primarily inhibits JAK1 and JAK2 and is used in the treatment of myelofibrosis, has also been reported to induce high rates of herpes zoster infection in Asian patients^{52,53}. Although the exact mechanism by which VZV reactivation occurs in the context of JAK inhibition is unclear, the downregulation of innate antiviral signalling through type I and II interferons is likely to be involved. In agreement with this hypothesis, in patients with SLE treated with the anti-IFN α monoclonal antibody sifalimumab for 52 weeks, herpes zoster cases were more common than in those treated with placebo (5.9% versus 0.9%, respectively)⁵⁴.

Whereas most opportunistic infections can sometimes occur in normal hosts, although less frequently or with less severity⁵⁵, PML is one of few infections that does not occur outside the setting of immunosuppression. To date, PML has been reported very rarely among patients treated with rituximab or other biologic drugs; nearly all such cases have occurred in patients with other risk factors for PML (such as cancer and lymphopenia). Given the apparent 'viral signal' observed with tofacitinib, it is notable that no PML cases have been reported. However, many thousands more patient-years of exposure would be required to identify such a case. One case of PML has been reported in a patient with myelofibrosis treated with ruxolitinib, a JAK2 inhibitor⁵⁶. Although it is not clear whether the drug caused PML, this finding raises potential concern given that antiviral responses signal through such pathways, and hosts carrying JAK mutations and consequent STAT1 deficiency can develop lethal viral and other types of infections⁵⁷⁻⁵⁹.

Non-viral opportunistic infections. Tuberculosis cases have been reported with tofacitinib, but no direct comparison is available between the risk associated with the use of tofacitinib and that of TNF blockers or other biologic agents. An increased risk of tuberculosis seems to be present with a dose of 10 mg tofacitinib twice daily compared with a dose of 5 mg twice daily, but, as with TNF blockers, the risk of tuberculosis is largely dependent on the background prevalence in the region where the drug is being used⁴⁹. Within Western Europe and North America, tuberculosis incidence within the RA development program was several folds greater than that in the general population, a trend similar to that seen with TNF blockers⁴⁹. Importantly, the tofacitinib

development program screened for tuberculosis before trial entry and allowed patients to enter into phase III trials if they started and complied with a 9-month isoniazid treatment. None of these patients developed active tuberculosis during the trial, and few patients showed elevation of liver transaminase levels despite concomitant use of isoniazid and tofacitinib⁴⁹. It should be noted that rifampin and tofacitinib interact, and therefore rifampin should not be used for the treatment of latent tuberculosis during tofacitinib therapy⁶⁰.

In the tofacitinib development program for RA, the incidence of opportunistic infections other than tuberculosis was 0.25 (0.18–0.36) per 100 patient-years. These infections included esophageal candidiasis ($n=9$), *pneumocystis jirovecii* pneumonia ($n=4$), CMV ($n=6$), pulmonary nontuberculous mycobacteria ($n=2$), *Cryptococcus* (pneumonia, $n=2$; meningitis, $n=1$), BK virus ($n=1$) and toxoplasmosis ($n=1$). Of note, nine cases of multidermatomal herpes zoster were included in this incidence rate⁴⁹. In post-marketing reports for tofacitinib, at least one case of histoplasmosis has been reported in the USA.

Very little information is available for other JAK inhibitors in development. Tuberculosis in patients treated with baricitinib and decernotinib has been reported^{17,18}. With baricitinib, all cases ($n=7$) occurred in countries with higher background prevalence of tuberculosis, with incidence generally 5–10-fold higher than that in the general population. No cases of tuberculosis, however, were reported in Europe, Japan or North America.

No serious cases of fungal or CMV infections with baricitinib therapy have been reported to date⁶¹. Safety data for this compound come from just over 4,000 person-years of exposure and are relatively limited compared with those for tofacitinib⁶², which draw on over 12,000 person-years of exposure. Only short-term data are available from phase II studies investigating ABT-494 and filgotinib in a relatively small number of patients with RA²⁴⁻²⁷. One case of oral candidiasis was reported with ABT-494 treatment and no cases of opportunistic infections with filgotinib use^{24,26,27}.

Gastrointestinal perforation. In a 2016 analysis of health plan data in the USA, an incidence of gastrointestinal perforation of 1.29 per 1,000 patient-years was found in patients with RA treated with tofacitinib⁶³. This rate was similar to that observed with tocilizumab in the same study, but the number of cases and exposure time for tofacitinib was limited, and the elevated relative risk was not significantly different compared with that of other biologics. In patients with RA receiving baricitinib, two cases of gastrointestinal perforations were reported (an incidence of 5 cases per 1,000 patient-years in the development program)¹⁸. Currently, no cases of gastrointestinal perforation have been reported in other development programs of JAK inhibitors in RA.

Pregnancy. Little information exists regarding the effect of JAK inhibition on pregnancy. Pregnant patients were excluded from trials investigating JAK inhibitors, and only a small number of patients treated with tofacitinib

became pregnant while receiving the drug⁶⁴. In all these cases, the drug treatment was stopped when the pregnancy was found. Among 31 pregnancies, 16 were carried to term, and only one had some malformations (pulmonary stenosis). Of the remaining patients, seven had spontaneous abortions whereas the others were lost to follow-up or had elective abortions⁶⁴.

Prevention of herpes zoster reactivation

To date, albeit with limited experience, the safety profile of tofacitinib and other JAK inhibitors seems to be similar to that of biologic agents, with the exception of viral diseases such as those caused by herpes zoster. Notably, herpes zoster is a vaccine-preventable disease, and the recognition that it disproportionately affects patients with RA has prompted efforts to improve vaccination and prevention among these patients.

Prevention of herpes zoster with use of Zostavax (Merck, USA), a live vaccine, can be considered in patients over 50 years of age, particularly in those with inflammatory autoimmune disease. In 2015, the ACR updated its recommendations to vaccinate all patients with RA aged ≥ 50 years where not contraindicated⁶⁵. The opportunity to prevent herpes zoster, however, is limited by the fact that many patients are also using biological therapies, and the attenuated live viral vaccine is contraindicated with the concomitant use of these agents. To date, this contraindication also extends to tofacitinib and should for other JAK inhibitors⁶⁰. It is unclear if this contraindication is relevant for TNF blockers or other biologic agents as it is based on expert opinion. Some observational data suggest that it might be safe to vaccinate patients who are being treated with TNF blockers⁶⁶, and a current clinical trial is addressing this question⁶⁷; nonetheless, the herpes zoster vaccine should remain contraindicated until conclusive data prove its safety in the biological setting.

Given the contraindications discussed above, the timing of vaccination should be before treatment with a biologic agent or JAK inhibitor. Patients should stop such immunosuppressive treatment for at least 1 month before vaccination and for 2–4 weeks afterwards^{68,69}, although the exact interval needed between vaccination and the resumption of immunosuppressive therapy is unknown. Studies have shown that patients have asymptomatic dissemination of herpes zoster after vaccination, and viral DNA is present in the saliva of a small percentage of individuals for up to 4 weeks after vaccination⁷⁰.

The first study investigating herpes zoster vaccination in RA involved patients treated with methotrexate who were randomly allocated to receive treatment with either placebo or tofacitinib 5 mg twice daily 2–3 weeks after vaccination. Vaccine immunogenicity was similar in the two

groups, although one patient developed dissemination of the vaccine 2 days after starting tofacitinib treatment⁷¹. Importantly, this patient was the only one in the study who lacked pre-existing VZV immunity. Checking VZV serology before vaccination, or simply waiting longer after vaccination (for example, 4 weeks instead of 2 weeks) before tofacitinib start would probably have prevented this occurrence. This study suggested, however, that patients with RA can respond adequately to vaccination and that subsequent tofacitinib treatment did not adversely affect immunogenicity.

Zostavax reduces the risk of herpes zoster and associated complications by two-thirds in the general population⁷². Theoretically, the protective effect of this vaccination might be reduced in patients with rheumatic disease, given their underlying immunosuppression. However, one observational study suggests that this protective effect is similar to that observed in the pivotal herpes zoster vaccination studies conducted in non-immunosuppressed individuals⁶⁶. The protective effect of herpes zoster vaccination has been shown to last more than 5 years on average, but to wane over time⁷³.

Other vaccines currently in development might prove useful to rheumatologists. For example, a trial published in 2015 showed that, during a 3-year follow-up, two doses of a nonlive, adjuvanted VZV subunit vaccine was effective in $>95\%$ of patients who were not immunosuppressed⁷⁴. Whether the efficacy of this vaccine would be as high in immunosuppressed populations, or whether disease flares could be caused by the adjuvant used in this vaccine, remains to be seen.

Conclusions

JAK inhibition offers a new therapeutic strategy for rheumatologists. Whereas the safety profile of JAK inhibitors to date is similar to that of biologic agents, specific differences exist with regard to cellular changes and to the risk of certain types of infections, most notably viral diseases such as herpes zoster. Apart from tofacitinib, safety data on JAK inhibitors are limited, but herpes zoster risk seems to be increased by all or most of these compounds despite their differential selectivity. Although no malignancy signals have been found to date, further studies and time are needed to evaluate this issue. For now, JAK inhibitors remain a promising class of oral therapeutics for which many adverse events, like those associated with use of biologic agents, are preventable through screening, vaccination or laboratory monitoring. As TNF blockers enabled the infectious disease community to advance understanding of the human immune response against tuberculosis, JAK inhibitors might very well do the same for another common latent pathogen, VZV.

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Haematopoietic stem cell transplantation for autoimmune diseases

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Abstract | Autologous haematopoietic stem cell transplantation (HSCT) is the only treatment that is able to induce long-term, drug-free and symptom-free remission in several refractory autoimmune rheumatic diseases. Over 3,000 HSCT procedures for rheumatic and nonrheumatic severe autoimmune diseases have been performed worldwide. Specific conditioning regimens are currently used to eradicate the autoreactive immunological memory of patients. Although *in vivo* immune cell depletion with antithymocyte globulin or anti-CD52 is the norm for many regimens, *ex vivo* selection of CD34⁺ stem cells from the graft is controversial. Following the extensive immune depletion associated with serotherapy and chemotherapy, HSCT effectively resets the immune system by renewing the CD4⁺ T cell compartment, especially the regulatory T cell population. The risk of transplant-related mortality (TRM) within the first 100 days should be weighed against the risk of disease-related mortality, and the careful selection and screening of patients before transplantation is essential. Systemic sclerosis is the first autoimmune disease for which HSCT has been shown, in a randomized, controlled trial, to be associated with increased TRM in the first year but a significant long-term, event-free survival benefit afterwards. In this Review, we discuss the immunological mechanisms of HSCT in various autoimmune diseases and current HSCT regimens. After carefully taking into consideration the risks and benefits of HSCT and alternative therapies, we also discuss the efficacy, complications and proposed indications of this procedure.

All procedures that involve the partial or total replacement of the haematopoietic system of a recipient with haematopoietic stem cells from any donor type or source (bone marrow, peripheral blood or cord blood) are defined as haematopoietic stem cell transplantation (HSCT)¹. The aim of HSCT in autoimmune disease is the eradication of autoreactive immune cells and the regeneration of a naive, self-tolerant immune system². Clinical remission in autoimmune disease after HSCT is the result of a true reconfiguration of the immune system instead of long-term immunosuppression.

A 1989 study showed that supralethal total-body irradiation of rats, followed by infusion of histocompatible allogeneic bone marrow from rats resistant to arthritis improved adjuvant-induced arthritis³. Furthermore, transplantation of autologous bone marrow in rats was as effective as allogeneic bone marrow transplantation from a rat strain that was not susceptible to adjuvant-induced arthritis⁴.

Case reports documented that HSCT resolved autoimmune disease in patients who had coincident haematological disease^{5–10}, and in 1994, an article reviewed results from seven patients with rheumatoid arthritis (RA) complicated by iatrogenic severe aplastic anaemia who underwent allogeneic bone marrow transplantation from HLA-matched siblings¹¹. Although three patients died as a result of the transplant, RA resolved in all seven patients¹¹. The first treatment with autologous HSCT in a patient with rheumatic autoimmune disease was described in 1996 (REF. 12). This patient, who had connective tissue disease and severe pulmonary hypertension, was denied lung transplantation, but benefited from HSCT. Following these studies, further autologous procedures were performed, many under the framework of the European Society for Blood and Marrow Transplantation (EBMT)/EULAR Autoimmune Disease Stem Cell Project¹³. The EBMT registry now comprises over 1,800 HSCT procedures performed to treat several

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Key points

- Haematopoietic stem cell transplantation (HSCT) requires a careful selection of patients according to autoimmune disease, and a consideration of therapeutic alternatives, risks and benefits, and the expertise of the transplantation team
- The need for graft manipulation before HSCT is uncertain
- Individualized conditioning regimens might provide increased long-term remission rates, and stem cell rescue could minimize the duration of neutropenia and improve the containment of viruses
- HSCT resets the immune system by renewing the CD4⁺ T cell compartment, especially within the T_{reg} cell population, and by restoring T cell receptor diversity and function
- In patients with systemic sclerosis, HSCT results in increased mortality within the first year but a considerable long-term, event-free survival benefit afterwards

types of severe autoimmune disease, including systemic sclerosis (SSc), RA, juvenile idiopathic arthritis (JIA), systemic lupus erythematosus (SLE) and Sjögren syndrome¹⁴.

In this Review, we describe the sequential steps of current HSCT regimens, such as collection and mobilization of stem cells and conditioning and selection of CD34⁺ stem cells (FIG. 1). We also discuss what is known about the immunological mechanisms of HSCT in various autoimmune diseases, including the role of lymphopenia and thymopoiesis in immune cell reconstitution, the role of regulatory T (T_{reg}) cells and T cell receptor (TCR) heterogeneity. We will then address unmet clinical needs in autoimmune disease, the evidence for therapeutic HSCT in such diseases and its adverse effects. Taking into consideration the risks and benefits of HSCT and alternative therapies, we will finally discuss the proposed indications for this procedure in rheumatic diseases.

Steps of autologous HSCT

Specific recommendations exist for each sequential step of HSCT in children and adults, including patient selection, chemotherapy-based mobilization, stem cell collection, conditioning regimens, stem cell infusion, supportive care during reconstitution of neutrophils and lymphocytes and post-transplant care (FIG. 1). Toxicity and risk of transplant-related death of HSCT varies according to the type of autoimmune disease being treated, the donor cells used and the intensity of conditioning regimens¹⁵. In all cases, safer but equally effective alternative treatments including biological therapies should first be considered¹⁵. If possible, the patient should be included in prospective clinical studies, ideally randomized controlled trials (RCTs), or otherwise

monitored in prospective non-interventional studies in a centre accredited by HSCT programmes¹⁵. However, it can be difficult to enrol patients into clinical trials owing to a lack of financial resources for non-industry-sponsored trials. Patients need to be carefully selected and extensively screened before transplantation to assess whether the potential benefit of transplantation outweighs its risks, as well as to be sure that the patient's condition is optimal for transplantation. An example of such a screening performed in the Netherlands in adult patients with SSc is outlined in BOX 1 (REF. 16). Collecting data on patient characteristics, HSCT regimen used and outcomes, as well as supporting data analyses from the respective working parties and institutions, is essential not only to monitor local clinical practices, but also to assess clinical outcomes for HSCT in autoimmune disease in general (the quality platform established by the Joint Accreditation Committee-International Society for Cellular Therapy (ISCT) & EBMT (JACIE) requires yearly outcome reports).

Autologous haematopoietic stem cells can be derived from peripheral blood or bone marrow. According to the EBMT recommendations¹⁵, cytokine-mobilized peripheral blood progenitor cells are the preferred choice for autologous HSCT because they enable a larger harvest of CD34⁺ stem cells and better engraftment than bone-marrow-derived stem cells, resulting in a more rapid reconstitution of the haematopoietic system¹.

Mobilization and collection of stem cells. When stem cells are mobilized for collection, a patient's disease should be closely monitored to prevent flares, a potential consequence of the necessary administration of granulocyte colony stimulating factor (G-CSF)¹⁵. The mobilization regimen recommended by the EBMT is cyclophosphamide (2–4 g/m²) plus uromitexan and careful hyperhydration, followed by administration of G-CSF (5–10 µg/kg)¹⁵. Of note, in a retrospective analysis the administration of cyclophosphamide at 2 × 2 g/m² (n = 16) and 1 × 2 g/m² (n = 17) were found to be equally effective for peripheral blood stem cell mobilization in patients with refractory autoimmune disease¹⁷. Duration of leukopenia and G-CSF treatment might be reduced by administering lower doses of cyclophosphamide (1 × 2 g/m²). This reduction is important because G-CSF is known to be toxic in patients with autoimmune disease¹⁸. If mobilization with G-CSF-based regimens is insufficient, an adequate harvest of stem cells can still be obtained by inhibiting the binding of CXC-chemokine receptor 4 (CXCR4) to stromal derived factor 1α¹⁹. Combined treatment with plerixafor (a CXCR4 antagonist) and G-CSF has been successfully used for autologous stem cell mobilization in patients with multiple myeloma or malignant lymphoma²⁰, but not yet in patients with autoimmune disease. However, this treatment might be associated with a lower number of CD34⁺ cells and should not be considered outside of clinical trials²¹.

After mobilization, peripheral blood stem cells are collected via leukapheresis; the target amount for infusion is 3–5 × 10⁶ CD34⁺ cells per kg. Therefore, 10 × 10⁶ CD34⁺ cells per kg should be collected to compensate

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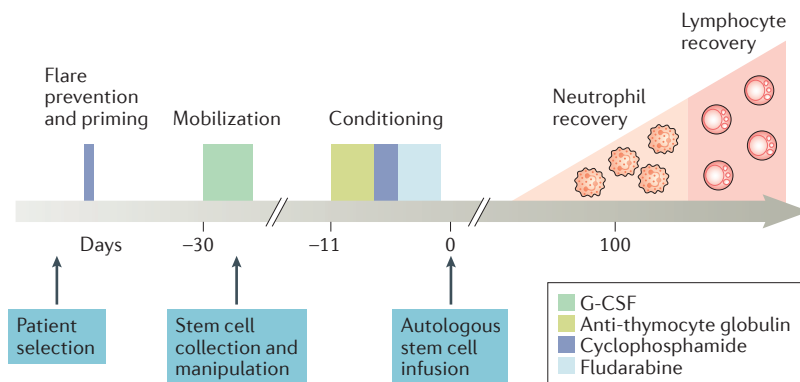


Figure 1 | Haematopoietic stem cell transplantation in patients with severe autoimmune disease. The schematic illustrates the timeline of haematopoietic stem cell transplantation (HSCT) according to the guidelines of the European Society for Blood and Marrow Transplantation¹⁵. Stem cells are mobilized by treatment with cyclophosphamide and granulocyte colony stimulating factor (G-CSF). Cyclophosphamide is also administered to prevent a possible flare of autoimmune disease caused by G-CSF. Stem cell collection is performed 4 or 5 days after G-CSF administration and approximately 4 or 5 weeks before autologous stem cell infusion. The patient is then discharged and readmitted after 1 or 2 weeks for immunoablative (intermediate intensity) conditioning, which consists of treatment with anti-thymocyte globulin, cyclophosphamide and fludarabine^{1,15}. In adult patients, cyclophosphamide dosage in the conditioning regimen is usually higher than that used in children, but fludarabine is not administered. The conditioning is followed by infusion of autologous (CD34⁺) stem cells, and the patient is discharged from hospital as soon as their neutrophil numbers have recovered, which generally occurs within 1–3 weeks after stem cell infusion. Most patients are severely lymphopenic for several months after HSCT while their immune system fully reconstitutes.

for the 50% loss of cells that results from selection and thawing procedures²². Irrespective of any graft manipulation, the EBMT recommends the reinfusion of a minimum dose of 2×10^6 CD34⁺ cells^{1,21}.

Conditioning. The conditioning regimens used in HSCT vary considerably and a consensus has not yet been reached. In autologous HSCT for patients with autoimmune disease, the conditioning regimens are immunoablative rather than myeloablative, and function to eliminate autoreactive T cells from the host as well as to deplete T cells (which are still present at the time of stem cell infusion) from the autologous graft with sufficient concentrations of antithymocyte globulin (ATG) or anti-CD52 (Campath-1H; Genzyme). The conditioning regimens can be classified as high-intensity, such as those including total-body irradiation or high-dose busulfan; low-intensity, such as those based on the use of cyclophosphamide, melphalan or fludarabine; or intermediate-intensity, which in most patients with autoimmune disease consist of the combination of ATG with either high-dose cyclophosphamide or other chemotherapeutic drugs¹⁵. Although ATG is a polyclonal antibody targeting T cells, it can also induce complement-independent apoptosis of naive, activated B cells and bone-marrow-resident plasma cells at clinically relevant concentrations²³. Long-lived plasma cells are known to support chronic inflammatory processes by the continuous secretion of pathogenic antibodies,

and can contribute to flares in autoimmune disease²⁴. Furthermore, long-lived plasma cells are not sufficiently eliminated by current therapies²⁴. However, in immunobliterated patients with SLE, not only autoantibodies but also protective serum antibodies against diphtheria, measles, mumps and tetanus are eliminated²⁵.

Among the many conditioning regimens reported, the Autoimmune Disease Working Party from the EBMT recommends 200 mg/kg cyclophosphamide with polyclonal or monoclonal serotherapy for adults, and 120 mg/kg cyclophosphamide, 150 mg/m² fludarabine and serotherapy such as ATG for children²². After conditioning, stem cells are reinfused at a minimum dose of 2×10^6 CD34⁺ cells per kg (REF. 1). Hospital discharge after HSCT occurs generally within the first 1–3 weeks after stem cell infusion, that is, when the number of neutrophils increases. However, most patients remain severely lymphopenic for several months after HSCT while their immune system fully reconstitutes. The reasons why an autoimmune disease does not immediately relapse during the recovery of lymphocytes derived from a patient's own immune system are explained later in this Review.

Selection of CD34⁺ stem cells. In addition to severe lymphocyte depletion and exposure of transplanted stem cells to ATG or alemtuzumab, further selection of CD34⁺ stem cells has been explored but remains controversial. It is still unclear whether manipulation of the graft by *ex vivo* depletion of T cells (including potentially autoreactive T cells) from the stem cell population is helpful in prolonging response to therapy. A pilot, multicentre, randomized trial compared the transplantation of haematopoietic stem cells depleted of T cells (on the basis of CD34⁺ cell selection) with transplantation of unmanipulated haematopoietic stem cells in a total of 33 patients with severe, refractory RA receiving a high-dose immunosuppressive regimen (200 mg/kg cyclophosphamide without serotherapy)²⁶. The rate of 70% improvement according to ACR criteria (ACR70) response was similar in the two groups²⁶. In another study, a retrospective analysis was performed on clinical and laboratory data from 138 patients with SSc at diagnosis, before and after HSCT²⁷. HSCT with CD34⁺ stem cell selection was performed in 47% of patients, 83% of which received prior ATG, whereas the other 53% of patients received unmanipulated cells; 100% of patients received serotherapy (ATG or Campath-1H). The overall survival, progression-free survival and incidence of relapse or progression between the two groups was not significantly different²⁷.

A 2013 study showed that the relapse incidence at 3 years post-HSCT in ten patients with SLE who received HSCT with CD34⁺ stem cell selection was lower than that of patients with SLE who received normal HSCT (11% versus 68%, respectively). However, low-intensity conditioning was performed in 50% of the patients undergoing normal HSCT but in only 10% of the patients receiving HSCT with CD34⁺ stem cell selection²⁸. Therefore, the role of additional serotherapy in the reduction of relapse incidence in patients receiving

Box 1 | Haematopoietic stem cell transplantation in diffuse cutaneous systemic sclerosis in the Netherlands**Indications**

Diagnosis of diffuse cutaneous systemic sclerosis (SSc) according to ACR/EULAR 2015 criteria AND

- disease duration ≤ 2 years since development of skin tightness
- modified Rodnan Skin Score (mRSS) ≥ 20
- involvement of the torso
- erythrocyte sedimentation rate >25 mm/h and/or haemoglobin concentration <11 g/dL without active scleroderma
OR
- disease duration ≤ 4 years since development of skin tightness
- mRSS ≥ 15
- substantial organ involvement (occurring or worsening in the 6 months prior to haematopoietic stem cell transplantation), including:
 - pulmonary involvement: diffusing capacity of the lung for carbon monoxide (DLCO) of predicted value, plus signs of interstitial lung disease
 - renal involvement: at least one of the following criteria; hypertension (systolic blood pressure ≥ 160 mmHg or diastolic blood pressure >110 mmHg (two consecutive measurements performed at least 12 hours apart)), persistent abnormalities in urine sediment (proteinuria, haematuria, casts), microangiopathic haemolytic anaemia, new onset of renal failure (increase of serum creatinine $>$ upper limit of normal levels)
 - cardiac involvement: at least one of the following criteria; reversible congestive heart failure, atrial or ventricular arrhythmias including recurrent episodes of atrial fibrillation or flutter, recurrent atrial paroxysmal tachycardia or ventricular tachycardia, second or third degree atrioventricular block, pericardial effusion

Contraindications (any of the following)

- Pregnancy or refusal of contraceptives
- Severe comorbidities
- Respiratory: mean pulmonary arterial pressure on echocardiography >50 mmHg or with right heart catheterization >25 mmHg, DLCO $<40\%$ (of predicted value), respiratory failure
- Renal: creatinine clearance rate <40 ml/min (measured or estimated)
- Cardiac: clinical indications of refractory heart failure; left ventricular ejection fraction $<45\%$ on a multigated acquisition scan or echocardiography, chronic atrial fibrillation requiring oral anticoagulants, uncontrolled ventricular arrhythmias, pericardial effusion with haemodynamic consequences as assessed by an experienced cardiologist
- Liver failure: persistent increase of serum transaminases or bilirubin to levels three times higher than normal levels
- Abuse of drugs or alcohol
- Presence of neoplasms or myelodysplasia
- Leukopenia $<4.0 \times 10^9$ per litre, thrombocytopenia $<50 \times 10^9$ per litre, anaemia <8 g/dL, CD4⁺ T cell lymphopenia $<200 \times 10^6$ per litre
- Therapy-resistant hypertension
- Therapy-resistant acute or chronic infection, including HIV, human T cell lymphotropic virus type 1 (HTLV-I) and HTLV-II (PCR positivity)
- Prior radiotherapy for an underlying lymphoid malignancy, total body irradiation or alkylating agents including cyclophosphamide
- Poor compliance of the patient as reported by the referring doctor

Adapted from REF. 16

HSCT with CD34⁺ stem cell selection cannot be ruled out. Furthermore, the selection procedure requires the harvesting of additional CD34⁺ cells, which considerably increases the costs of autologous HSCT¹⁵. Thus, even though CD34⁺ selection of stem cells is standard practice in many institutions, compelling evidence to justify its use is lacking. The EBMT states that no evidence exists to support *ex vivo* graft manipulation, although decisions can be made on the basis of individual patients and *ex vivo* graft manipulation should be the focus of future clinical trials¹⁵. As suggested by evidence from allogeneic HSCT studies, individualized ATG treatment might influence the outcomes. Indeed, an excessive ATG exposure can retard T-cell reconstitution and increase the risk of viral infections, whereas a low ATG exposure can increase the risk of autoimmune disease relapse²⁹.

Disease remission without stem cell infusion. It is interesting to note that a stem cell mobilization and conditioning regimen might be sufficient to induce disease remission on its own. Furthermore, it is unclear whether stem cell rescue is really required after high-dose cyclophosphamide (≥ 120 mg/kg over 2–4 days). Indeed, in a 1977 report, autologous haematopoietic recovery was observed in a patient with severe aplastic anaemia after an attempted allogeneic HSCT with high-dose cyclophosphamide conditioning without donor engraftment³⁰. A small pilot study performed 20 years later supported this finding, as durable remission was observed in seven out of ten patients with severe aplastic anaemia after high-dose cyclophosphamide therapy without HSCT³¹. An observational, retrospective study in a tertiary-care hospital showed haematological recovery in 140 patients

with various severe, progressive autoimmune diseases (including SLE, multiple sclerosis, myasthenia gravis, scleroderma and autoimmune haemolytic anaemia) receiving only high-dose cyclophosphamide (200 mg/kg for four consecutive days) without stem cell infusion, although these patients did not receive prior serotherapy³². It is unclear whether cryopreserved haematopoietic stem cells would have been superior to endogenous stem cells in resetting the immune system, but haematologic recovery following treatment with high-dose cyclophosphamide occurred in all of these patients³². In this study, the overall response rate (a decrease in disease activity together with a decrease or elimination of immune-modulating drugs) was 94%, and during a median follow-up of 38 months, 44% of patients remained progression-free³².

If disease relapses, high-dose cyclophosphamide can also be safely readministered to patients with refractory, severe autoimmune disease; the quality and duration of this second remission seems to be at least equal to those of the first remission³³. Although immunosuppression alone would be sufficient to induce remission of autoimmune disease, stem cell rescue minimizes the duration of neutropenia and enables the containment of viruses by the quick recovery of the innate immune system (natural killer cells and $\gamma\delta$ T cells), and subsequently, CD4⁺ and CD8⁺ lymphocytes.

Resetting the immunological clock

The rationale behind autologous HSCT is that after the profound depletion of immune cells, including autoreactive T and B cells, a new and naive immune system reconstituted from the stem cell graft will re-establish immune tolerance through the thymus. However, exactly how HSCT rewires an immune system that is out of control is still unknown³⁴. It is unclear, for instance, which cells need to be depleted and which ones are important to maintain. In addition, despite the conditioning regimen, not all potentially pathogenic T cells are completely depleted, as specific CD8⁺ and CD4⁺ T-cell clones can be still detected after HSCT^{35,36}. Although some of these T-cell clones were abundant before HSCT, they remain subdominant after treatment.

Autoreactive T cells can survive HSCT conditioning, which, if vigorously stimulated, might hinder the restoration of immune tolerance. In a 2005 study³⁵, patients with autoimmune disease had sustained disease remission after HSCT even though pre-existing dominant T-cell clones remained present after transplantation, suggesting that these cells were neither autoreactive nor able to induce disease activity within the new environment. It is important to mention that in early studies, patients with autoimmune diseases, especially those with multiple sclerosis, were usually treated with a very strong conditioning regimen before HSCT. Nowadays, conditioning regimens are less intensive, which might enable the survival of a number of T-cell clones.

Role of lymphopenia and thymopoiesis in immune cell reconstitution. Several factors could be involved in resetting the immunological clock (FIG. 2). The first mechanism responsible for T-cell reconstitution after

HSCT is lymphopenia-induced proliferation. The lymphopenic environment drives expansion of existing peripheral T-cell populations via the action of homeostatic cytokines and antigen stimulation. Importantly, given that only a few T cells survive intermediate-intensity conditioning, clonal expansion increases the absolute number of T cells, leading to T cell receptor (TCR) oligoclonality^{35,37}. The antigens driving this expansion can be self or foreign antigens, and might thus induce T-cell autoreactivity^{38–40}. Indeed, an association exists between lymphopenia and autoimmunity^{41–43}, and the expansion and activation of autoreactive T cells that survive conditioning might lead to adverse effects in HSCT.

The second phase of immune reconstitution is thymopoiesis. The thymus produces naive T cells with unique TCRs, and it is thereby responsible for establishing a heterogeneous TCR repertoire. The expansion of these naive T cells lasts longer than that of existing T cells mediated by lymphopenia-induced proliferation. Several clinical studies demonstrated that disease remission after HSCT is associated with increased thymus activation^{25,35,44,45}, including the renewal of a polyclonal TCR repertoire^{35,37,45} and reduction of central memory T-cell numbers³⁵. In the syngeneic, proteoglycan-induced arthritis (PGIA) mouse model, following bone marrow transplantation the CD4⁺ T cell compartment is gradually replaced by graft-derived and thymus-derived T cells that produce less proinflammatory cytokines than those that survived conditioning^{46,47}. In addition, T-cell-derived cytokine responses after HSCT were investigated in two small studies^{25,48}. After HSCT in patients with SLE, memory T cells produced IFN γ when stimulated with viral antigens but not autoantigens²⁵. In patients with multiple sclerosis, the number of T cells producing IL-17 decreased after transplantation compared with that of T cells producing IFN γ , suggesting a shift in the ratio between type 1 T helper and type 17 T helper cells⁴⁸. Together, these findings indicate a reduction in disease-associated CD4⁺ T-cell responses after HSCT. Given that the conditioning regimen affects a broad range of immune and nonimmune cells, it is likely that the production of proinflammatory cytokines and chemokines by these cells is also influenced.

The levels of CXC-chemokine ligand 10 (CXCL10), galectin 9 and TNF receptor superfamily member 1B highly correlate with active juvenile dermatomyositis⁴⁹. After HSCT, a slow but steady and sustained reduction in the levels of these plasma proteins was found, which correlated with improved clinical scores in patients⁵⁰. It is important to note that the conditioning regimens used in these studies are very heterogenic, which makes it difficult to gather conclusive data on immune reconstitution and mechanisms following HSCT. In addition, the majority of studies have focused on immune reconstitution in circulation, and, therefore, whether the same process occurs in the sites of inflammation is unclear.

Role of regulatory T cells in HSCT. Regulatory T (T_{reg}) cells have a suppressive role in peripheral immune tolerance and are important for immune homeostasis, autoimmunity and inflammation. T_{reg} cells are characterized by the expression of forkhead box protein P3

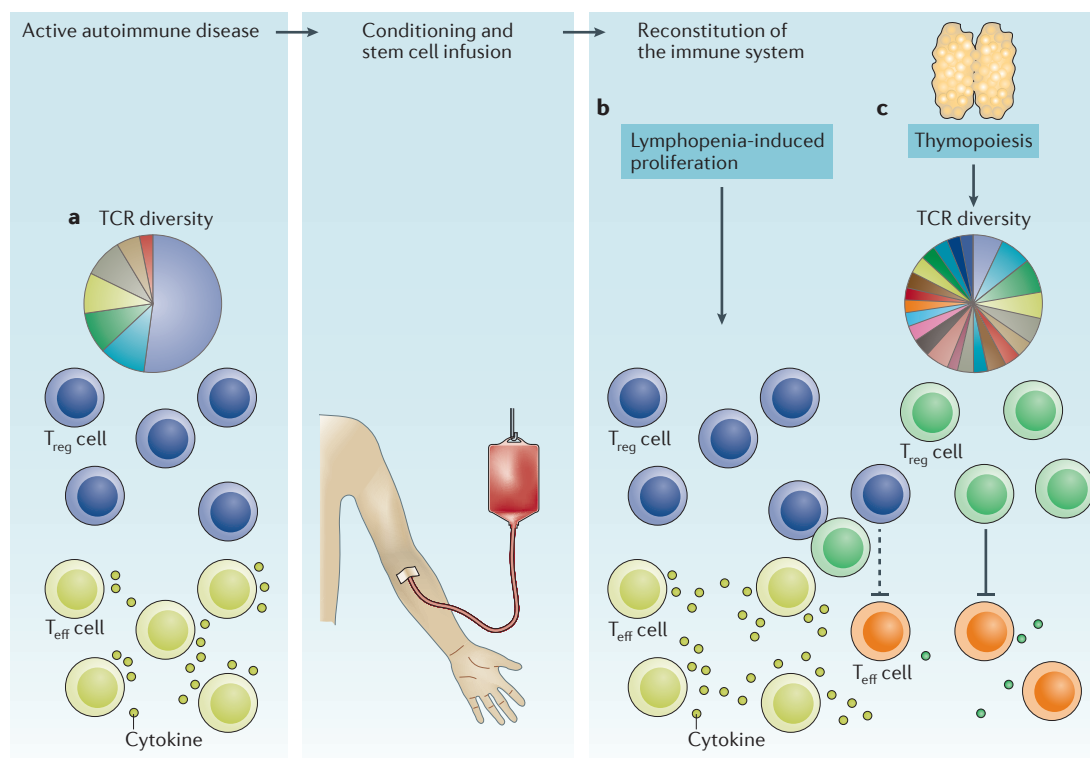


Figure 2 | Renewal of the CD4⁺ T cell compartment after autologous haematopoietic stem cell transplantation. **a** | The regulatory T (T_{reg}) cell compartment in patients with active autoimmune disease is oligoclonal before autologous haematopoietic stem cell transplantation (HSCT). **b** | After conditioning and stem cell infusion, the T cell compartment begins to be reconstituted. Owing to the lymphopenic environment, T cells proliferate (lymphopenia-induced proliferation), which leads to clonal expansion (blue cells). **c** | However, over time, the thymus starts to generate naive CD4⁺ T cells from the infused graft, and the T cell compartment largely consists of graft-derived T cells (green cells). After transplantation, T_{reg} cells display a renewed and restored T cell receptor (TCR) repertoire. Graft-derived T_{reg} cells have an enhanced suppressor activity compared with those that survived conditioning. Furthermore, graft-derived effector T (T_{eff}) cells produce less proinflammatory cytokines than T_{eff} cells that survived conditioning.

(FOXP3), which is important for their development, maintenance and function⁵¹. Findings from several animal and human studies^{52,53}, including those involving patients with Crohn's disease, multiple sclerosis and SLE, suggest that T_{reg} cell numbers increase after HSCT^{25,54,55}. In a study involving patients with SSc, the number of CD4⁺CD25^{hi} FOXP3⁺ T_{reg} cells was measured before and after transplantation and compared with that in healthy individuals⁵⁶. T_{reg} cell numbers in patients with SSc were reduced before HSCT, but 2 years after transplantation they returned to levels comparable to those in healthy individuals.

Two studies suggest that reconstituted T_{reg} cells express markers of recent thymic emigrants. First, in the PGIA mouse model, the number of naive T_{reg} cells in the graft-derived T_{reg} cell population increased after HSCT compared with that in the population of T_{reg} cells that survived conditioning⁴⁷. Second, Helios⁺ T_{reg} cells from patients who received HSCT express higher levels of naive markers (such as CD45RA and CD31 (also known as PECAM1)) than those from patients with active SLE⁵⁷. A few studies also investigated suppressive functions of reconstituted T_{reg} cells after HSCT. In patients with SSc, T_{reg} cells before HSCT displayed a less suppressive activity towards autologous effector T compared with T_{reg} cells

from healthy individuals, whereas after HSCT, T_{reg} cells were more suppressive compared with those from healthy individuals⁵⁶. In the PGIA mouse model, depletion of T_{reg} cells after HSCT led to an increase of disease activity, suggesting that these cells are crucial in preventing disease relapse⁵⁸. In addition, graft-derived T_{reg} cells have an increased suppressive function compared with T_{reg} cells that survive conditioning, indicating that renewal of the T_{reg} cell compartment is essential for long-term restoration of immune homeostasis and disease remission⁴⁷.

Infusion of T_{reg} cells in HSCT has been explored in murine models and humans. T_{reg} cell infusion during allogeneic HSCT reduces acute and chronic graft-versus-host disease^{59–63}, and clinical trials involving solid-organ transplantation are ongoing^{64,65}. Patients with autoimmune disease undergoing HSCT might also benefit from T_{reg} cell infusion, but no human studies have been performed yet to test this hypothesis. In the PGIA mouse model, the addition of T_{reg} cells to the bone marrow graft does not lead to positive clinical outcomes despite a decrease in proinflammatory cytokine production⁴⁷. At the same time, T_{reg} cell infusion is associated with a delay in the reconstitution of graft-derived T cells, indicating that this treatment might have adverse effects. In contrast with this finding, an enhanced T-cell reconstitution

is observed in patients treated with additional T_{reg} cells a few days before allogeneic HSCT^{59,66}. Therefore, T_{reg} cell therapy could have beneficial effects, but to date, little is known about the optimal setting, timing and dosing.

TCR heterogeneity before and after HSCT. As previously discussed, HSCT leads to renewal of the T cell compartment, with the thymus having an important role in the generation of a diverse TCR repertoire. Whether the heterogeneity of the TCR repertoire is also important for disease remission, or if a diverse TCR repertoire is important for T_{reg} cell function, is currently unknown. Data from experimental models suggest that T_{reg} cells with a restricted TCR repertoire have reduced functionality^{67,68}. Indeed, a T_{reg} cell compartment with polyclonal TCRs is more likely to have an antigen-specific clone than if it had a restricted TCR range. The activation and expansion of a diverse T_{reg} cell compartment have also been proposed to be more likely in the presence of a specific antigen⁶⁷. Therefore, an imbalance between T_{reg} cells with a restricted TCR and heterogeneous effector T cells might increase the risk of autoimmune disease. Before HSCT, T_{reg} cells from children with autoimmune diseases express an oligoclonal TCR repertoire⁴⁷. After HSCT, a complete renewal and increased heterogeneity of the T_{reg} cell compartment was observed in all of these patients except one, whose disease relapsed. Other cell populations also demonstrated an increase in TCR diversity⁴⁷. Thymus function declines during ageing, but in this study the increased TCR diversity after HSCT was measured in patients with JIA who probably still had a functional thymus⁴⁷. It would be interesting to analyse TCR diversity in T_{reg} cells and other populations from adult patients to learn if the same phenomenon occurs. In patients with multiple sclerosis undergoing HSCT, the CD4⁺ T cell compartment is mostly renewed whereas CD8⁺ T cells show an expansion of the existing TCR repertoire⁶⁹. In patients with multiple sclerosis, restoration of TCR diversity in the CD4⁺ T cell population has been suggested to predict clinical response following HSCT⁶⁹. However, given that TCR heterogeneity might be greater in T_{reg} cells than CD4⁺ T cells⁴⁷, the TCR repertoire of T_{reg} cells could be a strong predictor of clinical outcome following HSCT.

Reconstitution of B cells has also been reported to occur between 2 months and 1 year after HSCT^{25,70,71}. In patients with autoimmune diseases (such as RA, SSc and SLE) who underwent HSCT, an initial increase in the number of memory B cells was observed, followed by a decline of memory cells and an increase in naive B cells⁷¹. In patients with SLE, B-cell homeostasis was restored with the recovery of naive B cells within 12 months of HSCT, and disease-associated autoantibodies largely disappeared. Consistent with this finding, bone marrow transplantation in the PGIA mouse model leads to decreased levels of proteoglycan-specific IgG1 antibodies⁴⁶.

Unmet clinical need in autoimmune disease

The impact of a refractory rheumatic disease on patients is dependent on the duration and severity of symptoms and also on the damage caused by the therapies used. Since the second half of the twentieth century, DMARDs

such as methotrexate and cyclophosphamide have been widely used in the treatment of severe autoimmune disease. These DMARDs have even become the cornerstone in the treatment of autoimmune disease, usually combined with glucocorticoids⁷². However, such DMARDs are nonspecific and might cause serious or even life-threatening adverse effects. Autoimmune diseases have been treated much more effectively since the registration of the first biological response modifier (biologic) in November 1998, a specific drug that is produced by biological processes rather than chemical synthesis. However, despite the increasing numbers of biologics, treatment with these agents is still symptomatic rather than curative. Furthermore, a large proportion of patients will not benefit from, or become resistant to, any of these new drugs. Even in RA, the most common autoimmune disease with a prevalence of 0.24%⁷³, 28–34% of patients do not achieve 20% improvement according to ACR criteria (ACR20) in clinical trials when treated with etanercept^{74,75} or adalimumab⁷⁶. Moreover, 20–45% of all patients with RA discontinue anti-TNF therapy within 1 year⁷⁷, and 70–90% of patients still have active disease 1 year after switching to a second biologic such as CTLA4-Ig (abatacept), anti-CD20 (rituximab), or to a second anti-TNF therapy⁷⁸. Unfortunately, we are still unable to predict which patients will be refractory to which therapeutic agent, although timing seems to be important as early anti-TNF therapy in patients with RA correlated with improved outcomes⁷⁹. Other autoimmune diseases such as SSc are particularly refractory to various therapeutic regimens, including biologic therapies. No biologics have been approved for the treatment of Sjögren syndrome, SSc, dermatomyositis or polymyositis, and strikingly, these diseases are characterized by the presence of known autoantigens such as Ro and La in Sjögren syndrome, Scl-70 in SSc, histidyl-tRNA-synthetase in dermatomyositis and anti-SRP in polymyositis⁸⁰. This illustrates the difficulty in finding an effective treatment by blocking a single immune pathway, given the high degree of redundancy within the immune system⁸⁰. In patients with refractory autoimmune disease, quality of life is not only hampered by the disease itself but also by the cumulative toxicity of the immunosuppressants they take. Glucocorticoids are often part of this therapeutic strategy and lead to several adverse effects, such as weight gain, osteoporosis, avascular necrosis, glaucoma, type 2 diabetes mellitus, cardiovascular disease and serious infections⁸¹. Clearly, many patients with refractory autoimmune disease would benefit from more effective treatments characterized by fewer adverse effects and shorter duration.

Evidence for HSCT in autoimmune disease

The first HSCT for autoimmune disease was performed in 1995, and the Autoimmune Disease Working Party of the EBMT was launched in 1996 (REF. 13). EULAR collaborated progressively with the EBMT. Outside of Europe, the Center for International Bone Marrow Transplant Registry (CIBMTR) and the NIH in the USA interacted with large HSCT programmes in Australia, Brazil, China and the USA¹⁵. In 1997, a consensus report was written

on behalf of EULAR and the EBMT regarding stem cell transplantation in autoimmune disease, and an internationally coordinated clinical program was started¹³. Retrospective analyses from the EBMT autoimmune disease registry, the largest registry collecting HSCT data on autoimmune diseases, were followed by CIMBTR analyses. These studies, together with small, prospective phase I and II trials, supported the feasibility, safety and efficacy of HSCT in several severe, therapy-resistant autoimmune diseases¹⁵. These studies also led to large-scale phase II and III HSCT trials in several autoimmune diseases¹⁵. In 2012, it was estimated that around 3,000 patients with autoimmune diseases had been treated with HSCT worldwide¹⁵. TABLE 1 summarizes the results of HSCT in the EBMT registry and CIMBTR.

The database created by EBMT transplantation centres incorporated data from 1,273 patients with autoimmune diseases who underwent HSCT between 1996 and 2011 (REF. 15), whereas the CIMBTR database contains data from 368 patients with autoimmune diseases who underwent HSCT between 1996 and 2009 (REF. 82). Most HSCTs in the EBMT registry involved autologous grafts (1,209 patients), 9.8% of which were performed in children <18 years old. In the CIMBTR, autologous grafts were transplanted in 339 patients, of which 9.4% were children <21 years old. In the combined data from EBMT and CIMBTR databases, allogeneic HSCT was performed in 93 patients (6.0%), and in both registries the majority of these were children. The most frequent refractory autoimmune diseases in patients undergoing HSCT were SSc (363 patients; 22.1%), SLE (122 patients; 7.9%) and RA (86 patients; 5.6%)^{15,82}. In the EBMT registry, other frequent autoimmune disease indications were JIA (71 patients; 5.6%), vasculitis (29 patients; 2.2%) and polymyositis or dermatomyositis (16 patients; 1.3%)¹⁵. Other, nonrheumatic autoimmune disease were multiple sclerosis, autoimmune haematological conditions and Crohn's disease^{15,82}.

A more in-depth analysis of patients with autoimmune disease from the EBMT registry was performed in 2010, involving 900 patients who underwent a first autologous HSCT for a rheumatic (60%) or neurological (33%) autoimmune disease, and nine patients (1%) who received a second autologous HSCT⁸³. The median interval between time of diagnosis and performance of HSCT treatment for rheumatic disease was shortest for patients with SSc (30 months) and longest for those with RA (86 months)⁸³. During a median follow-up of 34 months, 12.3% of patients in the EBMT registry had died, whereas in the CIMBTR 10.3% of patients died during a median follow-up of 31 months^{82,83}. The overall survival rate for patients with SSc 3 years after HSCT was 80% in the EBMT registry and 83% in the CIMBTR^{82,83}. In the EBMT registry, 5 years after HSCT the overall survival rate was 76% for patients with SSc, 94% for patients with RA, 76% for patients with SLE and 82% for patients with JIA⁸³. In the EBMT cohort, 100-day transplant-related mortality (TRM) decreased considerably compared with that reported in 2001 in a smaller number of patients (6.6% versus 12%, respectively; TABLE 1)⁸⁴, and was comparable with that reported for the CIMBTR cohort (7.1%)⁸².

However, even a small difference in TRM is an important factor that needs to be taken into consideration when performing HSCT, since the transplantation itself is still the main cause of death (53.1% in the EBMT registry and 68.4% in the CIMBTR), with fatal infections as the leading cause of TRM according to both databases^{82,83}. Disease-related mortality, defined as the rate of deaths caused by the original rheumatic disease, was 38.7% in the EBMT registry and 28.9% in the CIMBTR. In patients with SSc, disease-related mortality was highest (13.1%), which, in contrast to other autoimmune diseases, was even higher than the TRM⁸³. The 5-year progression-free survival rate, defined as survival without evidence of relapse or progression in 5 years, was 55% for patients with SSc, 18% for patients with RA, 44% for patients with SLE and 52% for patients with JIA⁸³. Besides the disease indication, other factors associated with a positive outcome were the transplantation centres where HSCT was performed, a patient's age being <35 years, whether the HSCT was performed after December 2000, the use of peripheral blood stem cells and, surprisingly, a longer disease duration before HSCT⁸³.

Predictably, given such results, the next logical step was to investigate HSCT in SSc in a large phase III trial. Ideally, a double-blind, placebo-controlled, RCT is performed to test the efficacy of a therapy. However, the use of placebo is not an option in severe diseases that require HSCT, and blinding of patients and physicians is not possible because the precautionary measures required after HSCT should be known to all. It is important to be aware that these studies are unblinded, especially when subjective measures such as patient-reported outcomes and skin thickening are interpreted⁸⁵. However, the results of two phase III RCTs are consistent with data from registries, pilot studies and a small phase II RCT. The first RCT was ASSIST, a single-centre phase II trial performed in Chicago, Illinois, USA, in which ten patients were allocated to HSCT treatment and nine patients to 6 months of cyclophosphamide therapy. HSCT was more effective than cyclophosphamide regarding pulmonary function, skin thickness and quality of life⁸⁶. Eight out of nine patients in the cyclophosphamide group also underwent HSCT because of an insufficient response. Due to the small sample size, the two groups differed, with the control group receiving less cumulative cyclophosphamide than is usually administered in clinical practice, perhaps inflating the relative efficacy of HSCT⁸⁵. Remarkably, no deaths were observed during the study and even serious toxicity was not common. These results can be explained by either the experience of the clinical team or by chance, given the small number of patients and the short follow-up (maximum of 2 years after HSCT)⁸⁵. The trial was stopped prematurely for the benefit observed in the HSCT group. However, RCTs stopped early for benefit are associated with an overestimation of positive treatment effects⁸⁷.

The first phase III HSCT RCT in patients with SSc was the ASTIS trial⁸⁸, which demonstrated for the first time that in early diffuse cutaneous SSc, long-term poor prognosis can be altered by intensive immunosuppressive therapy. The primary end point of this trial was

Table 1 | Haematopoietic stem cell transplantation in patients with autoimmune disease

Parameters	EBMT	CIBMTR/ASBMT
Total population of patients with autoimmune disease undergoing HSCT^{15,82}		
Years of study participation	1996–2011	1996–2009
Number of patients	1,273	368
Number of female patients	815 (64%)	196 (58%)
Number of children*	119 (9.8%)	32 (9.4%)
Age (years)	35 (2.7–76)	39 (6–64)
Median time from diagnosis to HSCT (months)	62 (0–494)	52 (0–413)
Number of patients receiving autologous HSCT	1,209 (94.9%)	339 (92.1%)
Number of patients with systemic sclerosis	266 (20.9%)	97 (26.4%)
Number of patients with systemic lupus erythematosus	97 (7.6%)	27 (7.3%)
Number of patients with rheumatoid arthritis	78 (6.1%)	10 (2.7%)
Number of allogeneic HSCT patients (% of children)	64 (62.5%)	29 (55.2%)
Patients with autoimmune disease undergoing autologous HSCT^{82,83}		
Years of study participation	1996–2007	1996–2009
Number of patients	900	339
Median follow-up duration (months) [†]	34 (0.5–148)	31 (1–144)
Overall survival	85% at 5 years post HSCT	86% at 3 years post HSCT
100-day transplant related mortality	6.6%	7.1%
Disease-related mortality	4.8%	3.0%
5-year progression-free survival	43%	NA
Patients with autoimmune disease undergoing allogeneic HSCT^{82,94}		
Years of study participation	1984–2007	1996–2009
Number of patients	35	29
Overall 1-year survival	70%	58%
2-year transplant-related mortality [§]	22.1%	34.0%
2-year disease-related mortality	3.2%	3.4%
5-year progression-free survival	65%	NA

In addition to the disease risk, the risk of the transplantation procedure and the benefits of non-transplant strategies should be evaluated in individual patients by an experienced transplantation team^{1,101}. ASBMT, American Society for Blood and Marrow Transplantation; CIBMTR, Centre for International Bone Marrow Transplant Registry; EBMT, European Society for Blood and Marrow Transplantation. HSCT, haematopoietic stem cell transplantation. *Defined as <18 years old by EBMT and <21 years old by CIBMTR. [†]Follow-up duration in the CIBMTR registry was calculated among survivors. [§]Defined as death without relapse or progression of autoimmune disease within 100 days from autologous HSCT, or as related to the allogeneic HSCT including graft-versus-host disease.

event-free survival, defined as time from the randomization until the occurrence of major organ failure or death. From 2001 to 2009, 156 patients were randomly allocated to either HSCT (n = 79) or cyclophosphamide therapy (n = 77). During the first year, 11 deaths (13.9%), including eight transplant-related deaths, occurred in the HSCT group, whereas seven deaths (9.1%) occurred in the cyclophosphamide group, none of which were treatment-related. During a median follow-up of 5.8 years, 53 pre-specified events including deaths occurred, 22 in the HSCT group and 31 in the cyclophosphamide group. Irreversible organ failure events occurred three times in the HSCT group, and eight in the cyclophosphamide group, and the number of total deaths was 19 in the HSCT group and 23 in the cyclophosphamide group. Also the secondary end points, defined as the change

in the first 2 years of modified Rodnan Skin Score and generic health status measures (such as the Health Assessment Questionnaire) were significantly improved in the HSCT group ($P < 0.001$ for both). An increase in pulmonary function tests (forced vital capacity/vital capacity) was observed in the HSCT group, but without a significant change in diffusion capacity for carbon monoxide of the lung. The left ventricular ejection fraction did not significantly change in the two groups, although a modest but significant decrease in creatinine clearance was found in HSCT group. From this study⁸⁸, it was concluded that HSCT was associated with increased treatment-related mortality within the first year after treatment but improved long-term event-free survival. In addition, nonsmokers had the highest survival rate after HSCT⁸⁸.

The ongoing SCOT trial in North America not only has inclusion criteria that are very similar to those in the ASTIS trial, but is also characterized by an almost identical control group⁸⁹. In both trials, the populations studied were relatively similar in terms of extent of skin thickening, organ involvement and disease duration. Therefore, comparative analyses between the ASTIS and SCOT trials are possible, and these might help identify the optimal patient profile for HSCT candidacy and might also reveal if differences in transplant regimens have any effect on outcomes. Although previously underappreciated owing to the strict screening procedures used in trials, conditions such as gastric antral vascular ectasia have now been revealed to be relatively common⁸⁹.

HSCT might also become a therapeutic option for Behçet disease, which is life threatening and can be refractory to glucocorticoids and immunosuppressants. One systematic review described HSCT in 20 patients with Behçet disease, including nine with refractory disease (eight patients received autologous HSCT and one patient received allogeneic HSCT), and 11 with concomitant haematologic conditions⁹⁰. Of the nine patients with refractory Behçet disease, three patients with neurological involvement, two patients with pulmonary artery aneurysm and one patient with intestinal involvement achieved complete remission. All six patients with haematologic conditions, who also had gastrointestinal involvement as part of their Behçet disease, achieved complete remission of their gastrointestinal manifestations after HSCT⁹⁰.

Refractory JIA was a prominent indication for autologous HSCT around 15 years ago, but since the registration of multiple biologics, HSCT for patients with refractory JIA is now uncommon. One article described complete clinical remission in eight out of 20 patients after autologous HSCT. Among the remaining patients, seven partially responded to therapy, and five experienced disease relapse (occurring 7 years after HSCT in one patient)⁷⁰. During follow-up, the two patients whose disease relapsed died from infections developed after restarting immunosuppressive medications⁷⁰.

Autologous HSCT was also performed in 28 patients with SLE, who had a median age of 29 years (range of 16–48 years) and a median disease duration of 52 months (range of 9–396 months)²⁸; 60% of these patients had lupus nephritis. *Ex vivo* CD34⁺ stem cell selection was performed in 36% of patients, and the conditioning regimens used were either low ($n=10$) or intermediate ($n=18$). During a median follow-up of 38 months after HSCT, 5-year overall survival was 81%, disease-free survival 29%, relapse incidence 56% and non-relapse mortality 15%²⁸. Five deaths occurred within 2 years after HSCT, including three deaths caused by infection, one death caused by secondary autoimmune disease and one death caused by progressive SLE²⁸.

Adverse effects of HSCT in autoimmune disease

Before administration of cyclophosphamide for mobilization of stem cells and immunoablation, the risk of inducing infertility and premature menopause should be carefully considered¹⁵. Cryopreservation of semen,

oocytes or embryos, as well as hormone replacement therapy, should be offered when appropriate¹⁵. Patients with autoimmune disease might already have reduced organ function, which could increase the toxicity that results from conditioning⁹¹. Careful evaluation of the heart is also important, especially in patients with SLE and SSc. Indeed, cases of cardiac toxicity have decreased since the EBMT consensus statement clearly highlighted the potentially fatal levels of toxicity to the heart that can occur during HSCT for autoimmune disease⁹².

The immune system of patients with autoimmune disease is often substantially weakened by both the disease and chronic use of immunosuppressants. The mobilization of stem cells, conditioning regimen and T-cell depletion of the autologous graft are all associated with an increased risk of both latent and acquired infections¹⁵. Bacterial or fungal infections occur in the early post-HSCT phase during neutropenia, whereas prolonged lymphopenia can enable the reactivation of latent viruses and other opportunistic infections, endangering patients until their immune system has reconstituted⁹¹. Therefore, during aplasia all patients should receive broad-spectrum antibacterial, anti-fungal and anti-herpes prophylaxis for at least 100 days after transplantation. Furthermore, prophylaxis against the prevalent, opportunistic, but rapidly fatal bacterium, *Pneumocystis jiroveci* should be given to all patients with neutropenia (as is standard treatment in HSCT)¹⁵.

Autoimmunity can develop *de novo* during immune reconstitution after HSCT because of the loss of regulatory mechanisms within the immune system. The cumulative incidence of a secondary autoimmune disease was 9.8% at 5 years after HSCT, as revealed by specific questionnaires sent to EBMT transplantation centres to identify patients with newly developed autoimmune diseases⁹³. The effects of autologous HSCT on the endocrine system or frequency of malignancies are also of concern⁹¹.

Allogeneic HSCT has a higher risk of death compared with autologous HSCT, and it was 30% at 1 year among 35 patients with autoimmune diseases in the EBMT registry and 42% among 29 patients with autoimmune diseases in the CIBMTR^{92,94}. However, it is important to note that nowadays TRM for all indications has been reduced through individualized conditioning regimens and improved monitoring of infections. In addition, strict pre-transplantation screening before allogeneic HSCT should be mandatory, as it is for autologous HSCT. Although a complete clinical response was observed in 55% of patients with autoimmune disease who underwent allogeneic HSCT, and 23% of patients had at least a partial response, the TRM at 2 years (22.1%)⁹¹ was higher than that associated with autologous HSCT (6.6%)⁸³. In the CIBMTR, the TRM for allogeneic HSCT (34.5%) was also much higher than that for autologous HSCT (7.1%)⁹².

Allogeneic HSCT also causes more long-term complications than autologous HSCT, which can affect virtually all tissues and organ systems⁹⁵. These complications are due to graft-versus-host disease, toxicity or the original autoimmune disease itself. The occurrence of long-term adverse effects is associated with the conditioning regimen used, the use of total-body irradiation

and graft-versus-host disease. Patients receiving allogeneic HSCT also have a twofold–threefold increase in the risk of developing a solid tumour⁹⁶. However, novel transplantation strategies, such as individualized conditioning regimens to reduce toxicity and T-cell depletion, might overcome these limitations.

Individualized conditioning by monitoring the concentrations of drugs such as busulfan and ATG has been proven to influence TRM^{29,97,98}. The cellular source of allogeneic HSCT might also influence the risk of adverse effects. For example, cord-blood-derived samples are generally associated with a low probability of chronic graft-versus-host disease⁹⁹, but for patients treated with these samples, the precise dosing of ATG is challenging. Indeed, when exposure to ATG following HSCT is too high, the probability of immune reconstitution is reduced and TRM increased⁹⁷.

Allogeneic HSCT can be considered for the treatment of life-threatening forms of autoimmune diseases or relapsing autoimmune diseases occurring after an autologous transplantation^{15,100}. Within the paediatric Blood and Marrow Transplantation programme at the University Medical Centre Utrecht, Netherlands, an allogeneic HSCT study protocol for therapy-resistant autoimmune disease is used with an individualized conditioning regimen and cord blood as a cellular source. To date, three patients, including one with multiple sclerosis, one with relapsing polychondritis and one with SLE, are alive and disease-free 1–5 years after allogeneic HSCT.

Disease indications for HSCT

Patients with severe autoimmune disease that progresses despite standard or approved treatment can be considered for autologous HSCT¹. Various levels of evidence of long-term survival after autologous HSCT are

available for each specific autoimmune disease. In 2015, the EBMT published recommendations on the indications for HSCT, which were not based on a rigorous and extensive literature review, but were formulated according to prospective clinical trials, registry data and expert opinion¹. The American Society for Blood and Marrow Transplantation (ASBMT) established a task force consisting of multiple stakeholders such as payer representatives, HSCT experts and patient advocates to provide guidelines on ‘routine’ indications for HSCT¹⁰¹. Neither the ASBMT guidelines nor the EBMT recommendations should be used to determine whether HSCT should be performed in individual patients¹. Whether or not to proceed with HSCT is a clinical decision that is best made between the patient and the doctor after a careful consideration of alternative treatments and the risks and benefits of the procedure¹⁰¹. Pre-transplantation screening and cardiopulmonary evaluation are of vital importance to exclude patients at high risk of TRM¹. Besides a possible gain of survival, expected quality of life and potential long-term adverse effects after transplantation should be considered, especially in children. An overview of the indications of HSCT is provided in TABLE 2.

Conclusions

Autologous HSCT is the only treatment that is able to induce long-term, drug-free and symptom-free remission in several refractory autoimmune diseases. Over 3,000 HSCT procedures for severe autoimmune diseases have been performed worldwide. The sequential steps for autologous HSCT include careful patient selection, chemotherapy-based mobilization of stem cells, stem cell collection, conditioning regimens, stem cell infusion, supportive care during recovery of neutrophils and lymphocytes and post-transplant care. HSCT resets

Table 2 | Indications for autologous HSCT

Parameters	EBMT	CIBMTR/ASBMT
Severe systemic sclerosis	Indicated for certain subgroups of patients (non-Raynaud's disease duration <5 years and an mRSS ≥15, plus major respiratory, cardiac or renal involvement with documented evidence of onset or clinically significant worsening in the previous 6 months)	Developmental, but promising (HSCT for this indication is best performed in clinical trials)
Systemic lupus erythematosus	Optional for certain subgroups of patients (early in the disease course or after at least 6 months of standard therapy with mycophenolate mofetil or cyclophosphamide with still sustained or relapsed activity defined by the British Isles Lupus Assessment Group (BILAG) A score with either cardiovascular, neurologic, kidney, or pulmonary involvement, vasculitis or autoimmune cytopenias)	Developmental, but promising (HSCT for this indication is best performed in clinical trials)
Juvenile idiopathic arthritis	Optional for patients with polyarticular juvenile idiopathic arthritis (with no response to prednisone dose of 2 mg/kg/day for eight consecutive weeks, and inadequate response or intolerance to at least two DMARDs, including biological agents or unacceptable drug toxicity)	Standard of care, rare indication*
Juvenile systemic sclerosis	Optional for carefully selected patients (requiring special consideration and appropriate expertise in patient selection)	Standard of care, rare indication*

ASBMT, American Society for Blood and Marrow Transplantation; CIBMTR, Centre for International Bone Marrow Transplant Registry; EBMT, European Society for Blood and Marrow Transplantation. HSCT, haematopoietic stem cell transplantation. *Clinical trials and observational studies with sufficient number of patients are not currently feasible because of the very low incidence of this disorder. However, single-centre, multicentre or registry studies in relatively small cohorts of patients have shown that HSCT is effective, with an acceptable risk of morbidity and mortality. For patients with these diseases, HSCT can be considered as a treatment option after careful evaluation of the risks and benefits.

the immune system by renewing the CD4⁺ T cell compartment and the T_{reg} cell population, which is accompanied by an increase in the number of T_{reg} cells and the re-establishment of TCR diversity and function. The thymus is likely to have an important role in restoring this immune balance. Also, following transplantation, the B cell compartment becomes naive and the number of autoreactive antibodies decreases.

We believe that for any child or adult with a severe and therapy-refractory autoimmune disease, autologous HSCT can be considered, but only after a well-balanced evaluation by an experienced transplantation team to establish whether the risks associated with transplantation outweigh the burden of disease. It is unlikely that large clinical trials will be initiated in the near future to systematically investigate the role of allogeneic HSCT in autoimmune diseases. Given the high level of risk associated with this intervention, transplantation with cells from an allogeneic donor should be considered exceptional and only be performed at centres with substantial experience

in transplantation for patients with autoimmune disease. In 2013, allogeneic HSCTs in Europe were reported for 23 patients with autoimmune disease, compared with the 171 patients with autoimmune disease who received autologous HSCT¹⁰², indicating that a broader consensus in transplantation regimens and indications is needed to enable a rigorous analysis of the outcomes.

In addition to allogeneic HSCT, other cellular therapies, such as those using multipotent mesenchymal stromal cells (MSCs), might also have potential for autoimmune disease treatment. MSCs isolated from bone marrow and other body sites have specific immunomodulatory and anti-inflammatory properties, can be easily expanded to clinically relevant numbers and are now close to being used in the clinic¹⁰³. It is now important to start trials to examine the clinical efficacy of MSC infusion or injection compared with non-cellular immunosuppressive therapies. Furthermore, a careful patient selection is needed to gain insight into the safety and efficacy of MSC therapy¹⁰³.

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Author contributions

J.F.S. and E.M.D. researched data for article. F.V.W., J.B., J.K., J.V.L. and N.M.W. reviewed and edited the manuscript before submission. J.F.S., E.M.D., J.B. and J.K. wrote the manuscript. All authors contributed substantially to discussion of content.

Competing interests statement

J.K. is the co-founder and chief scientific officer of Gadeta. The remaining authors declare no competing interests.