EXPERT REVIEWS

T-cell-based immunotherapy of autoimmune diseases

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Dysregulated Th1/Th17 responses, frequently paralleled by a reduction or absence of Treg cells, are key features of autoimmune disease. Conventional therapeutic approaches for autoimmune disease treatment are largely based on nonspecific immunosuppression leading to substantial side effects. Recent developments in the area of CD4+ T-cell differentiation, together with experimental and preclinical findings with blockers of the IL-17 pathway and the use of Treg cell-based therapy, indicate that this CD4+ effector subset could represent effective targets to restore immune tolerance. Here, the authors summarize the recent progress in our understanding of the pathways and cues that drive CD4+ T-cell differentiation into specialized effectors, focusing on Th17 and Treg cells. The authors also discuss novel immunotherapeutic strategies based on targeting these T-cell subsets for autoimmune disease treatment.

Keywords: autoimmunity • CD4+ T-cell subset • cell differentiation • cellular therapy • regulatory T cells • Th17 cells

Overview of helper T-cell subsets

The immune system has developed throughout evolution as a potent means of defense against pathogens. The selective production of cytokines, which in turn recruit and activate or suppress other immune cells, is the principal mechanism by which CD4+ T cells orchestrate the protective immune response. CD4+ T cells are endowed with a dual function. On the one hand, they promote the clearance of invading pathogens by enabling antigen-specific immune responses, and on the other they turn off inappropriate effector responses, thereby preventing the clinical manifestations of autoimmune disorders. CD4+ T cells can be subdivided into two main groups based on their role during the immune response: effector cells, which are required for clearing pathogens from the organism, and regulatory cells, which control the duration and strength of pathogen-induced immune responses and hence avoid injury to healthy tissues.

Traditionally, effector CD4+ T cells were subdivided into Th1, Th2 and Th17 cells according to the expression of signature cytokines, master transcription factors and biological function [1]. Th1 cells express the transcription factor T-bet, secrete IFN-γ, TNF-α and IL-2 as main cytokines, and protect from viruses, mycobacteria and protozoa by promoting activation of macrophages and bacterial killing. Th2 cells express the transcription factor GATA3, secrete IL-4, IL-5, IL-9 and IL-13, and confer protection against extracellular parasitic infections. Th17 cells, which were identified in 2005 as a distinct CD4+ effector lineage [1–3], are characterized by expression of the transcription factor ROR-γt and production of IL-17, IL-21 and IL-22. Opposite to Th1 and Th2 cells, which were first identified as protective effector cells important for clearing intracellular and extracellular pathogens, respectively, Th17 cells were initially described in the context of autoimmunity as a potent pathogenic subset [4] and it was only later that their protective role against extracellular and intracellular bacteria as well as viruses and fungi was appreciated [5–7]. At variance with Th1-mediated protective responses, which mainly involve macrophage activation, a diagnostic feature of protective Th17 responses against intracellular pathogens is the recruitment of neutrophils and stimulation of antimicrobial peptide release by epithelial cells [8].

Recently, two other effector CD4+ subsets, termed Th9 and Th22, were described. Th9 cells express the transcription factor PU.1, secrete IL-9 and IL-10 as main cytokines and, similar to Th2 cells, mediate a protective response against helminthes. The pathogenic function of Th9 cells is still controversial as they are reported to contribute to inflammation in several autoimmune and allergic diseases but conversely were

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reported to promote Treg function [9]. Th22 cells are characterized by IL-22 (but not IL-17 or IFN-γ) production and their development is dependent upon the aryl hydrocarbon receptor. Similar to Th17 cells, Th22 cells play a key role in host defense against fungi and extracellular bacteria. In addition, they appear to contribute to skin homeostasis and pathology, based on their expression of the skin homing receptors CCR4 and CCR10 and their presence in inflamed skin tissue [10].

Tregs, which are responsible for immunological self-tolerance and homeostasis, were first described by Sakaguchi *et al.* in 1995 [11] as T cells expressing CD25 that possess immunosuppressive activity. To date several different types of Tregs have been described, including NKT cells, $CD8^+$ cells, TGF-β-producing cells, $CD4^-CD8^-$ T cells, γδ T cells, IL-10-producing $CD4^+$ T cells and $CD4^+CD25^+Foxp3^+$ T cells [12]. The latter are subdivided mainly into two distinct populations. The first, referred to as natural Treg (nTreg), develops in the thymus as a distinct lineage and as such is characterized by a stable phenotypic profile and function. The second, referred to as inducible Treg (iTreg), appears to be converted extrathymically from conventional $CD4^+$ T cells in response to microenvironmental inputs. Opposite to nTreg, the latter population, like other $CD4^+$ subsets, is endowed with high plasticity to optimize the immune response to invading microbes [13].

$Foxp3^+$ Tregs dampen T-effector responses both by direct inhibition of T-cell function and indirectly through the suppression of APC function. Specifically, T-cell suppression is achieved by secretion of suppressor cytokines (TGF-β, IL-10 and IL-35), IL-2 consumption, cytolysis and expression of surface molecules that induce cell-cycle arrest in the effector cells. Suppression of APC function by Tregs is achieved through multiple mechanisms that include: inhibition of APC function by preventing upregulation of costimulatory molecules (CD80, CD86); suppression of APC maturation and function through the binding of LAG3 to MHC class II on Tregs; decreased antigen presentation by restricting access of the effector cells to APC as the result of a prolonged interaction between Tregs and APCs, which is mediated by the Treg-associated receptor neuropilin 1 [14]. In this context, it has been shown that while antigen-specific Tregs affect the differentiation and/or expansion of effector T cells by inhibiting correct antigen presentation by APC, polyclonal Tregs affect antigen (Ag)-activated T-effector cell responses by inhibiting their egress from the lymph node; polyclonal Tregs have been indeed shown to downregulate sphingosine 1-phosphate receptor 1 on Ag-primed T-effector cells, thereby preventing their traffic to tissues [15].

For many years, both effector and regulatory $CD4^+$ T cells have been considered terminally differentiated lineages and as such characterized by a stable phenotype. However, recent evidence suggest that Th1, Th2, Th17 and Treg cells display plasticity, as witnessed by their ability to change the pattern of expression of both cytokines and master regulator transcription factors in response to external stimuli, suggesting that seemingly fully committed Th cells are actually a dynamic population [16]. An intriguing view of $CD4^+$ T-cell plasticity was proposed by Murphy and Stockinger [17], who suggest a model where, similarly to the energy levels of an electron in the atom, each $CD4^+$ subset transits from a higher energy (less stable) to lower energy (more stable) state. In this model, a possible hierarchy of stability is proposed according to experimentally observed transitions, with naive T cells being the least stable subset that has the possibility to decay into one of the other subsets depending on T-cell receptor (TCR) stimulation and the cytokine milieu, and Th1 and Th2 cells being the most stable subsets. Recent evidence has, however, demonstrated that reprogramming of stably committed Th2 cells to a hybrid phenotype that express GATA3 and T-bet as well as IL-4 and IFN-γ is possible [18], and that the programmed death 1 receptor signaling is responsible for converting human Th1 cells to a regulatory phenotype [19], suggesting that populations believed to be fixed are also flexible. Although these new findings argue a plasticity for Th1 and Th2 cells, Treg and Th17 cells maintain the highest instability of their differentiation program, which appears to be shaped by environmental cues and hence on the presence of microbes and signals from innate immune cells [20]. In this context, a plasticity of mouse Th17 cells toward a Th1-like profile has been reported under chronic inflammatory conditions such as those occurring in experimental autoimmune encephalitis (EAE), Type 1 diabetes (T1D) and colitis [20,21]. A cytokine-driven conversion of Treg to a Th1- or Th17-like phenotype has also been described. A switch of Treg to Th1-like cells occurs indeed both in healthy individuals in response to IL-12 and in subjects with relapsing/remitting multiple sclerosis (MS) where elevated IL-12 expression has been reported [22]. In addition, a conversion of Treg to Th17-like cells has been observed in the presence of fungal infections or in response to IL-6 and TGF-β, supporting the notion that the cytokine environment controls the plasticity of Th17 and Treg cells [20].

The central role played by the local microenvironment not only in the differentiation of $CD4^+$ T cells but also in the maintenance of their identity underscores the importance to elucidate the signals that control the flexibility of Th17 and Treg cells *in vivo* in order to understand the role of each specific subset in autoimmunity as well as for designing therapeutics.

Differentiation of Th17 cells

Upon encountering APC, naive $CD4^+$ T cells undertake a differentiation program that is initially tightly regulated by the TCR ligand and costimulatory molecules on the APC itself, as well as by the cytokine milieu, the composition of which is dependent on cytokine secretion by both activated APC and other innate immune cells. The cytokines present in the microenvironment are the main cues driving differentiation of $CD4^+$ T cells. To date IFN-γ and IL-12 are known to drive Th1 differentiation, IL-4 drives Th2 differentiation and IL-6 in combination with TGF-β drives Th17 differentiation [1]. Although IL-6 and TGF-β were initially shown to be necessary and sufficient to drive Th17 polarization in a mouse model [23], an IL-6-independent pathway of Th17 differentiation was later identified. Several groups showed that IL-21 together with TGF-β was able to induce Th17 cells in $IL\text{-}6^{-/-}$ mice [24] and that $IL\text{-}21^{-/-}$ T cells were markedly impaired in the induction of inflammatory Th17 cells characterized by the expression of IL-23R [25], suggesting a key role for IL-21 in

both Th17 differentiation and activation. Moreover, induction of antigen-specific Th17 cells has been shown to be abrogated in IL-1 receptor type I deficient mice, indicating that IL-1 is also implicated in Th17 cell differentiation [26]. Furthermore, IL-23, while dispensable for Th17 generation from naive CD4$^+$ T cells in the mouse, is required for Th17 cells to stabilize their phenotype and to acquire effector function [27].

In humans Th17 cells originate in response to IL-1β and IL-23 from a small subset of CD161$^+$ CD4$^+$ naive T cells, which constitutively express both IL-23R and the transcription factor RORC2, the human ortholog of mouse ROR-γt (Figure 1) [23]. The role of TGF-β in human Th17 differentiation is more debated. According to data obtained by two independent groups, TGF-β is not essential for human Th17 differentiation [23]; however, others have demonstrated that, as in the mouse, TGF-β is required for this process [28]. Romagnani *et al.* proposed that TGF-β does not play a direct role in human Th17 differentiation, but indirectly favors the expansion of Th17 cells by inhibiting the development of Th1 cells [29]. A significant step towards elucidating the cytokine milieu responsible for driving human Th17 differentiation has been achieved by shifting the studies from *in vitro* polarization experiments using different combinations of cytokines to the physiologically relevant *in vivo* setting of infection. Zielinski *et al.* recently demonstrated, using autologous monocytes pulsed with whole microbes, that human naive CD4$^+$ T cells differentiate towards two types of Th17 cells [30]. Specifically, *Candida albicans*-specific Th17 cells were shown to secrete IFN-γ in addition to IL-17, whereas *Staphylococcus aureus*-specific Th17 cells produced IL-10. This study provided the first evidence that the balance among polarizing cytokines induced by different microbes rather than their absolute amount is determinant in human Th17 cell differentiation. Moreover, it demonstrated that, while IL-6, IL-23 and IL-1β are required for Th17 differentiation in the presence of both pathogens, IL-1β is essential to promote the differentiation of *C. albicans*-specific proinflammatory Th17 cells that produce IL-17 and IFN-γ.

Orphan nuclear receptors ROR-γt and ROR-α orchestrate the Th17 program of differentiation in both humans and mice, leading to the transcriptional activation of the genes encoding IL-17, IL-22 and IL-23R in a STAT3-dependent manner [31]. While expression of ROR-γt is induced by IL-6, IL-21, IL-23 or TGF-β, the combination of TGF-β and IL-6 has been shown to synergistically upregulate the levels of *ROR-α* mRNA. Beside ROR-γ and ROR-α, other transcription factors have been shown to induce IL-17, IL-21 and IL-22 expression by cooperatively binding the same target gene such as *Runx1* and *BATF* [31–33]. IRF4 and aryl hydrocarbon receptor have been also demonstrated to be important for IL-17 and IL-22 expression, respectively [31].

Th17 cells in autoimmunity & IL-17/Th17-directed therapy

The first evidence that Th17 cells are implicated in the development of autoimmunity came from studies on EAE, a model for human MS [34]. The central role of Th17 in the pathogenesis not

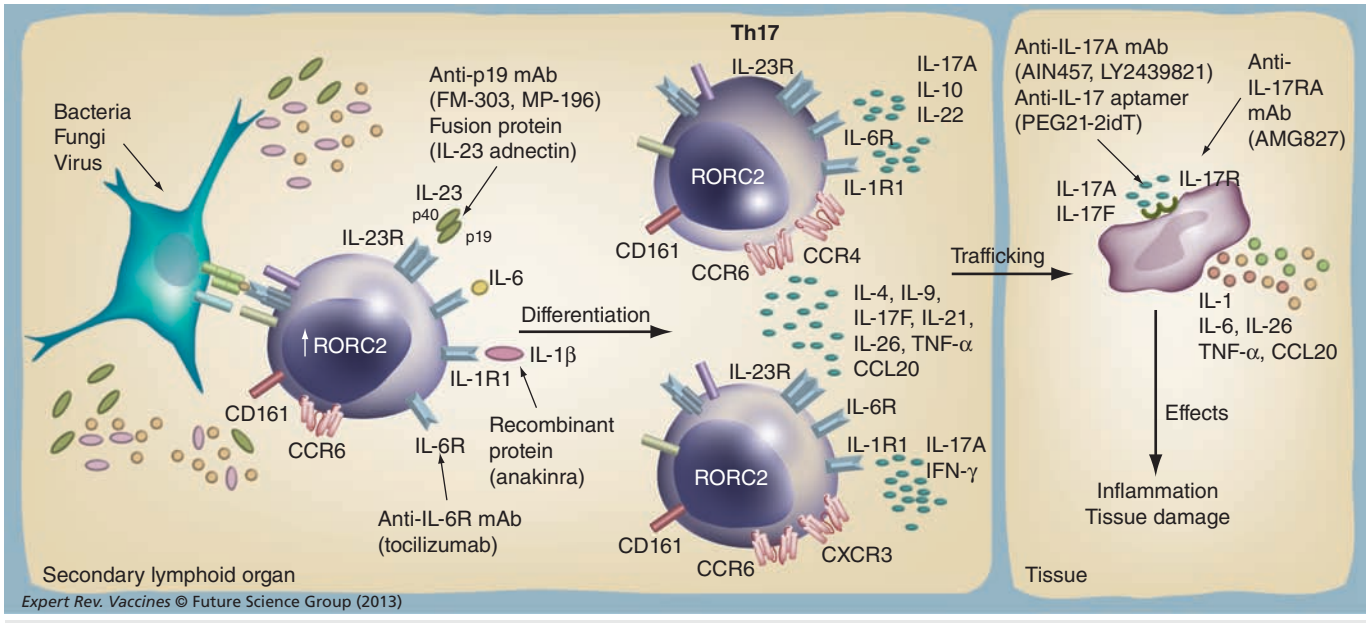

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Figure 1. Differentiation of human Th17 cells and Th17-based immunotherapies. T-cell receptor signals and cytokines present in the microenvironment drive CD4$^+$ naive T-cell differentiation in secondary lymphoid organs. Specifically, a small subset of human CD161$^+$ RORC$^+$ CD4$^+$ naive T cells differentiate towards Th17 cells in the presence of IL-23, IL-6 and IL-1β. Upon antigen stimulation, Th17 cells produce several cytokines and chemokines including IL-17A, IL-17F, IL-22, IL-10, IFN-γ, IL-4, IL-9, IL-21, IL-26, TNF-α and CCL20, which are responsible for Th17 responses. The Th17 cells compartment comprises two types of Th17 cells characterized mainly by the production of IL-17 and IFN-γ or IL-17 and IL-10, respectively. Ag-primed Th17 cells leave secondary lymphoid tissues and infiltrate into target tissues driven by CCR6/CCL20. There the release of Th17-derived cytokines induces inflammation and tissue damage. Specific biological therapies inhibiting Th17 differentiation and IL-17 effector function are shown.
mAb: Monoclonal antibody.

only of MS, but of several autoimmune and chronic inflammatory diseases, is now widely acknowledged. In addition to MS, elevated IL-17 and enhanced Th17 infiltration in inflamed tissue have been indeed described both in experimental autoimmune disease models and in human autoimmune diseases such as psoriasis, rheumatoid arthritis (RA), autoimmune diabetes and Sjögren's syndrome (Ss), as well as in chronic inflammatory diseases including inflammatory bowel diseases [35]. Since the proinflammatory cytokines released by Th17 cells have been identified as the major cause of tissue destruction in these pathological settings, several therapeutic approaches that target Th17 effector molecules or directly inhibit Th17 cells have been developed. Here we review the existing IL-17/Th17-directed therapies as well as the therapeutic potential of novel agents.

Keeping in mind the simplistic scheme of Th17 differentiation and their pathogenic function in autoimmunity depicted in Figure 1, the authors can summarize the Th17-based immunotherapies as strategies to control Th17 differentiation, Th17 trafficking and activities of the Th17 effector molecules.

Strategies to control Th17 differentiation

Deregulated production of proinflammatory cytokines such as TNF-α, IL-6 and IL-1β is a hallmark of autoimmune and chronic inflammatory diseases. Since this defect has been correlated to many clinical symptoms, biological therapies have been designed to target proinflammatory cytokines using specific blocking antibodies for either the cytokines or their receptors as well as recombinant receptor antagonists (i.e., anakinra, the human recombinant IL-1R antagonist).

However, notwithstanding the significant improvement in the quality of life, this approach may cause severe side effects because of the pleiotropic action of cytokines. The discovery that some proinflammatory cytokines are required to drive human $CD4^+$ T-cell differentiation, together with the identification of Th17 cells as the key players in autoimmunity, may pave the way to novel intervention strategies.

Blocking human Th17 differentiation could be achieved by inhibiting the effects of polarizing cytokines such as IL-6, IL-1β and IL-23 (Figure 1 & Table 1). Inhibition of IL-6 or IL-6R has been used extensively to treat chronic inflammatory autoimmune diseases, such as RA, systemic onset juvenile idiopathic arthritis (soJIA), CD and systemic lupus erythematosus (SLE), where a deregulated production of IL-6 has been described [35]. An example is tocilizumab (TCZ), a humanized monoclonal anti-IL-6R antibody developed to improve inflammation and joint destruction in RA. This drug is currently approved in Japan and Europe in adult patients with moderate to severe RA who do not respond to other treatments, and promising results have been obtained in Phase I trials of SLE [36]. At present, the precise mechanisms by which TCZ exerts its ameliorative effects in a number of autoimmune diseases is not completely understood since IL-6, like other proinflammatory cytokines, exerts pleiotropic effects. Since IL-6 is a key cytokine in the differentiation of both Th17 and Tregs and several autoimmune diseases are characterized by an imbalance in Th17/Tregs together with elevated levels of IL-6, TCZ may improve the symptoms by restoring the balance between Th17 and Treg. Recent evidence demonstrated that TCZ is indeed able to correct the Th17/Treg imbalance in patients with RA, resulting in clinical improvement. Importantly, TCZ treatment does not induce a significant decrease in the levels of serum IL-6, suggesting that the reduction of IL-6-induced inflammation is not the primary mode of action of this biological drug [37]. Despite the beneficial effects of TCZ treatment, a greater frequency of neutropenia, thrombocytopenia, hyperlipidemia and elevated transaminase values were observed in these patients compared with patients treated with placebo. Hence, further studies are required to better characterize the risk–benefit profile of monoclonal anti-IL-6R antibody therapy [38]

Results obtained with anakinra, a human recombinant IL-1R antagonist approved for the treatment of RA, resemble those obtained with TCZ both in its ability to correct the Th17/Treg imbalance and in its hepatotoxicity [39]. However, as for anti-IL-6R monoclonal antibody (mAb)-based therapy, further studies are required to assess the precise mechanisms of action of anakinra on Th17 cells.

To date biological drugs used to target IL-23 or the IL-23R, including ustekinumab and briakinumab, have been demonstrated to significantly improve the clinical manifestations of psoriasis and inflammatory bowel diseases but not MS [40]. Since p40 is the shared subunit of IL-12 and IL-23 and both the abovementioned antibodies are specific for p40, a possible explanation of this failure is the lack of specific inhibition of IL-23. The demonstration that IL-23 rather than IL-12 is the critical cytokine for autoimmune inflammation in the brain [34], together with the finding that IL-23 drives human Th17 differentiation [23], suggest that selectively targeting IL-23 could improve the therapy of all Th17-related autoimmune diseases, including MS. Preclinical studies using an anti-p19 mAb or peptide-based vaccines targeting the IL-23p19 subunit have indeed shown effectiveness in both the EAE model of MS and in collagen-induced arthritis (Table 1). Several pharmaceutical companies are now developing new drugs that specifically block IL-23R, including MP-196, FM-303 and IL-23 adnectin, for autoimmune disease treatment (Table 1).

Since ROR and particularly one isoform of ROR-γ, ROR-γt, have been shown to regulate Th17 differentiation [31], its selective blockade could represent a valid therapeutic approach to treat Th17-mediated autoimmunity. Accordingly, two independent studies reported for the first time that digoxin and SR1001, two synthetic ligands for ROR-γ and ROR-γ/ROR-α, respectively, are able to reduce ROR-γt transcriptional activity, Th17 cell differentiation and IL-17 production, as well as the severity of autoimmune disease in mice (Table 1) [41]. At present several ROR-selective modulators, both natural and synthetic, have been identified and shown to suppress Th17 cell development and function; however, a major drawback of these compounds is their toxicity and further studies are warranted [41].

Strategies to control Th17 trafficking

Different subsets of $CD4^+$ T cells express distinct patterns of chemokine receptors that allow their recruitment to the site of

Table 1. Th17-based immunotherapy in preclinical models and humans.

Drug	Target	Disease	Status	Ref.
Tocilizumab	IL-6R	Moderate rheumatoid arthritis	Approved	
Ustekinumab	IL-12R/ IL-23R (p40)	Psoriasis Crohn's disease, inflammatory bowel disease, colitis	Approved Phase III (NCT01369342)	[201]
Briakinumab	IL-12R/ IL-23R (p40)	Moderate-to-severe plaque psoriasis	Withdrawn Phase I (NCT01260844)	[201]
MP-196	IL-23R (p19)	Autoimmune disease	Clinical	[87]
FM-303	IL-23R (p19)	Inflammatory bowel disease	Discovery	[87]
IL-23 adnectin	IL-23R (p19)	Immune disorder	Discovery	[87]
Sekukinumab	IL-17A	Rheumatoid arthritis	Phase III (NCT01377012/NCT01640938/ NCT01350804)	[201]
		Psoriasis	Phase III (NCT01544595/NCT01640951/ NCT01406938/NCT01365455/NCT01412944/ NCT01392326)	
		Ankylosing spondylitis	Phase III (NCT01358175)	
		Refractory Behçet's disease	Phase III (NCT00995709)	
		Crohn's disease	Phase II (NCT01009281)	
		Multiple sclerosis	Phase II (NCT01051817/NCT01433250)	
Ixekizumab	IL-17A	Psoriasis	Phase III (NCT01474512/NCT01597245/ NCT01695239/NCT01646177/NCT01624233)	[201]
		Rheumatoid arthritis	Phase II (NCT00966875)	
AMG-827	IL-17R	Rheumatoid arthritis	Phase II (NCT01059448)	[201]
		Psoriatic arthritis	Phase II (NCT01516957)	
		Crohn's disease	Phase II (NCT01150890)	
Drug	**Target**	**Mouse model**	**Effects**	**Ref.**
Anti-p19 mAb	IL-23R (p19)	EAE	Block of both acute disease and EAE relapse	[88]
		CIA	Protective	[89]
SR1001	ROR-γ/ ROR-α	EAE	Delayed onset and reduced severity of EAE	[90]
Digoxin	ROR-γ	EAE	Delayed onset and reduced severity of EAE	[91]
PEG21-2idT	IL-17A	EAE	Inhibition of development of symptoms	[92]
		Induced rheumatoid arthritis		

CIA: Collagen-induced arthritis; EAE: Experimental autoimmune encephalomyelitis; mAb:Monoclonal antibody.
Data taken from [201].

inflammation. Th1 cells express CXCR3, CCR5 and CXCR6 whereas Th2 cells express CCR4, CCR8 and CRTH2, Th17 cells express CCR4 CCR6, CXCR3 and CXCR6, and Tregs express CCR5, CCR4 and CCR6 [42,43]. To date, CCR6 expression, together with IL-17 production, has been considered as the specific marker of the Th17 subset [42] However, it was recently demonstrated that among Th17 cells, referred to as IL-17$^+$CCR6$^+$, two distinct populations could be identified based on the expression of additional chemokine receptors. Specifically, CCR4 was found to be expressed in Th17 cells that secrete both IL-17 and IL-22 and CXCR3 in cells producing IL-17 and IFN-γ [42]. A functional link between CCR6 expression and the recruitment of pathogenic Th17 cells has been documented both in human autoimmune diseases and in the respective animal models, which include RA, psoriasis and EAE [42,44,45], suggesting that selective CCR6 antagonists might be an effective tool to target Th17 cells. Unfortunately, data obtained in mouse models do not consistently support the effectiveness of CCR6 inhibition. Inhibition of CCR6 signaling using neutralizing anti-CCR6 mAb or *CCR6* gene disruption has been reported to reduce the severity of EAE [42,46]. The lack of CCR6 expression in Treg has, however, been demonstrated to impair their recruitment to inflammed tissues, resulting in disease exacerbation in EAE and experimental glomerulonephritis [47,48]. These findings indicate that CCR6 expression *per se* is not necessarily pathogenic and that targeting CCR6/CCL20 may be beneficial only in autoimmune diseases

where CCR6$^+$ Th17 cells rather than Treg cells are implicated. Potentially targetable conditions are the first wave of pathogenic T-cell entry into the CNS in MS as well as in active MS, where Th17 cells play a prominent role [42]. An important consideration is that CCR6 is expressed not only by Th17 and Treg cells, but also by B cells, neutrophils and monocytes. Hence, targeting the CCR6/CCL20 axis to target Th17 cells will have to take into account unwanted effects both on Treg cells and on the other immune cells expressing CCR6.

Beside the chemokine/chemokine receptor axis, T-cell recruitment to inflammatory sites is tightly regulated by the expression of adhesion molecules on both the vascular endothelium and T cells. Integrins, including LFA-1 ($\alpha L\beta 2$) and VLA-4 ($\alpha 4\beta 1$), are one of the major families of adhesion molecules expressed on T cells and their interaction with specific ligands expressed on the endothelium (ICAM-1 and VCAM1, respectively) critically regulates leukocyte adhesion and spreading as well as transmigration of activated leukocytes into inflammatory sites [49]. VLA-4 has been proposed as the major adhesion molecule allowing entry of T cells in the CNS. Based on this consideration, neutralization of $\alpha 4$ integrin by the mAb natalizumab has been used in the treatment of MS. However, while clinical trials have documented the efficacy of this treatment, a significant number of patients developed progressive multifocal leukoencephalopathy, probably as the result of a defect in the recruitment of protective immune cells into the CNS, thereby limiting the use of this therapy [49]. Efforts are being made to understand the molecular cues mediating the infiltration into the CNS of pathogenic T cells, namely Th1 and Th17 cells, to design a selective drug for MS. In this context, Rothhammer *et al.* showed that the entry of Th17 cells into the brain is dependent on LFA-1 and occurs in the absence of VLA-4, whereas Th1 cells preferentially infiltrate using a VLA-4-dependent mechanism [50]. In addition, a higher expression of $\alpha 4$ integrin on Th1 cells compared with Th17 cells has been reported [50]. In agreement with a specific usage of LFA-1 by Th17 cells it was recently demonstrated that the adhesion to ICAM-1 of Th17 but not of Th1 cells was dependent on integrin activation by CCL20 [51], indicating that the entry of Th1 and Th17 cells into the CNS relies on different mechanisms. Taken together, these findings support the notion that specifically targeting Th1 and Th17 adhesion molecules may be advantageous in the treatment of autoimmune disease such as MS, where both subsets infiltrate into the CNS but with a different timing [42].

Strategies to control Th17 effector molecules

Th17 cells were originally identified as a novel CD4$^+$ T-cell subset able to produce the cytokine IL-17A, but were subsequently shown to secrete several other cytokines and chemokines, including IL-17F, IL-9, IL-21, IL-22, IL-26, TNF-α, IFN-γ, IL-4, lymphotoxin-β, IL-10 and CCL20. The inappropriate release of these proinflammatory mediators by Th17 cells has been shown to result in pathogenic inflammation and to be the cause of the pathological manifestations of a number of autoimmune disorders [52]. Since IL-17 is the signature cytokine of Th17, Th17-targeted therapeutic approaches based on direct blockade of IL-17 are currently the most promising therapeutics (Figure 1 & Table 1). The IL-17 cytokine family includes six members (IL-17A–F) and five IL-17R family members (IL-17RA–E) have been identified [53]. Th17 cells are believed to only produce IL-17A and IL-17F, hence these cytokines are currently being investigated as targets for Th17 immunotherapy. To date several neutralizing anti-IL-17A antibodies have been developed, including AIN457/sekukinumab (Novartis, Basel, Switzerland) and LY2439821/ixekizumab (Eli Lilly, IN, USA), and results obtained in recent clinical trials showed that both antibodies improved the clinical symptoms of RA and psoriasis and showed a good safety profile. AIN457 is currently in Phase III trials for psoriasis, RA and ankylosing spondylitis [54], has completed Phase II trials for CD and MS and Phase III trials for refractory Behçet's disease, and is in Phase II trials for asthma (Table 1). However, in a double-blind, randomized, placebo-controlled, proof-of-concept study conducted in patients with moderate-to-severe CD, Hueber *et al.* revealed unexpected ineffectiveness and higher rates of adverse events for AIN457 compared with placebo [55]. A possible explanation is that blocking IL-17 effector function in the gut may be detrimental since Th17 cells contribute to intestinal homeostasis. In support of this notion, it was recently demonstrated that increased numbers of circulating Th17 are present in patients with quiescent CD disease compared with active CD [56]. Collectively these data suggest that targeted inhibition of IL-17 may be an effective treatment for patients with RA and psoriasis, where Th17 has a prominent proinflammtory role, but not for patients with CD, where Th17-related cytokines appear to mediate protective responses.

Another monoclonal antibody in clinical development is AMG-827/brodalumab (Amgen, CA, USA), which is directed to the IL-17A receptor. AMG-827 has been shown to block signaling by both IL-17A and IL-17F as well as by the IL-17A/F heterodimer and to significantly improve plaque psoriasis, as assessed in a Phase II study [57]. AMG-827 is currently also in Phase II trials for psoriatic arthritis, asthma and RA (Table 1).

Recently, an aptamer-based therapeutic approach to block IL-17A has been described. In this context, it has been shown that a PEGylated form of the RNA aptamer Apt21-2 (PEG21-2idT) is able to prevent the development of autoimmunity in RA and EAE mouse models as well as to slow progression of arthritis after the onset of symptoms (Table 1). Since nucleic acid aptamers have high binding affinity and specificity and exhibit significant advantages in terms of size, synthetic accessibility and modification by medicinal chemistry compared with therapeutic antibodies, they represent an interesting class of modern pharmaceuticals and as such an anti-IL-17A aptamer holds potential as a therapeutic for autoimmune disease treatment [58].

Differentiation of Treg cells

Among immunomodulatory cell types, CD4$^+$CD25$^+$ Foxp3$^+$ cells represent a thymus-derived subset of T cells endowed with suppressive activity, referred as nTreg. To date Foxp3 is considered as a lineage-specification factor of this Treg population, based on the inhibitory effects of Foxp3 mutation or

deficiency on the differentiation and function of $CD4^+CD25^+$ cells [59]. Peripheral $CD4^+CD25^+$ cells have, however, been suggested to consist of a mixture of nTregs and iTregs, which originate from $CD4^+$ $CD25^-$ cells in response to environmental cues. While nTregs and iTregs can be distinguished with respect to their origin, they share similar phenotypes, both being $CD25^+CTLA\text{-}4^+GITR^+CCR4^+CD62L^+Foxp3^+$ and $CD45RB^{lo}$ (mice) or CD45R0 (human) cells (Figure 2) [13]. Since specific molecular markers to distinguish these two populations are still under investigation, the respective contribution of nTregs and iTregs to the overall Treg pool remains elusive.

The requirements for intrathymic nTreg differentiation from $CD4^+$ $Foxp3^-$ precursors include high-strength TCR stimulation by thymic self-antigen, costimulation of CD28 by CD80 and CD86 ligand expressed on APC as well as IL-2R signaling, all of which are instrumental to the expression of Foxp3. By contrast, while Foxp3 expression is also the end point of the iTreg developmental program, their generation, which occurs in the periphery, requires a suboptimal TCR stimulation, and the presence of both IL-2 and TGF-β. Moreover, the vitamin A metabolite retinoic acid has been shown to facilitate the differentiation of naive T cells to iTreg in the presence of TGF-β (Figure 2) [13].

Recently, expression of the transcription factor Helios was proposed as a signature of nTreg, based on a study aimed at the identification of specific molecular markers to better define these two populations [12]. Helios has, however, recently been shown to also be expressed in mouse $Foxp3^+$ iTregs [60], precluding its exploitation as a diagnostic marker for nTregs. Two recent reports provided evidence that the surface molecule, neuropilin 1, is expressed at high levels on nTregs but not in iTregs in mice, suggesting that it could be used to distinguish nTregs from other Tregs [61,62].

While Foxp3 expression in mouse cells is a marker of Tregs, the situation is quite different in humans. Indeed, not all human $CD4^+CD25^+Foxp3^+$ cells are Tregs, as both CD25 and Foxp3 can be transiently expressed in $CD4^+$ T cells following Ag stimulation without conferring suppressive activity to these cells [63]. It has been suggested that stable Foxp3 expression is required to establish Treg functionality [64]. To add complexity to this issue, Miyara *et al.* described three phenotypically and functionally

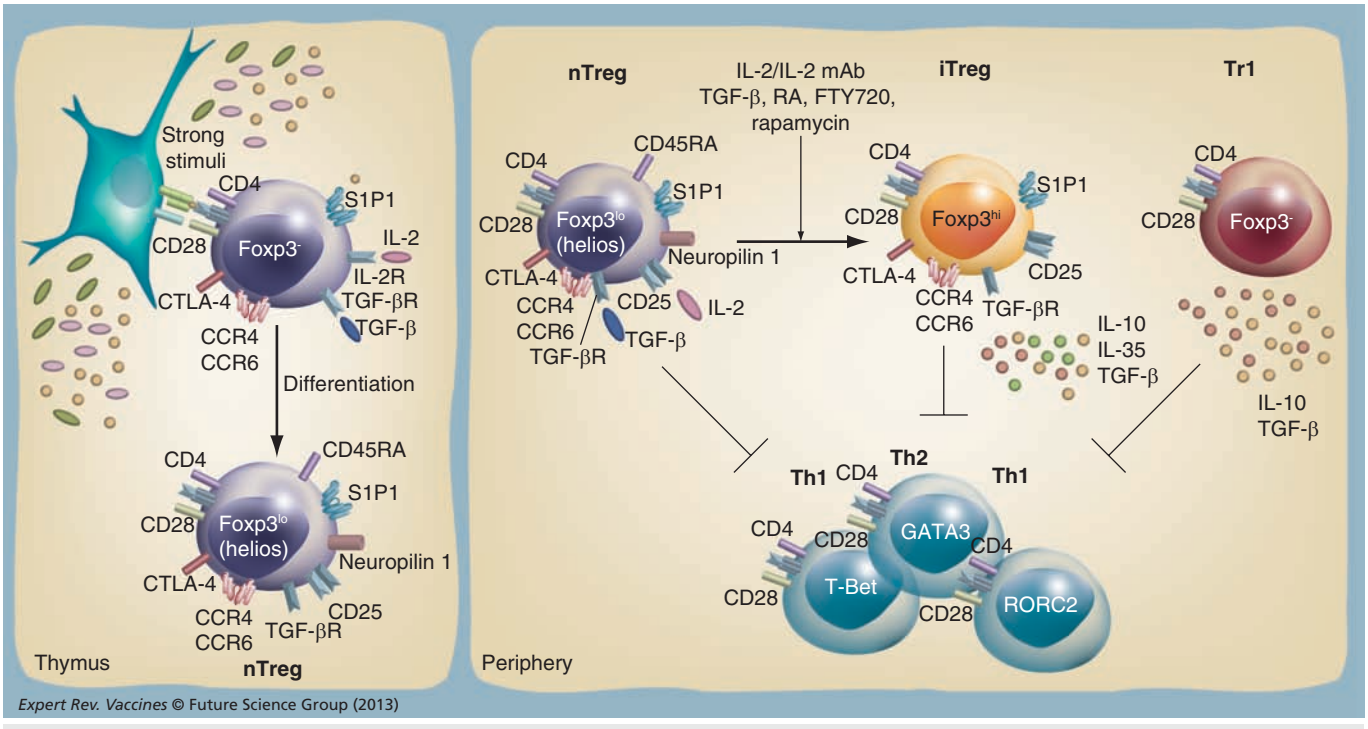

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Figure 2. Differentiation of $CD4^+$ $CD25^+$ $Foxp3^+$ (Treg) and $CD4^+$ $Foxp3^-$ (Tr1) Treg and their exploitation for cell-based immunotherapy. High-strength T-cell receptor stimulation by self-antigens, CD28 costimulation and IL-2R signals, all of which induce Foxp3 expression, are required for intrathymic Treg differentiation from naive $CD4^+$ T cells. Both naive $CD4^+$ T cells and thymic-derived Tregs (nTregs) leave the thymus and upon Ag stimulation can differentiate into iTregs in secondary lymphoid organs. In the periphery, the total Treg pool comprises both nTregs and iTregs, which are characterized by similar phenotypes ($CD4^+CD25^+CTLA\text{-}4^+GITR^+CCR4^+CD62L^+$ $Foxp3^+$). The expression levels of Foxp3 and CD45RA as well as expression of neuropilin 1 have been suggested as potential markers of nTregs and iTregs. nTregs are defined as $CD45RA^+$ $Foxp3^{lo}$ $neuropilin^+$ and iTregs as $CD45RA^-$ $Foxp3^{hi}$ $neuropilin^-$. In addition, while human iTregs secrete immunomodulatory cytokines including IL-10, TGF-β and IL-35, no cytokine production by nTregs has been reported. Specific biological therapies promoting expansion of Tregs both *in vivo* and *in vitro* are shown. An additional $CD4^+$ regulatory T-cell population in the periphery is the Tr1, which does not express Foxp3. No specific cell-surface markers have been reported for Tr1 cells, hence they are identified by their unique cytokine profile, characterized by high levels of IL-10 and TGF-β, low levels of IL-2, variable levels of IL-5 and IFN-γ and absence of IL-4. Similar to nTregs and iTregs, Tr1 cells preserve immunological self-tolerance and homeostasis through the control of effector $CD4^+$ T-cell responses.
iTreg: Induced Treg; mAb: Monoclonal antibody; nTreg: Natural Treg; RA: Retinoic acid; Tr1: T-regulatory type 1.

different human Treg subpopulations based on the expression of CD45RA, a marker of naive cells and the levels of Foxp3 [65]. The three subpopulations include $CD45RA^+Foxp3^{lo}$ resting/naive Treg cells and $CD45RA^-Foxp3^{hi}$ activated/effector Treg cells, which are both suppressive *in vitro*, and the nonsuppressive $CD45RA^-Foxp3^{lo}$ T cells that secrete proinflammatory cytokines including IL-17A. According to Miyara *et al.* effector Treg cells that are able to suppress the proliferation and the function of polarized activated T cells derive mainly from resting/naive Treg cells, which once egressed from the thymus differentiate into activated/effector Treg cells following Ag-dependent TCR stimulation [66]. In addition, they suggest that $CD45RA^-Foxp3^{hi}$ activated/effector cells can also derive from Ag-activated $CD45RA^-Foxp3^-CD4^+$ naive T cells or $CD45RA^-FOXP3^{lo}$ nonTreg cells.

Mouse and human Tregs differ also in their cytokine requirements. Suboptimal TCR signals in combination with TGF-β and IL-2 are essential for the induction of $Foxp3^+$ Tregs in the mouse [13,67]. At variance, TGF-β in combination with TCR stimulation is not able to induce human T cells endowed with suppressive activity despite its ability to increase Foxp3 expression [68]. However, it has recently been shown that combinations of IL-2, TGF-β and retinoic acid, or of TGF-β and rapamycin, but not retinoic acid or rapamycin alone, were able to induce human iTregs, suggesting that TGF-β may also be implicated in the differentiation of human Treg cells [12].

Tregs in autoimmunity & Treg-based therapy for autoimmune diseases

Failure of Treg function, resulting from intrinsic Treg defects, resistant effector T cells or Treg deficiency, are well-documented features of autoimmunity both in mice and humans [66]. In addition, a role for cytokines secreted by APC in the resistance of effector T cells to Treg-mediated suppression was recently demonstrated, suggesting that the functionality of Tregs goes beyond their mere numbers [69]. While intrinsic Treg defects have been described in T1D, antineutrophil cytoplasm antibody (ANCA)-associated vasculitis and in MS, altered Treg frequencies frequently associate with SLE or RA [66]. Contrasting results have been reported regarding the correlation between Treg frequency and clinical outcome in MS [70]. In T1D patients, the resistance of effector T cells to Treg-mediated suppression is the most evident alteration, notwithstanding a reduction in the suppressive activity of Tregs. At present, the impairment in Treg function in SLE is believed to result from the proinflammatory properties of APC in these patients. Yan *et al.* demonstrated that high levels of IFN-α derived from APC of SLE patients are responsible for the defect in Treg-mediated suppression [71]. Thus, while different mechanisms may underlie the altered functionality of Treg cells in individual autoimmune diseases, collectively these studies underscore the importance of Treg in the control of tolerance and autoimmunity.

The potent suppressive function of Tregs towards effector T cells, together with the demonstration that defects in the frequency and/or function of Treg cells are causally related to autoimmunity, highlight this subset of $CD4^+$ T cells as a major therapeutic target. Ideally, the enhancement of Treg function could be achieved using two alternative approaches: first, promoting Treg development and survival *in vivo* by targeting specific receptors and/or cytokines required for their differentiation and function, as well as professional APC, which are instrumental for their priming and activation; second, isolating and expanding *in vitro* Ag-specific Tregs, which, once adoptively transferred in patients, could restore the proper balance between effector and regulatory cells.

Promoting Treg development & survival in vivo

Agents that induce expansion of Tregs *in vivo* appear ideal for the treatment of autoimmunity. Based on their ability to induce Foxp3 expression, a number of cytokines (e.g., IL-2 and TGF-β), receptor agonists (e.g., anti-CD28 and anti-CD3 mAbs) and small molecules (e.g., retinoic acid) have been assessed as therapeutics to restore self-tolerance in autoimmune diseases (Figure 2 & Tables 2 & 3). Cytokines that mimic Treg activity, including IL-10 and TGF-β, have been considered as an alternative therapeutic approach.

IL-2 therapy has been long used to enhance antitumor immunity [72]. With the discovery that IL-2 is also a critical factor for Treg development and survival, this cytokine is currently being explored as a tool to promote Treg cell expansion *in vivo*. An increase in the frequency of Treg cells and an enhancement of their function could be achieved in naive mice by stimulating IL-2R with specific agonists. However, similar to other pleiotropic cytokines, the use of IL-2 *in vivo* is likely to result in adverse effects. The IL-2R is a trimeric complex consisting of CD25 (IL-2Rα), CD122 (IL-2Rβ) and CD132 (IL-2Rγ). Of these, Treg cells preferentially express CD25 while NK and $CD8^+$ T cells express CD122. A recent attempt to specifically target the Treg population has capitalized on this specificity in the expression profile of the IL-2R subunits to use IL-2/anti-IL-2 mAb complexes that prevent IL-2 binding to CD122. An example is the IL-2/JES6-1 complex, which has shown beneficial effects associated with an increase in the frequency and function of Treg cells in mouse models of EAE and myasthenia gravis, underscoring the potential of IL-2-based therapy for the treatment of autoimmune disease [73].

In humans, IL-2-based therapy has been shown to increase Treg cell numbers in cancer patients and in active chronic graft-versus-host disease patients; however, it is still unclear whether the therapeutic effects of IL-2 in humans are mainly dependent on its activity on Tregs or instead result from the activation of other immunoregulatory cells. Other potential cellular targets of IL-2, including the recently identified innate-like lymphoid cells and NK cells, have been demonstrated to express IL-2R [73], and the beneficial effects of daclizumab, an IL-2R agonist currently in Phase III clinical testing for MS, have been primarily ascribed to the expansion of regulatory NK cells. Based on the promising results obtained in the mouse, human analogs of the murine IL-2/anti-IL-2 mAb complexes are currently being developed (Tables 2 & 3).

An increase in the numbers of Tregs following *in vivo* administration of TGF-β or retinoic acid has been demonstrated in mice, highlighting these molecules as potential therapeutics also in humans (Table 2). Moreover, rapamycin, an immunosuppressive

drug extensively used to prevent graft rejection in humans, has been shown to promote Treg development and enhance Treg function through the inhibition of effector cell proliferation in T1D patients (Table 2 & 3). Interestingly, in the absence of mTOR activity human naive $CD4^+$ T cells differentiate to $Foxp3^+$ Treg cells [74]. Since rapamycin inhibits the PI3K–Akt–mTOR pathway, it may be considered as a promising drug to control Treg function (Tables 2 & 3).

The sphingosine-1 phosphate receptor 1 (S1P1) has been recently demonstrated to promote Treg development and function through the activation of the PI3K–Akt pathway and Foxp3 expression. Consistent with this function, the S1P1 agonist FTY720 increases the number and enhances the suppressive activity of $CD4^+CD25^+$ Tregs *in vitro*, highlighting this immunomodulatory drug as a promising agent to restore Treg functionality (Table 2).

An alternative approach to treat autoimmunity is based on histone deacetylase (HDAC) inhibitors. HDACs deacetylate not only histones, but also nonhistone proteins involved in a wide range of cellular processes including transcription, translation, DNA repair, cell cycle and cell structure, thereby tuning their activity [75]. Based on the demonstration that Foxp3-dependent gene expression is associated with its acetylation [76], inhibitors of HDACs are being tested for their ability to modulate the function of Treg cells [77]. Promising initial results show that HDAC inhibitors promote Treg development and function in animal models of autoimmune disease [77]. Interestingly, Tao *et al.* [78] demonstrated that HDAC9 is instrumental for Treg development and accordingly T cells from $HDAC9^{-/-}$ mice show increased suppressive activity both *in vivo* and *in vitro* [77]. Furthermore, HDAC9 deficiency was recently correlated to protection from systemic autoimmmunity [79].

Notwithstanding the excellent results obtained in animal models of autoimmune disease using therapeutics that enhance Treg *in vitro*, all these treatments are not highly specific for Treg cells. Hence, their exploitation in humans is at present precluded in the absence of a more complete understanding of their effects *in vivo*.

Isolating & expanding in vitro *Ag-specific Tregs*

In mice the transfer of both polyclonal and Ag-specific iTreg has been demonstrated to prevent and suppress disease in a lupus-like syndrome model, T1D, EAE and collagen-induced arthritis (Table 2) [12]. Moreover, it is now clear that among $CD4^+CD25^+Foxp3^+$ cells, antigen-specific iTreg appears to have a higher therapeutic potential compared with nTreg. It was indeed recently shown that, unlike nTregs, iTregs generated *in vitro* are able to suppress Th17-mediated diseases when transferred in mice, indicating that their functionality is maintained [80]. Moreover, MBP-specific Treg have been shown to be more protective compared with polyclonal Treg in MBP-induced EAE and to prevent disease relapse (Table 2). Recently, adoptively transferred Ag-specific Treg cells generated by co-transduction of purified $CD4^+CD25^+$ T cells with specific *TCR* genes were shown to ameliorate both bone destruction in a mouse model of arthritis and nephritis in a lupus-prone mice, opening the possibility to target Treg to tissue-specific antigen in autoimmune conditions [81].

However, this therapeutic approach is far from being applicable to human diseases. A first issue is that, at variance with the mouse, where Tregs can be isolated from $CD4^+CD25^{hi}$ cells based on Foxp3 expression, there are no specific Treg markers in humans, which prevents the isolation of Tregs from effector cells. Efforts to purify human Tregs are currently based on two different approaches. The first stems from the demonstration that human Treg are CD127 negative and that expression of this surface marker is inversely correlated with Foxp3 expression [82]. The second relies on the notion that Treg cells originated from the $CD45RA^+$ $CD4^+$ $CD25^+$ T-cell compartment are easily expanded *in vitro* and maintain their suppressive function after expansion compared with antigen-experienced $CD45RO^+$ Tregs [83]. Hence, human Tregs could be isolated by cell sorting as $CD4^+$ $CD25^+$ and $CD27^{lo/-}$ or $CD4^+$ $CD25^+$ $CD45RA^+$.

Table 2. Treg-based immunotherapy in preclinical models.

Drug	Target	Mouse model	Effects	Ref.
Rapamycin	mTOR	NOD	Expansion of nTreg/prevention of disease	[93]
TGF-β	TGF-βR	BALB/c and DO11.10	Increase in Treg number	[94]
FTY720	S1P1	BALB/c and C57BL/6 (*in vitro*)	Increase in Treg number	[95]
IL-2/anti-IL-2 mAb complexes (mouse IL-2/JES6-1)	CD25	EAE	Expansion and increased function of Treg	[96]
		Myasthenia gravis	Expansion and increased function of Treg	[97]
Adoptive transfer	**Mouse model**		**Effects**	**Ref.**
$Foxp3^+$ $CD4^+CD25^+$	EAE, NOD, CIA, lupus-like model		Prevent development and suppress disease	[12]
$Foxp3^+$ $CD4^+$ $CD25^+$, MBP-specific	EAE		Prevent disease relapse	[98]
$CD4^+$ $CD25^{hi}$ $CD127^{lo}$	Humanized mouse skin allograft model		Prevent disease	[99]
	Humanized mouse model of atherosclerosis		Prevent disease	[100]

DO11.10 transgenic mice expressing a TCR with specificity for chicken OVA peptide 323–339.
CIA: Collagen-induced arthritis; EAE: Experimental autoimmune encephalomyelitis; mAb: Monoclonal antibody; NOD: Non-obese diabetic; nTreg: Natural Treg.

Table 3. Treg-based immunotherapy in humans.

Drug	Target	Disease	Effects/status	Ref.
Rapamycin	mTOR	Type 1 diabetes mellitus (*in vitro*)	Increase in Treg function	[101]
IL-2/anti-IL-2 mAb complexes (BAY50-4798)	CD25	HIV	NCT00059462	[201]
Adoptive transfer	**System**	**Disease**	**Status**	**Ref.**
$CD4^+$ $CD25^{hi}$ $CD127^{lo}$	Human	Type 1 diabetes mellitus	Phase I (NCT01210664)	[201]
Tr1	Human	Crohn's disease	Phase I/II	[102]

mAb: Monoclonal antibody.
Data taken from [201].

At present the efficacy of human $CD4^+$ $CD25^+$ and $CD27^{lo/-}$ Treg adoptive transfer therapy has been demonstrated in a humanized mouse model of atherosclerosis and alloimmune-mediated injury of human skin grafts (Tables 2 & 3). However, a recent report demonstrated that expansion of $CD4^+$ $CD25^+$ and $CD27^{lo/-}$ Treg *in vitro* leads to enhanced frequencies of IL-17, IFN-γ and IL-2-producing cells, indicating that copurification of contamining proinflammatory effector cells may be a critical issue in the development of Treg-based cellular therapies in humans. Interestingly, these authors identified as useful markers for Treg purification CD127 and CD49d/VLA-4, the α4 subunit of the α41-integrin, which play a key role in the homing of hematopoietic progenitors to bone marrow as well as in lymphocyte migration [49]. Both CD127 and CD49d are indeed absent in immunosuppressive $Foxp3^+$ human T cells, thereby allowing purification of untouched Treg cells by negative selection [84]. $CD45RA^+$ could also exploited to isolate human Tregs with high suppressive activity, with the added advantage that umbilical cord blood contains a high number of these cells [85]. Nevertheless, the correlation between suppression function and memory ($CD45RO^+$) or naive ($CD45RA^+$) phenotypes of Tregs remains to be clarified.

Although purification and expansion of Tregs is still problematic, results obtained in preclinical models are encouraging and clinical trials to assess the safety and efficacy of Treg cell-based therapy in systemic autoimmune diseases are warranted. At present a Phase I clinical trial is recruiting participants to assess safety and feasibility of *ex vivo*-expanded human autologous $CD4^+$ $CD25^+$ and $CD27^{lo/-}$-polyclonal Tregs in T1D (Table 2).

An alternative method to generate human regulatory cells with the desired antigen specificity is based on the use of Tr1 cells. Tr1 cells are human $CD4^+$ $Foxp3^-$ cells that have been shown to suppress both naive and memory T-cell responses through secretion of the immunosuppressive cytokines IL-10 and TGF-β (Figure 2) [83]. At variance with Tregs, Tr1 cells can be induced *ex vivo* and only produce IL-10 following Ag stimulation, suggesting that Tr1 cells of the desired antigen specificity can be easily generated *in vitro* [83]. At present Tr1 cells are identified and isolated by their unique cytokine profile, characterized by high levels of IL-10 and TGF-β, low levels of IL-2, variable levels of IL-5 and IFN-γ and absence of IL-4 (Figure 2). The feasibility of Tr1-based immunotherapy is supported by the results of a Phase I/II trial in patients with CD showing safety and lack of generalized immunosuppression (Table 3). Importantly, this approach does not appear to suffer from a major drawback encountered when Tregs are expanded *in vitro* and then reinfused in the host – that is, limited stability. Andolfi *et al.* demonstrated that overexpression of IL-10 in human $CD4^+$ cells results in a population with a Tr1 cytokine profile that is stable and functional *in vivo*, further supporting the potential of Tr1-based therapies [86]. Unfortunately, as discussed above for human $Foxp3^+$ Tregs, human Tr1 cells cannot be distinguished from other $CD4^+$ T-cell subsets by specific markers, making the isolation of a pure Tr1 population problematic.

Although Treg-based therapy might be of interest as a therapeutic option for several autoimmune diseases, key issues remain to be addressed. First, specific molecular markers that allow the elimination of contaminating pathogenic cells need to be identified. Second, a method that permits sufficient numbers of Treg cells to be obtained needs to be developed. Moreover, it is essential to better assess the safety of Treg cell-based immunotherapy; it is known that Treg cells can convert into the Th17 pathogenic cells under highly inflammatory conditions *in vivo*. In addition, stable suppressive Tregs could theoretically lead to general immunosuppression.

Expert commentary & five-year view

Defects in the regulation of the immune response by Treg together with the discovery of the detrimental role of Th17 cells in autoimmunity have prompted the development of therapeutics aimed to enhance Treg function or suppress Th17-mediated responses. While a number of strategies to inhibit IL-17/Th17 responses have been developed and their efficacy has been demonstrated both in preclinical animal models and in a number of human autoimmune disorders, recent evidence suggests that the role for Th17 and IL-17 in autoimmune disease is more complex that previously believed. First, Th17 cells also exert a protective role against several pathogens, especially at the mucosal surfaces, and inhibition of Th17 responses may be beneficial only in autoimmune diseases where the pathogenic role of Th17 clearly dominates over the protective one. Second, Th17 cells possess a high flexibility under different inflammatory settings in terms of cytokine production, making their identification and characterization difficult. Furthermore, Th17 cells and Tregs share some markers such as CCR6 expression as well as factors that control their differentiation (TGF-β), and therapeutics designed to inhibit these molecules may result in worsening the disease as a result of Treg function inhibition. While Treg manipulation in autoimmune disease appears promising,

the lack of specific molecular markers to isolate and expand this population remains the major problem. Another important issue to be faced is the possibility that Treg cells may convert *in vivo* into pathogenic cells such as Th17 cells. A better understanding of the specific role of Th17 and Treg cells autoimmunity as well as the identification of specific molecular markers will bring us closer to developing safe and effective targeted cellular therapy.

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Key issues

- The differentiation of helper T-cell subsets is dynamically regulated by the local microenvironment.
- The balance among polarizing cytokines induced by different microbes rather than their absolute amount is determinant in human Th17-cell differentiation.
- Th17 and Treg cells are endowed with high plasticity and flexibility in their differentiation program and this must be taken into account in the development of therapeutics designed to target these populations.
- Th17 responses are implicated both in host defense and in the development of autoimmunity and a better understanding of their pathogenic or protective role in each autoimmune disease is required to develop safe and effective therapeutics.
- Defects in the frequency and/or function of Tregs are a feature of autoimmunity.
- Human Treg cells are a heterogeneous population and specific molecular markers are still unknown. Furthermore, Th17 cells and Treg share some molecular markers as well as factors that control their differentiation.
- Promising results have been obtained in animal models of autoimmune disease using biologicals that increase Treg numbers *in vitro*, including IL-2/anti-IL-2 complexes, rapamycin and HDAC inhibitors. However, at present the lack of specificity of these therapeutics towards Tregs limits their translatability to the context of human autoimmunity.
- Human Treg or Tr1 adoptive transfer therapy has yielded encouraging results in preclinical models; however, purification and expansion of both Tregs and Tr1 cells is still problematic due to the lack of specific molecular markers.

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