RHEUMATOLOGY

Review

Treating arthritis by immunomodulation: is there a role for regulatory T cells?

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Abstract

The discovery of regulatory T cells almost 15 years ago initiated a new and exciting research area. The growing evidence for a critical role of these cells in controlling autoimmune responses has raised expectations for therapeutic application of regulatory T cells in patients with autoimmune arthritis. Here, we review recent studies investigating the presence, phenotype and function of these cells in patients with RA and juvenile idiopathic arthritis (JIA) and consider their therapeutic potential. Both direct and indirect methods to target these cells will be discussed. Arguably, a therapeutic approach that combines multiple regulatory T-cell-enhancing strategies could be most successful for clinical application.

Key words: Rheumatoid arthritis, Juvenile idiopathic arthritis, Regulatory T cells, Treatment, Pro-inflammatory cytokines, Effector T cells, Combination therapy.

Introduction

RA and juvenile idiopathic arthritis (JIA) are autoimmune diseases characterized by destructive joint inflammation. In the chronic phase of the diseases, a non-remitting activation of cells and expression of soluble mediators of especially the innate immune system dominates the inflammatory process. The resulting synovial inflammation is characterized by non-specific infiltration of both lymphocytes and innate immune cells, such as synoviocytes, macrophages and neutrophils. The importance of this innate immune activation in chronic arthritis is underscored by the success of interventions with biologicals that target non-specific effector mediators such as TNF-α. In contrast, interventions directed against CD4$^+$ T cells have been disappointing. This has led to the assumption that T cells are of less importance in the chronic phase of RA and JIA [1].

Data obtained over the last years, however, have shed new light on the role of T cells in regulation of the inflammatory response. This line of research started almost

15 years ago with the discovery of so-called regulatory T cells (Treg) [2]. This exciting discovery raised expectations for novel ways of treating arthritis by targeting these Treg. Their presence and function in RA and JIA, and the questions still surrounding their potential therapeutic application will be discussed in this review.

Treg

Treg are capable of suppressing effector cell proliferation and cytokine production, and play an important role in immune homeostasis. Several subtypes of CD4$^+$ Treg have been identified that can be either naturally occurring, derived from the thymus or induced in the periphery. These subtypes of Treg are depicted in Table 1 together with their supposed mechanism of action. Natural Treg constitutively express the IL-2 receptor (CD25) and require IL-2 for their survival and function [2, 3]. These cells are further characterized by the transcription factor FOXP3, which controls the development and suppressive function of the cells [4–6]. CD25$^+$FOXP3$^+$ Treg can suppress via multiple mechanisms, probably depending on the context *in vivo* [7]. These cells are critical in preventing autoimmune disease in animal models [3], which is confirmed in humans by the fact that patients with mutations in the *FOXP3* gene suffer from immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), characterized by autoimmune disease in multiple organs [8, 9]. More recently, it has been established in both mice and humans that Treg can also be induced in

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Table 1 Subtypes of CD4^{+} Treg and supposed mechanism of action

Term	Markers	Origin	Mechanism of action
Natural Treg	CD25 FOXP3 CTLA-4 GITR	Thymus	Cell–cell contact and suppressive cytokines
Adaptive Treg	CD25 FOXP3 CTLA-4 GITR	Induced in the periphery	Cell–cell contact and suppressive cytokines
Tr1	–	Induced in the periphery	IL-10 production
Th3	–	Induced in the periphery	TGF-β production

the periphery upon antigen encounter. These cells can be not only FOXP3^{+} [10–13], but also FOXP3^{−}, such as T regulatory 1 (Tr1) cells that depend on IL-10 for their development and function [14, 15] and T helper 3 (Th3) cells, producing TGF-β [16]. CD25^{+}FOXP3^{+} Treg are highly important in the control of autoimmune arthritis both in experimental models [17–19] and in human disease [20]. Therefore, we will further refer to this specific CD25^{+}FOXP3^{+} subset by the term Treg and we will discuss the potential of these cells as a target for immune intervention in arthritis.

Presence, phenotype and function of Treg in arthritis patients

Given the convincing evidence that Treg play a critical role in preventing experimental autoimmune arthritis, numerous groups have studied the presence, phenotype and function of Treg in patients with RA and JIA (summarized in Table 2) [20–28]. When analysing these data, it should be kept in mind that several studies were performed before FOXP3 was identified as a marker for Treg. In these studies, Treg were identified based on (high) CD25 expression, which is a less definitive marker for Treg compared with FOXP3. In addition, FOXP3 can also be up-regulated in effector cells during activation [29] and this makes it difficult to distinguish Treg from activated effector T cells in patients with ongoing autoimmune inflammation.

Nevertheless, the majority of studies suggest that Treg numbers in the periphery are not reduced in arthritis patients compared with healthy controls [22, 23, 26, 28]. Instead, Treg are enriched at the site of inflammation, since increased levels of these cells are found in the SF compared with peripheral blood [20, 21, 24–26, 28]. These SF-derived Treg show enhanced expression of FOXP3 mRNA, cytotoxic T lymphocyte antigen 4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor (GITR), HLA-DR, CD69 and OX40 [20, 25, 26, 28] and are more efficient in inhibiting effector cell activation [20, 25, 26]. In contrast, reduced suppressive function has been reported for peripheral blood-derived Treg from RA patients in some [22, 23, 27], but not all studies [24, 26]. Thus, there is still conflicting evidence on the suppressive function of Treg in arthritis, which can result from the different test systems used to analyse the suppressive function of the cells.

For obvious technical reasons, all the above studies investigated Treg-mediated suppression in vitro. However, in vivo the local pro-inflammatory environment can interfere with the suppressive function of the cells. High levels of pro-inflammatory cytokines are present in the inflamed synovium of RA and JIA patients, including IL-6, IL-7, IL-15 and TNF-α [30–32]. In addition, human CD25^{hi} cells express the TNF receptor, TNF receptor II (TNFRII) and expression of this receptor is up-regulated on cells from RA patients [27]. As a result, TNF-α can act directly on Treg and, in line with this, it was shown that pre-incubation of Treg with TNF-α reduces FOXP3 expression and abrogates suppression [27]. Other pro-inflammatory cytokines, IL-6, IL-7 and IL-15, can also interfere with Treg function [25, 33, 34], or even worse facilitate the conversion of Treg into IL-17 producing effector cells [35–37]. Finally, monocytes and dendritic cells from the site of inflammation express elevated levels of CD80, CD86 and CD40 [34, 38] and this enhanced expression of co-stimulatory molecules might also interfere with Treg-mediated suppression [34]. Thus, though Treg function in patients with RA and JIA is still incompletely understood, data from both animal models and human disease indicate that Treg play an important role in controlling autoimmune arthritis. As such, these cells form a promising treatment option for arthritis patients. Here, we will discuss several strategies to target these cells, both directly and indirectly.

Direct approaches to enhance Treg function

There are several methods available to directly target Treg for the treatment of autoimmune disease. These include expansion and induction of Treg in vitro followed by reinfusion into the patient, or in vivo by immunomodulatory compounds.

Ex vivo expansion of Treg

Treg can be isolated and expanded ex vivo by anti-CD3/anti-CD28 stimulation in the presence of IL-2 [39, 40]. With this protocol up to 3000-fold expansion can be reached without loss of suppressive function. Moreover, the cells have a higher inhibitory potential compared with directly isolated Treg, even in co-cultures with pre-activated effector cells [39]. Therefore, expanded Treg could have enhanced suppressive capacity in ongoing immune

TABLE 2 Presence, phenotype and function of Treg in arthritis

Disease	Markers used to identify Treg	Peripheral blood			SF			Reference
		Presence	Phenotype	Function	Presence	Phenotype	Function	
JIA	CD4+CD25high	↓	FOXP3 mRNA=	NA	⇑	FOXP3 mRNA↑ / HLA-DR MFI, CTLA-4 MFI, GITR MFI CD69+⇑	↑	[20]
JIA	CD4+CD25+ a	NA	NA	NA	⇐	FOXP3 mRNA⇑	⇐	[25]
RA	CD4+CD25+CD27+ b	NA	NA	NA→	⇐	NA	–	[21]
RA	CD4+CD25high	=	NA	NA→	NA	NA	NA	[22]
RA	CD4+FOXP3high a	=	CTLA-4 MFI↓	→	NA	NA	NA	[23]
RA	CD4+CD25+CD127low b	Early active RA → / stable RA =	NA	=	⇐	NA	NA	[24]
RA	CD4+CD25+	↑	CD69+ HLA-DR+ OX40+ GITR+= CTLA-4 MFI	=	⇑	CD69+ HLA-DR+, OX40+ GITR+, CTLA-4 MFI⇑	⇐	[26]
RA	CD4+CD25high	NA	FOXP3 mRNA→ TNFRII+ GITR+↑ CD69+ =	→	NA	NA	NA	[27]
RA	CD4+CD25+	=	NA	NA	⇐	CTLA-4+ GITR+, OX40+ CD69+↑⇑	–	[28]

aUsed for phenotyping. bUsed for functional assays. cSuppressive, but functionality not compared with peripheral blood. =: not changed; ↑: increased; ↓: decreased, compared with peripheral blood of healthy controls; ⇑: increased compared with paired peripheral blood of patients; NA: not analysed; MFI: Mean Fluorescence Index; +: percentage of positive cells.

responses *in vivo* and be useful in the treatment of auto-immune disease. In favour of this argument, it has been shown that *in vitro* expanded Treg survive upon transfer *in vivo* and reverse pathology in new-onset diabetic mice [41]. Similarly, in experimental lupus, adoptive transfer of expanded Treg delayed the progression to severe renal disease, resulting in prolonged survival [42].

However, a potential hazard with expanding Treg for therapeutic purposes is the outgrowth of contaminating effector cells, since it is difficult to distinguish Treg from activated effector cells. This risk can be reduced by adding rapamycin to expansion cultures, which selectively allows for regulatory T-cell proliferation and survival, while depleting effector cells [43, 44]. Still, expanded Treg can also convert into effector cells themselves. Using the same protocol as described before, Hoffmann *et al.* [39], discovered that, although FOXP3 purity at the start of culture was almost 100%, subpopulations of Treg lost FOXP3 expression and suppressive capacity. Further-more, these cells started to produce effector cytokines, such as IL-2 and IFN-γ. Only cells that co-expressed CCR7 and CD62L after expansion showed a stable Treg phenotype and these cells could be generated by select-ing the CD45RA+CD4+CD25high subpopulation for Treg expansion [45]. In addition, Tran *et al.* [46] identified latency-associated peptide and IL-1 receptor type I/II (CD121a/CD121b) as markers to purify stable Treg after expansion *in vitro*. However, under certain circumstances, FOXP3+ Treg can also convert into effector cells, after transfer *in vivo* [47]. This could pose a risk for worsening, instead of dampening inflammation, especially in autoim-mune disease [48]. Therefore, the stability of *ex vivo* expanded Treg should be further investigated and conditions reinforcing this stability need to be thoroughly determined. In addition, expansion of antigen-specific Treg can enhance efficacy [41] and reduce general immune suppression and this approach should, therefore, be explored in humans as well. Altogether, standardized protocols have to be developed to allow for reliable ex-pansion of Treg with compliance to the Good Manufactur-ing Practices approved by the US Food and Drug Administration. Only then will clinical application in a large cohort of patients become feasible.

In vitro induction of Treg

In addition to expansion of already existing Treg, Treg can also be induced *in vitro* from non-Treg. This method cir-cumvents the difficulty of obtaining high numbers of nat-ural Treg required for expansion. Treg induction works well in mice in which CD4+CD25− cells activated in the pres-ence of TGF-β develop into FOXP3-expressing cells with suppressive capacity that is maintained after transfer *in vivo* [11]. However, TCR stimulation of human CD4+CD25− cells can also result in transient expression of FOXP3 [49]. Furthermore, activation-induced expres-sion of FOXP3 in humans does not confirm a regulatory phenotype and can even coincide with IL-2 and IFN-γ pro-duction [50]. Therefore, *in vitro* induction of Treg is far more complicated in humans compared with mice and it still

TABLE 3 Immunoactive compounds with Treg-enhancing capacity

Compound	Effect on Treg	Results in experimental autoimmune disease	Results in human autoimmune disease
Anti-CD3 antibodies	Treg induction	Complete and sustained remission in recent-onset diabetic NOD mice [56]	Long-term improved insulin production in new-onset Type 1 diabetes [52, 53] Short-term improvement in the number of inflamed joints in PsA [54]
Neuropeptides	Treg induction, expansion and enhanced function	VIP reduces development of CIA and established disease in DBA/1J mice [57, 63] Urocortin reduces severity of established CIA in DBA/1J mice [64]	–
Retinoic acid	Treg induction	Reduced incidence of diabetes in NOD mice with established insulitis [70] Improved bodyweight and reduced colon inflammation in TNBS-induced colitis [71] Reduced severity and incidence of CIA in DBA/1J mice [72]	–
HDAC inhibitors	Enhancement and stabilization of FOXP3 expression leading to enhanced function	TSA and SAHA prevent bodyweight loss and histological damage in DSS-induced colitis [80, 81] TSA reduces development of renal pathology in lupus-prone NZB/W F1 mice [82] VPA reduces the incidence and severity of CIA in DBA/1J mice [83]	–

NOD: non-obese diabetic; TNBS: trinitrobenzene sulfonic acid; DSS: dextran sodium sulphate.

needs to be established which culture conditions reinforce stable FOXP3 expression and suppressive function.

In vivo expansion and induction of Treg with immunomodulatory compounds

Next to expanding and inducing Treg *in vitro*, several immunoactive agents can be used to enhance Treg function *in vivo*. The most extensively studied Treg-enhancing agents will be discussed in this section and are summarized in Table 3.

Anti-CD3 antibodies. The immunosuppressive efficiency of mAbs against CD3 was initially established in the transplantation field, where they prevented allograft rejection. After humanizing the antibodies into non-Fc-receptor-binding antibodies that are not mitogenic, application in the treatment of autoimmune disease was tested as well [51]. In new-onset Type 1 diabetes patients, treatment with humanized CD3 antibodies led to preserved β-cell function and reduced insulin need [52, 53]. Also in rheumatic disease, efficacy of anti-CD3 treatment was confirmed: in a Phase I/II trial in patients with PsA, administration of huOKT3γ1 led to a 75% improvement in the number of inflamed joints in six out of seven patients [54]. Studies in experimental diabetes further elucidated the mechanisms involved in immune suppression by anti-CD3 antibodies. These studies revealed that short-term disease improvement was achieved by elimination of pathogenic effector cells and Th2 polarization. However, long-term beneficial effects depended on a non-depleting, Treg-inducing activity of the antibody [55, 56]. Thus, CD3-specific antibodies are capable of inducing Treg and have already been proved to be safe and effective in patients with autoimmune disease. As such, they may provide a valuable treatment option for RA and JIA as well, which should be further investigated.

Neuropeptides. Vasoactive intestinal peptide (VIP), an immune-regulatory neuropeptide, has been shown to have suppressive effects in experimental autoimmune disease, including CIA. This suppressive effect was accompanied by inhibition of pro-inflammatory cytokines and chemokines and immune deviation towards Th2 responses [57, 58]. However, similarly to the CD3-specific antibodies, it has become clear that VIP is capable of enhancing Treg numbers and suppressive function as well [59], presumably via the induction of tolerogenic dendritic cells [60–62]. In CIA, administration of VIP increased both the absolute number and percentage of Treg, leading to lower arthritis scores [63]. Another neuropeptide, urocortin, also reduced disease severity in this model via the induction of Treg [64]. Although clinical trials in human autoimmune disease are still awaiting, neuropeptides could be of therapeutic value, due to their Treg-enhancing capacity.

Retinoic acid. All-trans retinoic acid (ATRA) is an active metabolite of vitamin A that regulates various cellular functions, including lymphocyte proliferation and differentiation. Recently, several research groups have found that ATRA induces Treg, while simultaneously inhibiting Th17 development [65–68]. Therefore, ATRA might be able to restore the balance between Treg and pathogenic Th17 cells that is thought to be disturbed in autoimmune pathology [69]. In experimental models of diabetes [70] and colitis [71], ATRA treatment improved clinical outcome by inducing Treg. ATRA-mediated induction of Treg has not been investigated in arthritis models; however, ATRA administration has been shown to reduce severity and incidence of CIA. This beneficial effect was accompanied by a decrease in pro-inflammatory cytokines and collagen-specific antibodies [72]. Given the therapeutic effects of ATRA in experimental arthritis and its potent Treg-enhancing capacity, it would be valuable to further explore this mechanism for the treatment of arthritis. In addition, Treg induced *in vitro* in the presence of ATRA are resistant to conversion into FOXP3$^-$ cells [73] and Treg expanded in the presence of ATRA have enhanced suppressive capacity [74]. Therefore, ATRA can also be used to optimize protocols for the *in vitro* expansion and induction of Treg.

Histone deacetylase inhibitors. The *FOXP3* gene is subject to epigenetic modifications, including acetylation mediated by histone acetyltransferases (HAT) that increases the negative charge of histones in the nucleosome. This leads to an open chromatin structure, allowing for gene transcription [75]. The described induction of Treg by ATRA probably depends on this modification, since acetylation of the *FOXP3* promotor is enhanced in ATRA-treated cells [76]. Acetyl groups can also be removed by histone deacetylases (HDACs), introducing a positive charge that leads to tight DNA binding and reduced transcription [75]. In addition, FOXP3 can directly interact with HAT and HDAC at the protein level [77] and a very recent study shows that hyperacetylation of FOXP3, reciprocally controlled by the acetyltransferase p300 and the HDAC SIRT1, prevents poly-ubiquination and subsequent proteasomal degradation of the protein [78]. Agents counteracting HDAC activity, so-called HDAC inhibitors, can therefore both increase *FOXP3* gene transcription and prevent protein degradation, thereby enhancing and stabilizing FOXP3 expression.

Two HDAC inhibitors, MS-275 and suberoylanilide hydroxamic acid (SAHA), have been shown to induce FOXP3 expression and suppressive function in human CD4$^+$CD25$^-$ cells *in vitro* [79]. Exposure to another HDAC inhibitor, nicotinamide, increased the number of FOXP3$^+$ cells in CD4$^+$ cell cultures as well as the amount of FOXP3 per cell and the suppressive capacity of CD4$^+$CD25$^+$ cells [78]. Also *in vivo,* administration of HDAC inhibitors leads to increased numbers of FOXP3$^+$ T cells with enhanced suppressive capacity. Moreover, treatment with HDAC inhibitors reduces pathology in dextran sodium sulphate-induced colitis [80, 81], lupus-prone mice [82] and experimental arthritis [83], by enhancing Treg function. Trichostatin-A (TSA) treatment even improved already established colitis and HDAC inhibitors have been shown to reduce the down-regulating effect of IL-6 on FOXP3 [84]. This makes them attractive candidates for the treatment of ongoing inflammation. Interestingly, several HDAC inhibitors are now being developed for application in human autoimmune disease based not on their capacity to enhance Treg function, but on their more familiar anti-inflammatory and immunosuppressive capacities. Among those agents is hydroxamic acid, which is being tested for therapeutic application in arthritis [85]. In addition, HDAC inhibitors can be used to stabilize FOXP3 expression in induced or expanded Treg since they prevent conversion of these cells into Th17 cells [35].

Antigen-specific induction of Treg by mucosal tolerization with self-antigen

The above-described methods are all based on enhancing the polyclonal Treg population. However, these non-specific approaches might lead to increased risk of infections and cancer, due to general immune suppression [86]. These unwanted side effects can be avoided by antigen-specific induction of Treg. This can be achieved by mucosal administration of self-antigen, which is a powerful way of inducing Treg towards a specific antigen [87]. Oral or nasal administration of self-antigens works well in animal models of arthritis, leading to delayed onset of disease and reduced severity [88–90], presumably via the induction of Treg [91–93]. Moreover, beneficial effects of oral antigen administration have also been described in already established disease, making a therapeutic application in humans feasible [94, 95].

However, results from animal models have been difficult to translate into humans. Clinical trials have shown that oral administration of antigen is safe [96–101]; however, in many cases only small improvements were found [97, 98], or only a minority of the patients responded to treatment [100]. These disappointing results are presumably caused by the fact that the disease-triggering antigen in humans is less clear and at the time of intervention multiple antigens are involved, due to epitope spreading [102]. Still, through bystander suppression, Treg specific for one antigen can also suppress immune responses towards other antigens that are presented in the same vicinity [103, 104]. This can be achieved by the production of non-specific, inhibitory cytokines, such as IL-10 and TGF-β by the induced Treg [88, 91, 92]. As a result, mucosal tolerization with self-antigen could work in human disease as well, as long as an immunogenic antigen is used that is presented at the same location as the self-antigens driving the immune response. A special class of proteins, termed heat shock proteins (HSPs), are promising antigens for this Treg induction via mucosal tolerization.

HSPs

HSPs are a set of evolutionarily conserved chaperones that are up-regulated under conditions of cellular stress,

for instance during infection and inflammation [105]. As a result, they are abundantly present at the site of inflammation in RA and JIA [106, 107] and, because of their unique features HSPs are very immunogenic [108–110]. Therefore, these antigens are good candidates for mucosal tolerization in autoimmune disease, since they trigger T-cell responses and are highly present at the site of inflammation. Moreover, studies with cells from JIA patients suggest that HSPs might have a natural role in controlling inflammation via the induction of regulatory responses [111–114].

Several HSP family members have been shown to be protective upon mucosal administration in experimental arthritis, even in already established disease [115], probably via the induction of Treg [116, 117]. Moreover, nasal administration of a mycobacterial HSP peptide inhibited adjuvant arthritis, but also arthritis induced by an unrelated, non-microbial stimulus [118]. Thus, HSPs suppress experimental arthritis irrespective of the initial trigger and are effective in already established disease. This makes them suitable for therapeutic application in human arthritis. Studies with OM-89, an extract of *Escherichia coli* used for the treatment of RA, provide the first evidence that HSP could be effective in the treatment of human arthritis. Multicentre placebo-controlled trials with OM-89 showed that it ameliorates RA with few side effects [119, 120]. Later on, analysis of the OM-89 content revealed that it contained HSP [121] and oral administration in animal models led to HSP-directed T-cell responses [122]. Therefore, HSP is thought to be responsible for the therapeutic effect of OM-89 in arthritis. More direct evidence comes from a pilot Phase II trial with a peptide derived from *E. coli* HSP, dnaJP1. Oral administration of this peptide in RA patients was well tolerated and led to enhanced IL-4 and IL-10, and reduced TNF-α and IFN-γ production towards the peptide. Furthermore, dnaJP1-induced expression of FOXP3 in CD25^{bright} cells was increased following treatment [123]. Subsequently, the clinical efficacy of this approach was studied in a placebo-controlled Phase II trial enrolling 160 patients with active RA. Again treatment was safe and well tolerated and reduced TNF-α responses towards dnaJP1 were found. Furthermore, a difference in the ACR20 and ACR50 score between treatment and placebo groups suggested clinical efficacy [124].

Indirect approaches to enhance Treg function

In addition to the above-described strategies that target the Treg population directly, indirect approaches can also be taken to enhance Treg function in patients with autoimmune disease. These include reducing the pro-inflammatory environment and enhancing responsiveness of effector cells to suppression.

Inhibition of pro-inflammatory cytokines

As described above, the *in vivo* pro-inflammatory environment at the site of inflammation in patients with autoimmune disease can have profound negative effects on Treg function. Therefore, dampening the ongoing inflammation, for instance by inhibiting pro-inflammatory cytokines, can indirectly lead to better Treg-mediated suppression. This is clearly shown by two studies that examined Treg function in RA patients before and after anti-TNF-α (infliximab) therapy. Both studies reported impaired Treg function before therapy, which was completely restored after infliximab treatment [22, 27]. Probably, neutralizing the high TNF-α levels in these patients directly reduced the down-regulating effect of TNF-α on Treg [27], thereby restoring their suppressive function. However, it is also possible that, instead of reconstituting the suppressive function of already existing Treg, anti-TNF-α therapy actually induced a new Treg population with enhanced regulatory potential [125].

Enhancing the responsiveness of effector cells to suppression

Indirect improvement of Treg function can also be achieved by enhancing responsiveness of effector cells to suppression. In Type 1 diabetes, inflammatory bowel disease and lupus, effector cells are refractory to inhibition by Treg [126–129]. Also in the SF of JIA and RA patients, effector cells appear to be less responsive to suppression compared with their peripheral blood counterparts [20, 26]. Elucidating the cause of this resistance to suppression and subsequent targeting will enhance Treg-mediated inhibition and restrict uncontrolled activation of effector cells. Several studies suggest that this can, at least partially, be achieved by blocking the production of pro-inflammatory cytokines. In experimental autoimmune encephalomyelitis (EAE), it was found that Treg isolated from the CNS could suppress effector cells from the spleen, but failed to inhibit effector cells isolated from the site of inflammation. When analysing these CNS effector cells, they were found to produce high levels of IL-6 and TNF-α. Furthermore, adding both these cytokines to naïve effector cells reversed their responsiveness to suppression. Thus, the increased resistance of effector cells at the site of inflammation in EAE mice is caused by TNF-α and IL-6 produced by these cells [130]. Another study describing the negative effects of IL-6 on Treg-mediated suppression also found that IL-6 acts on effector cells rather than on Treg [33]. Similarly IL-7, known to reduce Treg-mediated suppression, is expected to target effector cells as well [34], since expression of the IL-7 receptor (CD127) is low on Treg [131]. Therefore, blocking these pro-inflammatory cytokines will reduce the resistance of effector cells to suppression and thereby enhance control of inflammation by Treg.

Combination therapy

So far, we have described multiple approaches that can be taken to target Treg function in patients with autoimmune disease (Fig. 1), including direct, antigen-specific induction of Treg by tolerization with self-antigen. In addition, inhibition of the inflammatory response increases the responsiveness of effector cells to suppression and

Methods of enhancing Treg function in arthritis patients. Treg can be enhanced in arthritis patients via different methods: (**A**) isolation and *ex vivo* expansion of natural Treg or (**B**) *in vitro* induction of Treg from non-Treg (n-Tr), followed by reinfusion into the patient; (**C**) *in vivo* induction and expansion of Treg by anti-CD3 antibodies (αCD3), HADC inhibitors (HADCi) and neuropetides, such as VIP; (**D**) mucosal tolerization with self-antigen, preferably HSP; (**E**) indirect improvement of Treg function by enhancing the responsiveness of effector cells to suppression and blocking pro-inflammatory cytokines.
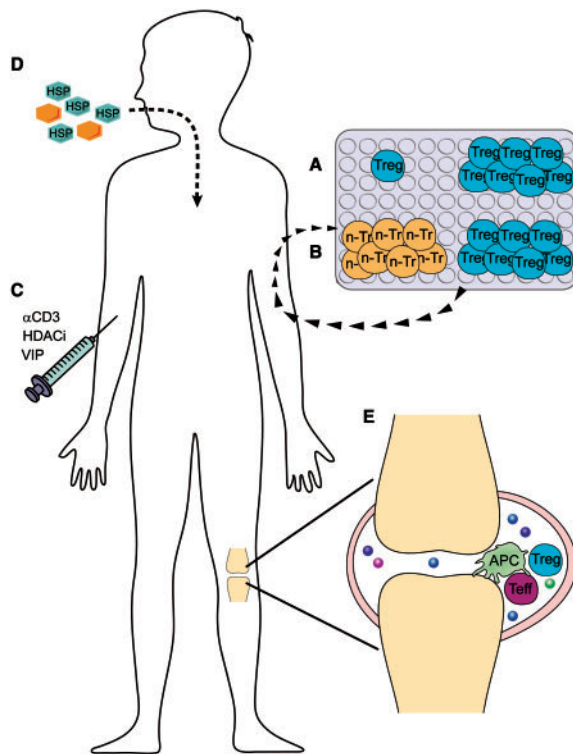
reduces the down-regulating effect of pro-inflammatory cytokines on Treg, thereby indirectly enhancing Treg function. It is therefore expected that clinical outcome can be enhanced by a combination of both these direct and indirect strategies. This is nicely illustrated by a study in which antigen-specific induction of Treg was combined with anti-TNF-α therapy in adjuvant arthritis. Both nasal administration of HSP60 peptide as well as a single dose of anti-TNF-α (etanercept) treatment, led to a small and insignificant reduction in arthritis scores. However, combining the two therapies resulted in a highly significant improvement of disease, as shown by lower arthritis scores and reduced joint destruction [132]. In addition, in several models of autoimmune diabetes, mucosal tolerization with islet antigen induced Treg and prevented development of disease, but was incapable of reversing established disease. Bresson *et al.* [133] now show that, when combined with a suboptimal dose of anti-CD3 therapy, intranasal administration of proinsulin peptide reverses recent-onset diabetes. Also in humans

there is evidence for enhanced effectiveness of Treg induction, when combined with anti-inflammatory treatment. In the previously described trial with dnaJP1 in RA patients a synergistic clinical effect was found in patients receiving HCQ, a drug with potent anti-inflammatory properties [124]. Together, these data clearly demonstrate that combining Treg induction with anti-inflammatory treatment enhances clinical outcome. In addition to increased effectiveness, dampening the ongoing inflammation might also be crucial in preventing adverse effects, as it has been shown that in a pro-inflammatory environment TGF-β produced by Treg drives Th17 differentiation [37, 134] and Treg can convert into Th17 cells themselves [35–37].

Autologous bone marrow transplantation as a multifactorial therapeutic approach

One very powerful therapy applied for the treatment of refractory autoimmune disease is autologous bone marrow transplantation (aBMT). The idea of using aBMT in the treatment of autoimmunity stems from observed remission in patients transplanted for co-existing haematological malignancies and from efficacy in experimental models [135]. Initially, the mechanism of action was thought to depend on the elimination of autoreactive lymphocytes by intensive immune ablation, followed by the development of a new tolerant lymphocyte population after aBMT. However, more recently it has become clear that induction of Treg is also important in the clinical efficacy of aBMT [136]. aBMT has been used in the treatment of RA and systemic JIA patients who are unresponsive to other treatments [135, 137]. Especially in systemic JIA patients, this approach has been successful, leading to long-lasting, drug-free remission in 53% of the patients and a partial response in 18% of patients [137]. In a follow-up study of JIA patients receiving aBMT, it was demonstrated that in addition to a more tolerogenic response observed in effector T cells, Treg were affected as well. The low Treg levels before treatment were restored after aBMT and even after long-term follow-up the numbers of Treg were significantly increased compared with pre-treatment [138].

The importance of Treg in aBMT has also been investigated in experimental models of autoimmune disease. In EAE, pseudo-autologous BMT prevented relapses and resulted in increased levels of CD25^{bright} cells and FOXP3 mRNA expression [139] and in CIA, co-transfer of purified Treg with the graft enhanced clinical outcome [18]. Furthermore, in proteoglycan-induced arthritis, it was found that depletion of CD25$^+$ Treg after pseudo-autologous BMT abrogated disease remission induced by aBMT [140]. This last result clearly demonstrates a key role for Treg in the clinical efficacy of aBMT, next to elimination of autoreactive T cells and reduced inflammation, caused by immune suppression. aBMT is therefore a good example of how a multifactorial approach targeting Treg, effector T cells and ongoing inflammation is highly

effective, even in the treatment of severe, systemic autoimmunity. It also shows that intensive immune ablation followed by aBMT provides an environment that is optimally suited for the development of Treg and might provide a window of opportunity for the induction of antigen-specific Treg.

Conclusion

Treg play a critical role in controlling autoimmune disease and several strategies are now being explored to target these cells for therapeutic purposes. For patients with RA and JIA, Treg provide a valuable new treatment option, since current therapies, such as anti-TNF-α therapy, cause a rather general immune suppression and do not induce sustained remission. As a result, side effects occur and life-long treatment is required. To enhance Treg function, the cells can be expanded and induced *in vitro* followed by adoptive transfer. However, these protocols have severe drawbacks, especially the risks associated with conversion of Treg into effector cells, and the costs and complexities associated with cellular therapy. Alternatively, Treg can be induced *in vivo* by immunomodulatory compounds and some of these agents have already been tested in patients.

Also, to avoid risks associated with general immune suppression, antigen-specific induction of Treg provides a potential safe and efficient approach, for which HSPs are promising candidate antigens. These proteins induce Treg that specifically recognize antigen at the site of inflammation, thereby avoiding systemic immune suppression. Clinical trials have shown that HSP treatment is safe and induces clinical improvement. Since the majority of studies indicate that Treg are not deficient in arthritis patients, but are functionally compromised by their pro-inflammatory environment, the efficacy of this approach can be optimized by inhibiting the ongoing inflammation in these patients. This is illustrated by a synergistic effect of Treg induction and anti-inflammatory treatment in both patients and experimental models. When combined with HSP treatment, only a single dose of anti-TNF-α therapy is sufficient to reduce pathology in experimental arthritis. The possibility of lowering the dose of anti-inflammatory treatment will have great impact on patient care, since it reduces the side effects associated with life-long drug administration. Therefore, Treg targeted approaches may significantly add to therapies that are in the clinic for arthritis today and deserve thorough future investigation.

> ### Rheumatology key messages
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> - Treg are attractive targets for immune modulation in RA and JIA.
> - Antigen-specific induction of Treg will reduce side effects associated with general immune suppression.
> - Combination therapy has enhanced clinical efficacy and reduces the risk of adverse effects.

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