*Neutralization of pro-inflammatory cytokines by monoclonal antibodies represents a novel therapeutic strategy for restoring the suppressive functions of regulatory T cells and induction and/or expansion of regulatory T cells in order to reinforce tolerance mechanisms in rheumatoid arthritis.*



# Rescuing CD4+CD25+ regulatory T-cell functions in rheumatoid arthritis by cytokine-targeted monoclonal antibody therapy

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**CD4+CD25+ regulatory T cells (Tregs) play a crucial role in controlling the development of autoimmune diseases such as rheumatoid arthritis (RA). However, despite an increased number of Tregs, the persistence of inflammation in the rheumatoid joints suggests that Tregs are unable to suppress ongoing disease, perhaps due to an inhibition of their functions by pro-inflammatory cytokines. Treatment of RA patients with anti-TNF-α monoclonal antibodies such as infliximab and adalimumab has been found to induce and restore the functions of Tregs. Thus, manipulation of the pro-inflammatory environment in the inflamed synovia via neutralization of inflammatory cytokines by monoclonal antibodies could represent a novel therapeutic strategy for restoring the suppressive functions of Tregs and induction and/or expansion of Tregs in order to reinforce tolerance mechanisms.**

The immune system is subject to multiple regulatory mechanisms. In addition to the deletion of autoreactive T cells in the thymus, various processes function to prevent destructive immune responses against self-antigens. One of these regulatory mechanisms involves suppressive T cells. To date, three prominent subtypes of suppressive T cells have been characterized in the CD4+ T-cell population: T regulatory 1 cells (Tr1), which exert suppressive function in an IL-10-dependent fashion; TGF-β-producing Th3 cells and naturally occurring CD4+CD25+ regulatory T cells (Tregs) [1–4]. Among these populations, CD4+CD25+ Tregs have been extensively studied, and there is considerable evidence that these cells contribute to both the maintenance of self-tolerance and the prevention of excessive responses against infection [1,4]. CD4+CD25+ Tregs inhibit proliferation and cytokine production of conventional T cells, and a deficiency of these cells is associated with autoimmunity in mice and humans. CD4+CD25+ T cells with regulatory activity are generated during intrathymic development (hence their description as 'natural' Tregs), but can also be induced in the periphery from CD4+CD25− T cells during the course of normal immune response [1]. The transcription factor forkhead box P3 (FoxP3) plays a major role in governing the functions of Tregs [5–8].

The mechanisms by which CD4+CD25+ Tregs suppress immune responses remain a subject of debate. Studies of CD4+CD25+ Treg activity *in vitro* have generally indicated that suppression of T-cell responses requires direct cell–cell contact, but soluble factors, particularly TGF-β and IL-10, have also been implicated in Treg activity [1]. Moreover, Tregs may act on multiple target cells. Thus, although some studies suggest that Tregs can act directly on responding T cells [1,4,9], there is evidence that effects of Tregs on natural killer cells, NKT cells, monocytes, B cells and dendritic cells (DCs) are also important [10–15]. The precise delineation of the function of Tregs is, therefore, of great importance for understanding the pathogenesis of autoimmune diseases. Moreover, the ability to modulate such regulatory mechanisms might provide novel therapeutic opportunities in autoimmune disorders such as rheumatoid arthritis (RA).

## Rheumatoid arthritis: a major autoimmune disease

RA is a complex, debilitating, chronic, systemic autoimmune disease characterized by an immunological, inflammatory and

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mesenchymal tissue reaction in the synovium accompanied by polyarticular synovitis, ultimately leading to the progressive destruction of articular and periarticular structures. It is one of the most prevalent autoimmune diseases, affecting up to 1% of the adult population in Western countries and causes significant morbidity and disability. Thus, RA imposes a substantial economic burden on both patients and society. The current worldwide market for RA drugs exceeds US$ 14 billion annually [16].

Despite major advances, the etiology of the disease is still not completely understood. Environmental factors, such as mechanical stress and smoking, can predispose individuals to disease [17]. In addition, there is evidence that the tendency to develop RA is genetically inherited. Most notably, RA is strongly linked to certain haplotypes of HLA class II antigens, specifically HLA-DRB1*0404 and DRB1*0401 [18]. Since the main function of HLA class II molecules present on antigen-presenting cells is to present antigenic peptides to CD4+ T helper cells, this association suggests that RA could be caused by unidentified arthritogenic antigens [19]. In this respect, both exogenous antigens, derived from viruses, bacteria and fungi, and endogenous antigens, including citrullinated protein, human cartilage glycoprotein 39, and heavy chain binding protein, have been implicated [20]. It is, therefore, suspected that certain infections, as well as environmental and genetic factors, contribute to the immune dysregulation and inflammation that play major role in the pathogenesis of RA.

## Pathogenesis of rheumatoid arthritis: interplay of vicious cellular and cytokine networks

Although it is not clear how RA is triggered, the deleterious process of joint destruction is mediated by activation of intracellular signaling pathways that results in the production of pro-inflammatory cytokines, chemokines, growth factors, expression of co-stimulatory molecules and their ligands, and adhesion molecules leading to the recruitment of inflammatory cells and the self-perpetuation of inflammation [21,22]. Although the role of inflammatory cells in the pathogenesis of RA has been well established, the specific contribution of resident cells, especially those of mesenchymal origin within the synovial membrane, and activation of innate immune system through Toll-like receptors have become the subject of current intense study. The central role of these cells in the progression of RA is underlined by their involvement in the crucial pathophysiological features: recruitment of inflammatory cells and of periarticular factors such as adipocytokines that contribute to joint inflammation, hyperplasia of synovia and joint destruction.

RA is accompanied by a markedly increased cellularity of the synovial membrane. Prominent features include infiltration of macrophages and T cells, proliferation and expansion of fibroblasts, activation of endothelial cells and neovascularization within the synovium. Although less abundant, DCs and B cells are also observed in synovial membrane. In contrast, synovial fluid is enriched with neutrophils, although macrophages, DCs and T cells are also present.

Monocytes, macrophages, DCs, fibroblasts and T cells release numerous cytokines upon stimulation that in turn results in the stimulation of neighboring cells and contributes to the inflammatory environment in the rheumatoid joint [23]. Elevated concentrations of these inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-8, IL-15, IL-17, IL-23 and IL-32, are detected in synovial fluid from patients with active RA. These cytokines contribute to the activation of macrophages, synovial fibroblasts, chondrocytes and osteophytes, and stimulate cell proliferation, adhesion molecule expression and the secondary release of other cytokines [24]. Thus, the vicious cycle of cytokines and local cellular interactions drive the long-term cellular proliferation and destruction of the rheumatoid joint.

## Level of CD4+CD25+ regulatory T cells in rheumatoid arthritis: does the number matter?

A breakdown in immune tolerance that regulates the CD4+ cell-mediated inflammatory process is central for the initiation and progression of RA [25]. The chronic nature of the inflammatory process in RA suggests that immune regulation in the joint is perturbed. This perturbed regulation might be due to an excessive inflammatory process that overpowers tolerance mechanisms. The net result is an aberrant inflammatory response against the healthy self-tissue that, under physiological conditions, is not normally subject to immunological aggression.

To address this issue, a series of recent articles have analyzed the role and function of Tregs in experimental models and in patients with RA, both at early and chronic stages of the disease [26–34]. These studies have suggested that Tregs have a role in regulating the inflammatory process in RA and that defects in Treg function might contribute to the development and/or maintenance of inflammation in RA.

Analysis of the frequency of peripheral blood CD4+CD25+ Tregs in RA patients has yielded contradictory results [35]. Although some papers have reported an increased frequency of peripheral blood Tregs [36], others have demonstrated either no difference in the frequency of Tregs compared to healthy donors or a decreased level of peripheral blood Tregs [30–32,34]. These conflicting results might be in part, to the different methodologies used to analyze the Treg populations. Some researchers considered $CD25^{high}$ cells as Tregs, whereas others analyzed the total population of CD25+ T cells. Notably, analysis of $CD25^{high}$ cells, which represent a much more enriched population of Tregs than that of CD25+ cells, has generally indicated that the number of Tregs in peripheral blood is decreased in RA. Moreover, the frequency of $CD25^{high}$ cells in the peripheral blood has been shown to be decreased compared to healthy donors in related inflammatory joint diseases, such as juvenile idiopathic arthritis (JIA) [37].

In contrast, however, there is clear evidence that the frequency of CD4+CD25+ Tregs is higher in the synovial fluid than in peripheral blood of patients with RA [29,31,32,36]. These results are consistent with those observed in other arthopathies such as JIA and spondylarthropathies [31,37]. The reasons for the increased frequencies of Tregs in inflamed synovia are not known. In addition to preferential homing to synovia from peripheral blood, it is possible that Treg population expands within the synovia. However, the persistence of inflammation in the rheumatoid joints despite the increased number of Tregs indicates that these cells are ineffective in controlling the inflammatory response. One possible explanation is that Tregs in the joint are defective in mediating their suppressive, anti-inflammatory activity.

## Compromised function of CD4+CD25+ regulatory T cells in rheumatoid arthritis: a pro-inflammatory cytokine-driven process?

Consistent with the general features described for these cells [4], CD4+CD25+ Tregs isolated from patients with active RA show the expression of FoxP3, an anergic phenotype upon TCR stimulation, and an ability to suppress the proliferation of effector T cells from synovia and from peripheral blood *in vitro* [29–32,36]. However, these Tregs are able to neither suppress pro-inflammatory cytokine secretion from activated T cells and monocytes nor confer a suppressive phenotype on 'conventional' T cells [30,33]. A recent study by Lipsky and colleagues demonstrated that TNF-α, one of the major inflammatory cytokines in the inflamed joint, inhibits the suppressive function of both naturally occurring CD4+CD25+ Tregs and TGF-β1-induced CD4+CD25+ Tregs [33]. The mechanism of this inhibition was shown to involve signaling through TNFRII, which is constitutively expressed on unstimulated Tregs and the expression of which is upregulated by TNF-α. TNF-mediated inhibition of suppressive function was associated with a decrease in the expression of FoxP3 mRNA and protein by CD4+CD25$^{high}$ Tregs isolated from patients with active RA [33]. The results suggest an interaction between the innate and adaptive immune systems, in which TNF-α, a product of the innate immune compartment, could promote immune reactivity by limiting the action of Tregs.

## Defining the role of CD4+CD25+ regulatory T cells in the pathogenesis of rheumatoid arthritis: lessons from experimental models

Using several experimental animal models, including collagen-induced arthritis, antigen-induced arthritis and K/BxN-transgenic models, it has been demonstrated that depletion of CD25+ T cells in animals before or after the induction of arthritis leads to increased cellular and humoral responses and an exacerbation of arthritis [26,27,38,39]. Conversely, transfer of CD4+CD25+ Tregs at the time of induction of arthritis has been shown to decrease the severity of disease, with a preferential accumulation of the transferred cells in the inflamed joint [26,28]. Taken together, the results from experimental models and clinical trials suggest that Tregs might have a potential therapeutic application in the early stages of arthritis. Notably, however, the transfer of Tregs appears to be unable to cure established chronic arthritis in animal models [30,33,40,41]. Along with the clinical data showing elevated Treg cell numbers in RA joints, the implication is that increasing Treg frequency is not sufficient to suppress ongoing inflammation in RA. Therefore, an important question is whether treatments that could augment Treg function would be effective in treating the disease. Given the results cited above indicating that inflammatory cytokines impair Treg function, one strategy would be to inhibit the activity or production of these cytokines.

## Rescuing the function of regulatory T cells in rheumatoid arthritis: a role for inflammatory cytokine-neutralizing monoclonal antibodies?

The emerging knowledge of the importance of inflammatory cytokines, such as TNF-α, in the pathogenesis of rheumatic diseases has provided a strong rationale for targeting them by neutralizing monoclonal antibodies (MAbs). The therapeutic efficacy of infliximab, a chimeric anti-TNF-α MAb and adalimumab, a fully human IgG1 MAb in combination with methotrexate, is now well established in RA [42–45]. Consistent with the negative effect of TNF-α on Treg function, treatment with anti-TNF-α MAbs was shown to restore the capacity of Tregs to inhibit the T-cell proliferation and cytokine production, and to confer a suppressive phenotype on 'conventional' T cells [30,33]. Furthermore, anti-TNF-α administration was shown to be associated with a significant rise in the number of peripheral blood Tregs in RA patients responding to this treatment, which correlated with a reduction in C-reactive protein level [30]. The increase in the frequency of peripheral blood Tregs in responding RA patients might also be due to an enhancement in Treg viability [46]. Treatment with anti-TNF antibody was also shown to result in increased FoxP3 mRNA and protein expression by CD4+CD25$^{high}$ Tregs and decreased expression of TNFRII [33].

In addition to modulating direct effects of TNF-α on Tregs, it is possible that anti-TNF therapy also neutralizes other functions of TNF-α linked to the trafficking and distribution of Tregs. In fact, anti-TNF-α therapy is associated with a large spectrum of anti-inflammatory activities such as downregulation of (i) C-reactive protein and vascular endothelial growth factor (a potent angiogenic factor that promotes migration and proliferation of endothelial cells), (ii) pro-inflammatory cytokines including TNF-α, IL-6, IL-1α and IL-1β, (iii) infiltration of inflammatory cells, (iv) expression of co-stimulatory molecules on activated B cells and (v) the endothelial cell activity [47,48]. Besides neutralizing soluble TNF-α, anti-TNF-α MAbs might also induce complement-mediated cellular lysis through interaction with cell-bound TNF-α.

## Neutralization of TNF-α leads to the induction of regulatory T cells

Further investigation into the effect of anti-TNF-α therapy in RA patients has suggested that this treatment leads to the generation of a newly differentiated population of Tregs [49]. Thus, infliximab therapy was shown to give rise to a CD4+CD25$^{high}$FoxP3+ Treg population, which exerted its suppressive function via TGF-β and IL-10. The absence of CD62L expression on these cells marked them as phenotypically distinct from the naturally occurring Tregs present in healthy individuals and patients with active RA. Of note, infliximab also induced *in vitro*, in a TGF-β-dependent manner, the differentiation of CD62L− Tregs from CD4+CD25− T cells that had been isolated from active RA patients. In spite of the potent suppressor capacity displayed by this CD62L− Tregs, the natural CD62L+ Tregs remained defective in infliximab-treated patients [49]. Therefore, the rise in the frequency of peripheral blood Tregs in responding RA patients and the restoration of the capacity of Tregs to inhibit proliferation and cytokine production and convey a suppressive phenotype to 'conventional' T cells [30,33] might be mediated by these newly differentiated Tregs.

## Other possible cytokine targets for rescuing regulatory T-cell function in rheumatoid arthritis: rationale and clinical development

As the focus of the therapeutic approach in RA has now shifted from disease control toward the induction of remission, targeting pro-inflammatory cytokines to reinforce immune homeostasis has attained a major priority. Thus, restoring the suppressive

functions of Tregs and induction and/or expansion of Tregs hold considerable potential as a treatment for RA. The evidence gathered from studies using anti-TNF MAbs suggests that Tregs can be induced by targeting specific pro-inflammatory cytokines for the restoration of tolerance in RA patients.

On the basis of our understanding of the role of cytokines in cellular communication and in the immunopathogenesis of RA, neutralization of several inflammatory cytokines can be considered for ameliorating the function of Tregs. Recent findings in mice have revealed that TGF-β, normally an anti-inflammatory cytokine with an ability to induce FoxP3+ Tregs from non-Treg populations [50], can contribute to the generation of immunopathogenic IL-17-secreting T cells in the presence of pro-inflammatory cytokines [51–53]. Although it is not known whether the same cytokines control the generation of human IL-17-secreting T cells, these findings raise the possibility that diversion of T cells from IL-17-secreting pathogenic cells to suppressive Tregs could be accomplished by the neutralization of pro-inflammatory cytokines, and in particular IL-6, TNF-α and IL-1. Interestingly, clinical studies have demonstrated that anti-TNF therapy, in addition to neutralizing TNF-α, can also suppress the production of both IL-6 and IL-1 [47,48], although future studies will be required to determine the spectrum of activities of individual neutralizing MAbs to IL-6 and IL-1 on Tregs.

Administration of IL-7 to humans leads to the expansion of CD8+ and CD4+ cells but a relative decrease in CD4+ Tregs [54]. Also, IL-7 and IL-15, which are present in the synovial fluid of JIA patients, have been found to abrogate the suppressive activity of Tregs *in vitro* [55]. Similarly, IL-21, another member of the common γ-chain-related cytokines, has been shown to render CD4+ T cells resistant to Treg-mediated suppression [56]. Interestingly, upregulation of the IL-21R has been observed on the inflamed synovial membrane and the tissue of RA patients and hyper-responsiveness of synovial T cells to IL-21 signaling has been demonstrated [57]. Thus, IL-21 enhances the activation and proliferation of synovial T cells from RA patients and induces the secretion of other inflammatory cytokines such as TNF-α and IFN-γ.

Furthermore, IL-32, a recently discovered cytokine, might also be considered as a therapeutic target to expand Tregs. IL-32 is found in synovial tissue of patients with active RA, and its level of expression has been shown to correlate with the severity of the disease [58]. Interestingly, IL-32 also induces the secretion of other key pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6, which influence the function of Tregs.

Research and clinical trials are required to determine how neutralizing MAbs to key pro-inflammatory cytokines such as IL-7, IL-15, IL-17, IL-21 and IL-32 affect Treg activity. Interestingly, a recent clinical trial demonstrated that treatment with HuMax-IL-15, a human anti-IL-15 MAb, significantly reduces disease activity in RA patients. The benefit was associated with an inhibition of IL-15-induced release of IFN-γ and of the expression of an activated phenotype by synovial mononuclear cells [59]. Although not demonstrated directly, the beneficial effect might also be linked to restoring Treg activity, as IL-15 has been shown to inhibit the function of Tregs in JIA patients [55].

Clinical trial designs in RA are well established and standardized. Evidence for an anti-inflammatory effect of MAbs (compared with placebo) can be obtained with a three-month, or longer, study using the criteria defined by the American College of Rheumatology (ACR) [42–45,47,48]. In such trials, the immunomodulatory effects of neutralizing pro-inflammatory cytokines on Tregs could be investigated using standards set by other studies [30,33,49]. Since signaling pathways of several pro-inflammatory cytokines are distinct (e.g. IL-15 vs TNF), combined MAb therapy might also be explored.

Finally, despite the expected benefits of an anti-inflammatory cytokine approach with respect to the direct inhibition of inflammatory effects and indirect pro-Treg effects, a note of caution should be interjected regarding the therapeutic use of such reagents. Blocking inflammatory cytokines could lead to several adverse events in patients as observed in the case of TNF inhibitors [60]: development of serious infectious diseases and difficulty in clearing these once they develop (e.g. tuberculosis, aspergillosis, histoplasmosis, coccidioidomycosis, listeriosis, Pneumocystis *carinii* pneumonia, cryptococcal infections and cytomegalovirus) lupus-like reactions, lymphomas, worsening of congestive heart failure and inflammatory demyelinating disease of the central nervous system. Striking a balance between suppressing a damaging inflammatory response and allowing for protective immunity to infection will be a key challenge.

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