The impact of biological therapy on regulatory T cells in rheumatoid arthritis

Rachel Byng-Maddick and Michael R. Ehrenstein

Abstract
Regulatory T cells (Treg) are functionally defective in patients with RA. Restoring their function may not only control inflammation but also restore tolerance in these patients. Biologic therapies have been tremendously successful in treating RA. Here we review numerous reports suggesting that these immunomodulatory therapies have an impact on Treg and that this may contribute to their beneficial effects. Better understanding of their mode of action may not only lead to improvements in therapies and sustained remission but also enable the development of biomarkers of response, which would be the first steps towards personalized medicine.

Key words: regulatory T cells, rheumatoid arthritis, biologic therapy, anti-TNF therapy.

Introduction
There has been significant progress in the understanding of RA pathogenesis over the past few decades, leading to the introduction of several new biologic therapies. These therapies have shed light not only by proving that their targets are important in disease, but also in numerous studies dissecting the immunopathogenesis of RA. One such mechanism that governs immune homeostasis involves the Treg. Treg are important mediators of peripheral immune tolerance, modulating many aspects of the innate and adaptive immune response, including T effector cells, NK cells and antigen-presenting cells. Restoration of tolerance is likely to be key in curing RA. In this paper we review evidence for modulation of Treg function in patients with RA receiving biologic therapy and discuss whether restoration of functional Treg may be an important approach in treating RA.

The role of Treg in autoimmune disease
Treg have been implicated in the pathogenesis of many autoimmune diseases, including RA, SLE and ANCA-associated vasculitis [1–3]. Treg are essential for self-tolerance, thus in autoimmune disease it is thought that a lack of or dysfunction of these cells occurs, resulting in a breakdown of immunological tolerance and abnormal immune responses to self-antigens [4].

In humans, Treg constitute 5–10% of the CD4+ T cell population in the peripheral blood of healthy individuals. These cells express high levels of CD25 (IL2Rα), cytotoxic T lymphocyte-associated antigen 4 (CTLA4) and glucocorticoid-induced TNF receptor family-related protein. They are characterized by the stable expression of the lineage-specific transcription factor Forkhead Box P3 (FoxP3), which is pivotal for Treg function and homeostasis [5]. CD4+ Treg are generated in both the thymus and the periphery; the latter are typically known as induced Treg. Many different subpopulations of induced Treg have been identified based on phenotypic and functional properties [6]. Thymic and induced Treg are increased during inflammation, working to suppress aberrant responses via different mechanisms, utilizing soluble and membrane-bound factors. Treg exert suppressive effects, mainly on CD4+ and CD8+ T cells, but also have some control of B cells, NK cells, dendritic cells and other antigen-presenting cells. The exact mechanism of their regulatory effects is not known, although it is thought to be via regulatory cytokines IL-10 and TGF-β, and cell–cell contact via CTLA4 and the membrane glycoprotein LAG3 [7, 8]. It has also been shown that Treg
can control B cell responses via a Fas-dependent mechanism [9].

Both mouse models and human disease have demonstrated that Treg play an important role in the prevention of autoimmune disease. Sakaguchi et al. [10] first showed that following adoptive transfer of CD4+CD25hi cells to thymectomized mice, spontaneous autoimmune disease could be prevented. Scurfy mice have a frameshift mutation in the FoxP3 gene and develop extensive lymphoproliferative disease and widespread inflammation [11]. In humans, mutations in the FoxP3 gene result in the IPEX syndrome, which leads to catastrophic multisystem autoimmune disease which is invariably fatal [12, 13].

Overview of the pathogenesis of RA

Disturbances in both adaptive and innate immunity contribute to the pathogenesis of RA [14]. It is thought that, initially, dendritic cells, macrophages and activated B cells act as antigen-presenting cells to present autologous antigens to T cells [15]. Monocytes and macrophages are central to driving inflammation [16] and are activated in RA [17, 18], infiltrating inflammatory sites such as the synovium [19]. This leads to the expansion of autoantigen-specific T cells in the joints and lymph nodes that secrete IL-2 and IFN-γ [20]. B cells also contribute to pathogenesis through the production of autoantibodies and cytokines, which may further stimulate the production of pro-inflammatory cytokines (including TNF) through Fc receptors and complement activation [20]. Activation of B and T cells further stimulates macrophages to produce pro-inflammatory cytokines such as TNF, IL-1 and IL-6.

Disturbed Treg function in RA

In active RA, Treg are unable to suppress Th1 responses, including the production of IFN-γ [21]. In addition, Treg do not effectively suppress B cells in RA [9]. Moreover, B cells (unlike T cells) are resistant to the effects of Treg in RA, indicating that restoration of Treg function may not be sufficient to restore tolerance.

A possible mechanism for defective Treg in RA is their lack of CTLA4 expression [22]. In mice, CTLA4-deficient Treg cells have impaired suppressive activity on T effector cells [23]. CTLA4-expressing Treg are able to reduce CD80 and CD86 expression on antigen-presenting cells, which may in turn inhibit T cell co-stimulation.

It is unclear whether the relative number of Treg in the peripheral blood of RA patients is altered. There does seem to be increased numbers of Treg in the RA synovium, although they are greatly outnumbered by effector T cells [24, 25]. In animal models of rheumatic disease, autoimmune responses can be controlled by re-establishing the T effector:Treg cell ratio. Therefore, in humans, Treg frequency and function have been studied as biomarkers for response to treatment. A recent study assessing T cell subsets in treatment-naïve patients with early RA found that CD4+CD25hi cells are not a useful biomarker of response to MTX, because the frequency of these cells remained reduced, unlike that of naïve T cells [26]. However, in response to anti-TNF therapy, Treg number and function do seem to be restored [27]. Therefore, the role of Treg as potential biomarkers of disease state or outcome in humans remains controversial.

Th17 cells have also been implicated in the pathogenesis of RA due to their expression of a number of pro-inflammatory cytokines, including IL-17. IL-17 acts in synergy with TNF to induce chemokine and cytokine production from synovial fibroblasts, resulting in destruction of cartilage [28]. Treg and Th17 cells may develop from the same precursors under distinct cytokine conditions [29], and a subset of IL-17-producing CD4+FoxP3+ Treg cells can be generated upon polarization by cytokines such as IL-6 [30]. In RA, the balance between Th17 cells and Treg is shifted towards an increased number of Th17 cells with less functional Treg [31]. Both rely on TGF-β for their induction, but in the presence of other pro-inflammatory cytokines such as IL-6, a Th17 response is favoured. Treg are likely to play a significant role in preventing and/or reducing the pro-inflammatory state. Restoring the balance and efficacy of Treg may be important in the treatment of diseases such as RA.

Anti-TNF therapy and Treg

TNF-α is a key pro-inflammatory cytokine involved in the pathogenesis of RA, although its role in this context is not completely understood. Cytokines, including TNF-α, are present at the site of inflammation and may influence the differentiation and function of Treg. However, it is unclear whether TNF-α disables or potentiates Treg function. Treg are known to express TNF receptor II, but contradictory results prevent clear understanding of the downstream consequences of the signalling through this receptor. For instance, recently it has been shown that FoxP3 is dephosphorylated by TNF-α, leading to defective function [32], whereas others have found that TNF-α promotes Treg expansion [33–36].

Anti-TNF therapies have transformed the management and disease progression of inflammatory arthritis. There are two main types: mAbs to TNF (infliximab, adalimumab and golimumab) and a soluble TNF receptor (etanercept). Certolizumab is a Fab fragment of a humanized TNF-α mAb.

Treg in patients with RA have impaired function [21] and are outnumbered by pathogenic effector cells. Therefore, re-establishing and expanding a population of functioning Treg may contribute to the resolution of inflammation and the restoration of tolerance. Following treatment of RA patients with infliximab, a novel population of Treg cells expressing low levels of CD62L were identified [27]. These cells mediate suppression of T effector cells via TGF-β and IL-10, resulting in reduced IFN-γ production [27]. Patients clinically responding to the fully humanized anti-TNF antibody adalimumab also have an increased percentage of FoxP3+ cells with restored regulatory function. These cells are able to suppress and resist conversion to Th17 cells by control of monocyte-derived IL-6 production [31]. However in patients responding to etanercept, a soluble TNF receptor
with similar clinical efficacy, Treg cell number and function remain similar to those of patients with active RA. This implies that defective Treg may not be the sole cause of inflammation in RA. Our data also suggest that anti-TNF antibody therapy and etanercept act via different mechanisms to restrain inflammation in RA, with the former harnessing Treg to aid in the resolution of disease. In clinical practice, switching to an anti-TNF therapy with a different mechanism of action in a patient with active RA may sometimes be an effective approach [37].

Anti-TNF therapy may also have an effect on T effector cells. It is thought that protein kinase B (PKB/c-Akt) hyper-activation in inflammatory T cells contributes to T effector unresponsiveness to suppression in JIA [38]. PKB/c-Akt activation positively regulates pro-inflammatory cytokine production, including that of TNF and IL-6 [39]; in addition, TNF can induce PKB/c-Akt activation [38]. Therefore, using anti-TNF therapy to block this positive feedback loop may explain why etanercept is able to reverse the resistance of T effector cells to suppression, thereby re-establishing Treg-mediated control of CD4+ and CD8+ T cells [40]. In addition, a recent study showed that 4–8 weeks after anti-TNF therapy, Th1 prevalence was higher than baseline in patients treated with etanercept and infliximab, while it was stable in the adalimumab group [41]. Th2 prevalence was higher in the anti-TNF antibody therapy groups, but remained stable in the etanercept group. Similarly, Treg numbers increased, but Th17 cells remained unchanged [41]. This alteration in T cell subsets may lead to a more favourable balance of pro- and anti-inflammatory cytokines.

The restoration of Treg function with anti-TNF antibody therapy but not etanercept may also help to explain the differential risk of granulomatous disease, particularly re-activation of latent tuberculosis during and after anti-TNF therapy. Although both mechanisms of TNF blockade confer similar clinical efficacy in inflammatory arthritis, anti-TNF antibody therapy increases the risk of active mycobacterial infection by 7– to 17-fold compared with etanercept [42]. Treg downregulate the Th1 and Th17 response required for anti-mycobacterial defence, leading to compromise of the granuloma causing increased disseminated and extrapulmonary disease, which is clinically seen in 50% of patients [43].

### B cell depletion therapy and Treg

Over the past decade growing evidence has emerged underlining the pathogenic role of B lymphocytes in RA [14], particularly due to the efficacy of rituximab [44] (a chimeric mAb to CD20), causing transient B cell depletion for ~6–9 months [45].

Although the main function of B cells is the production of antibodies, they also have other actions that can potentiate or regulate the immune response. B cells interact directly with T cells by antigen presentation and co-stimulation through CD80/86. This leads to the production of cytokines and the formation of germinal centres in tertiary lymphoid tissue [46]. Therefore, therapy that depletes B cells may also have an effect on the T cell compartment.

Analysis of peripheral blood samples of patients with RA treated with rituximab at different time points did not demonstrate any significant change in the frequency of whole T cell populations. Although there was a transitory decrease in Treg during the first month of therapy, later their numbers normalized [45, 47]. However, Hamel et al. [48] found that B cell depletion is correlated with a decreased percentage of T effector cell function, which is correlated with an increase in CD4+CD25+FoxP3+ cells and improvement in the clinical severity of arthritis.

In other CTDs, including SLE, ITP and granulomatosis with polyangiitis, patients with reduced numbers of Treg prior to therapy have demonstrated increased numbers of functional Treg following rituximab, and successful B cell depletion [49–51].

Other strategies for targeting B cells include blockade of B-cell activating factor (BAFF), the cytokine B cell activation factor of the TNF family. This is essential for B cell survival and activation, but T cells may also express the BAFF receptors (TACI and BR3), suggesting that they too may play a role in T cell responses [52]. Addition of exogenous BAFF from antigen-presenting cells in vitro to human and mouse T cells provides co-stimulatory signals [53]. BAFF-transgenic mice have increased numbers of peripheral FoxP3+ Treg [54]. BAFF is thought to play a key role in the pathogenesis of SLE, but to date little evidence supports a role for this cytokine in RA. Belimumab is a humanized mAb to soluble BAFF, but its effects on Treg are unknown.

### IL-6 blockade and Treg

The pro-inflammatory cytokine IL-6 plays an important role in the pathogenesis of inflammatory disease, leading to cartilage and bone destruction, and constitutional symptoms including the fever, fatigue and anaemia of chronic disease [55]. High levels of IL-6 have been found in sera and SF of patients with active inflammatory arthritis [56]. IL-6 blockade in experimental animal models, by either inhibition of signalling via the gp130 pathway or an IL-6 knockout gene, has led to significant improvement in inflammatory arthritis [57, 58]. IL-6 blockade in human patients with polyangiitis, patients with reduced numbers of Treg and improvement in the clinical severity of arthritis.

In vitro studies have shown that TGF-β is required for differentiation of naive T cells into Th17 and Treg cells. However, addition of IL-6 to culture medium prevents Treg development, while promoting Th17 cells, thereby promoting inflammation [29]. Murine studies also support the hypothesis that IL-6 suppresses Treg induction and potentiates the development of Th17 cells in the presence of TGF-β, IL-1β, IL-21 and IL-23 [60]. Patients with active RA have high numbers of Th17 cells, thus the ratio of Treg to Th17 is low. Following a clinical response to tocilizumab therapy, the Th17:Treg ratio is restored by an increased
number of Treg [61, 62]. This suggests that the efficacy of tocilizumab may be due to its ability to correct the impaired balance of Th17 and Treg cells.

**Abatacept (CTLA4-Ig) and Treg**

CTLA4 (CD152) is an essential negative regulator of T cell responses, thus preventing autoimmunity. It is expressed on the membrane of activated T cells—both conventional T cells and Treg—but its precise mechanism of action has not been clearly elucidated. CTLA4 inhibits T cell activation by reducing IL-2 production and IL-2 receptor expression, and by arresting the T cells at the G1 phase of the cell cycle [63]. It has also been shown that CTLA4 can remove the CD80 and CD86 co-stimulatory molecules (B7-1 and B7-2) from antigen-presenting cells via trans-endocytosis [64]. CTLA4 is ~70% homologous to the stimulatory molecule CD28, and both receptors compete for binding to CD80 and CD86. It is known that CD28 plays an important role in the survival and expansion of Treg. It is possible that CTLA4 expression also attenuates CD28 signals on Treg, although it is not clear how this affects thymic selection, proliferation and survival [7].

CTLA4-deficient or knock-out mice develop a fatal lymphoproliferative disorder together with autoimmunity, leading rapidly to death, indicating a vital role in T cell homeostasis and or the development, activity and function of Treg cells [65, 66]. The relationship between CTLA4 and Treg suggests they share mechanisms of immune tolerance. FoxP3 is not required for CTLA4 induction, nor is CTLA4 required for the expression of FoxP3 [66]. However, CTLA4 ligation on Treg does lead to augmentation of function, whereas on T effector cells this inhibits their action. It has been reported that CTLA4 expression by Treg cells from RA patients is significantly reduced compared with healthy controls. Therefore, restoring CTLA4 expression and function in Treg could represent a novel therapeutic approach for patients with this condition [67].

Abatacept is a soluble fusion protein consisting of the extracellular domain of human CTLA4 and a fragment of the Fc portion of human IgG1. It interrupts the co-stimulatory interaction between CD28 on T cells and CD80/86 on antigen-presenting cells, thus inhibiting effective T cell activation and proliferation. Both abatacept (CTLA4-Ig) and CTLA4 on CD4+CD25+ cells bind to B7 on molecules, and modify dendritic cells to express indoleamine 2,3 dioxygenase, a tryptophan-catabolizing enzyme that induces and activates CD4+CD25+ Treg [68].

Patients with RA treated with abatacept were shown to have significantly fewer CD4+FoxP3+ cells in their peripheral blood after 12 weeks of treatment, but the remaining cells had enhanced activity and this was correlated with reduced disease activity [69]. In mouse models this was thought to be via a TGF-β-dependent mechanism [68]. However, in vivo blockade of co-stimulation does not result in de novo generation of Treg, but does lead to proliferation of functional, suppressive FoxP3 cells [70].

**Relationship of newer biologic therapies with Treg**

**Anti-CD3 antibody therapy**

Anti-CD3 therapy has been used as an immunosuppressive agent in prevention of rejection following organ transplantation. A short course may also be used therapeutically to induce remission in type I diabetes mellitus in NOD mice [71]. In mouse models of arthritis, administration of anti-CD3 therapy leads to an increased proportion of CD4+ and CD8+ Treg [72] and a transient downregulation of the TCR on remaining T cells [73]. In vitro, these potently suppress Th1 and Th17 responses [72]. Teplizumab, a humanized anti-CD3 therapy, has also been used in the treatment of new onset diabetes mellitus, with clinical responders developing an increased proportion of induced CD8+ Treg [74]. Modified anti-CD3 has been used in phase I/II clinical trials of both RA and PsA, but full details of their effects are awaited.

**Anti-CD4 antibody therapy**

CD4 is a co-receptor for the TCR on CD4+ helper cells and Treg. A CD4-specific mAb is currently in early phase clinical trials for psoriasis and RA [75]. These mAbs work either by depleting autoreactive CD4+ T effector cells, which generally suppresses the immune system, or by specifically activating Treg, thereby inducing tolerance. Non-depleting anti-CD4 therapy (YTS177) used in mouse models of inflammatory arthritis (SKG) has prevented onset of inflammatory disease and can halt progression of erosive disease [76]. It was also able to prevent in vitro Th17 polarization, favouring peripheral conversion of naïve T cells to FoxP3+ T cells.

Tregalizumab (BT-061) is able to activate the suppressive properties of naturally occurring T-reg, but not of conventional T cells [77]. It is an agonistic humanized mAb that binds to CD4 on T cells (Th cells and Treg), causing phosphorylation of the ZAP70 protein kinase associated with the TCR complex. It activates Treg, enabling suppression of proliferation and cytokine secretion of CD4+ and CD8+ T cells in vitro [78]. Tregalizumab has been tested in phase II clinical trials and has been associated with rapid and sustained improvements in RA [79].

**Anti-IL-17 antibody therapy**

IL-17 is a pro-inflammatory cytokine that is mainly produced by Th17 cells. However, in the inflammatory state, Treg are also capable of producing IL-17 [30]. IL-17 plays a central role in RA, activating numerous cell types, including monocytes, macrophages, chondrocytes and osteoblasts. This cytokine also acts in synergy with TNF-α to induce chemokine and cytokine production from synovial fibroblasts, resulting in cartilage destruction [28]. Elevated levels of Th17 cells and IL-17 are seen in the periphery of patients with RA [28, 80]. Overexpression of IL-17 in murine knees leads to joint inflammation, bone erosion and cartilage proteoglycan loss [81], whereas blockade, deficiency or neutralization improves inflammatory symptoms [82].

Several anti-IL-17 or anti-IL-17 receptor antibodies are currently in development for the treatment of autoimmune
inflammatory disease. Both the anti-IL-17 antibody secukinumab [83] and the anti-IL-17RA antibody brodalumab [84] significantly improved moderate-to-severe psoriasis, although disappointingly these results have not been replicated in RA [85, 86]. Although the IL-17/IL-23 pathway is implicated in the pathogenesis of RA, it may not be the main driver of the pro-inflammatory cascade. Alternatively, IL-17 could act synergistically with other pro-inflammatory cytokines in RA. In mouse models of inflammatory arthritis, IL-17 expression led to joint inflammation, cartilage destruction and bone erosion. These effects were markedly enhanced when IL-17 was co-expressed with TNF [81]. Therefore blockade of IL-17 may have little impact on inflammation if other cytokines persist. It remains unclear whether these treatments have a direct effect on Treg.

**Direct Treg therapies**

In RA it has been shown that Treg are unable to effectively suppress inflammatory responses, and that the balance between Treg and T effector cells has been altered.

*In vitro* Treg are expanded by antigenic stimulation and IL-2 [87]. Therefore, low doses of IL-2 have been trialled to increase Treg numbers in various diseases, including graft-vs-host disease [88] and hepatitis C-induced cryoglobulinaemic vasculitis [89], and together with rapamycin in type I diabetes mellitus [90]. Although there was some clinical improvement and circulating Treg numbers did improve during the infusion, these effects were not long lasting. In RA, the main Treg abnormality occurs within the joints; however, systemic or IA IL-2 has not yet been tried in this disease.

**Conclusions**

It is widely recognized that Treg are important in preventing autoimmune disease and are likely to contribute to the resolution of inflammation. However, there is still controversy and debate as to their exact role in patients. From this review of the literature, it is apparent that most biologic therapies appear to have an impact on Treg, and restoration of Treg function has been reported, particularly with anti-TNF antibody therapy. However, restoration of Treg function may not be sufficient if target cells are resistant to suppression. Understanding how biologic therapy affects pathogenic and Treg subsets could lead to the generation of biomarkers of response. For instance an *in vitro* assay based on Treg induction could be developed to predict which patients will respond to specific therapies. Ultimately, it is likely that a combination approach may be the most successful, involving suppression of the inflammatory response together with optimizing Treg function in order to restore tolerance and cure disease.

**Funding:** No specific funding was received from any funding bodies in the public, commercial or not-for-profit sectors to carry out the work described in this manuscript.

**Disclosure statement:** The authors have declared no conflicts of interest.

**References**


