VIVE LA RÉSISTANCE!?

HOW T CELLS ESCAPE REGULATION IN AUTOIMMUNE INFLAMMATION

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HOE T-CELLEN ONTSNAPPEN AAN REGULATIE
IN AUTO-IMMUUNZIEKTE

(met een samenvatting in het Nederlands)

Proefschrift

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CONTENTS

GENERAL INTRODUCTION

To establish effective protection against infection, the immune system must recognize and respond to diverse types of pathogens, such as viruses, bacteria and parasites. T cells play an important role in this defense against microorganisms and express a wide variety of T cell receptors (TCR) by which they specifically recognize antigens expressed by different pathogens. However, the process by which these diverse T cell receptors are created is random and therefore inevitably results in the generation of T cells specific for self (auto-) antigens. These auto-reactive T cells can recognize and attack the bodies own tissue upon activation and thereby cause tissue damage resulting in autoimmune pathology. To prevent these harmful autoimmune responses, important regulatory mechanisms are at place. First of all, by a process called central tolerance, T cells that recognize self antigens with high affinity are eliminated in the thymus. However, some self-reactive T cells escape central tolerance and therefore additional regulation takes place in the periphery to prevent these cells from causing harm. Regulatory T cells are key players in peripheral tolerance by controlling unwanted activation of auto-reactive T cells. However, also peripheral tolerance is not flawless and in some individuals under specific environmental and genetic circumstances autoimmune disease develops.

JUVENILE IDIOPATHIC ARTHRITIS

CHARACTERISTICS AND SUBCLASSES OF DISEASE

Juvenile idiopathic arthritis (JIA) is one of the most common autoimmune diseases in children, affecting 16 to 150 children per 100.000 and by definition starting before 16 years of age[1]. JIA is thought to develop in genetically susceptible individuals in response to environmental factors, possibly including infections[2]. Similar to rheumatoid arthritis (RA) in adults, JIA is characterized by chronic inflammation of the joints[3]. However, different from RA, juvenile arthritis can be divided into several subclasses of disease, including the following subtypes[3;4]. In ***oligoarticular JIA*** a maximum of 4 joints is affected, usually involving the larger joints of the legs, such as knees and ankles. ***Polyarticular JIA*** includes 5 or more joints within the first 6 months of disease and next to the larger joints often the smaller joints of the hands and feet are involved. This subtype of JIA usually displays a rather progressive course of disease. In contrast, oligoarticular JIA has a better prognosis and can even be self-limiting. However, it can also extend to more than 4 joints after the first 6 months of disease, more resembling polyarticular JIA, and is then classified as ***extended oligoarticular JIA***. Finally, ***systemic JIA*** is characterized by extra-articular features such as spiking fever and skin rash. It is now clear that this subclass of JIA involves abnormalities in inflammatory pathways of the innate immune system and is therefore considered to be an acquired autoinflammatory disorder instead of an autoimmune disease[5]. Since this thesis focuses on T cell regulation during autoimmune inflammation, only patients with oligoarticular, extended oligoarticular or polyarticular JIA were included in the experiments described.

PATHOLOGY OF JOINT INFLAMMATION

As in most other human autoimmune diseases, the initial trigger causing inflammation in JIA and RA is unknown and might involve one or more environmental factors[1;6;7]. In addition,

joint pathology is a complex process, involving multiple mechanisms of inflammation and is still incompletely understood. Nevertheless, due to some trigger innate cells, such as macrophages and fibroblasts become activated and migrate into the synovium lining the joint. Upon activation these cells start to produce cytokines and express adhesion molecules that allow for continued ingress of immune cells[6]. What follows is an extensive infiltration of multiple immune cells, including granulocytes, monocytes/macrophages, B cells and high levels of $CD4^+$ and $CD8^+$ T cells, mostly with an activated memory phenotype[1;3]. These infiltrating cells produce large quantities of proinflammatory cytokines and chemokines, such as, IL-6, IL-15, TNFα, IFNγ, CCL3, CCL11 and CXCL8[6;8;9]. Production of these cytokines and chemokines further drives the inflammatory process by inducing cell activation and proliferation and attracting more immune cells to the site of inflammation[10]. In addition, proinflammatory cytokines enhance osteoclast maturation and activation and induce matrix-degrading enzymes, leading to bone and cartilage degradation[6;10]. Due to this tissue damage, self-antigens are released and further drive the ongoing inflammation independent of the initial trigger by activating auto-reactive T cells[2;6;7;10].

THERAPIES FOR JIA

Depending on the course of disease different types of therapy are used to treat JIA[3]. As a first-line therapy, to quickly relief patients from symptoms, usually ***non steroidal anti-inflammatory drugs (NSAIDs)*** are administered, such as naproxen and ibuprofen. However, these dugs do not modify the underlying disease process nor affect the long-term outcome of disease. ***Disease modifying anti-rheumatic drugs (DMARDs)*** do target the disease more effectively and thus may help to limit damage and long-term morbidity. ***Methotrexate (MTX)*** is the most frequently used DMARD in JIA. However, treatment with MTX causes considerable side effects in a large proportion of patients[11]. ***Corticosteroids*** affect multiple organ systems and as a consequence can have numerous side effects as well, including growth retardation. These agents are therefore rarely administrated systemically, only in patients with systemic JIA or in severe polyarticular JIA patients who do not respond to other therapies. However, corticosteroids are often applied intra-articularly in oligoarticular JIA patients where they repress the local inflammation in the joint, but are unable to cause side effects systemically.

BIOLOGICALS

In recent years, a tremendous progress has been made in understanding the underlying mechanism of autoimmune pathology and as a result more specific therapies to treat autoimmune disease have been developed. These so-called biologicals target critical mediators of the inflammatory response, for instance TNFα and IL-6. The rationale for blocking TNFα in the treatment of arthritis came from initial experiments showing that blockade of TNFα in synovial cell cultures abrogated the production of multiple proinflammatory cytokines, including IL-1, IL-6, IL-8 and GM-CSF[12]. This led to the concept that TNFα plays a key role in coordinating the proinflammatory cytokine cascade in the inflamed synovium. Since then several agents have been developed to block TNFα for the treatment of arthritis and their efficacy and safety have been thoroughly tested in animal models and clinical trials[12]. Both infliximab and adalimumab,

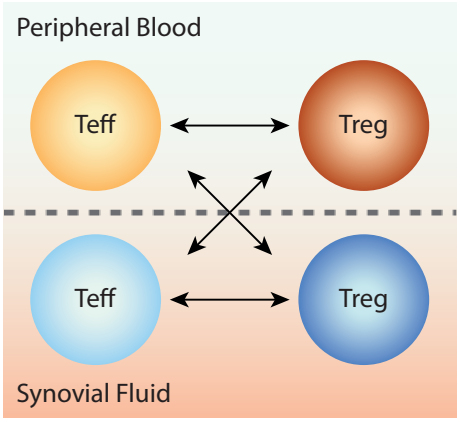
monoclonal antibodies against TNFα, and etanercept, the soluble TNF receptor, are used in the treatment of RA[13]. Etanercept is also frequently applied in JIA, especially in patients with polyarticular disease[3]. In addition to TNFα targeting biologicals, efficacy of a humanized anti-IL-6 receptor antibody, tocilizumab, has been verified in clinical trials and is now approved for the treatment of RA and systemic JIA[14]. Both anti-TNFα and anti-IL-6 clearly have direct anti-inflammatory effects on the innate immune system, however, these agents might also target the adaptive immune response[15-19].

Although many patients respond well to treatment with biologicals, there are also patients who do not respond, patients who only show partial remission upon therapy or patients that become resistant during treatment. Moreover, no permanent remission is achieved and patients relapse after discontinuation of therapy[6]. As a result, life-long treatment is required. This is associated with considerable side effects due to general immune suppression, possibly including malignancies and infections[13]. Therefore, the search for new and improved treatment options for arthritis as well as other autoimmune diseases continues.

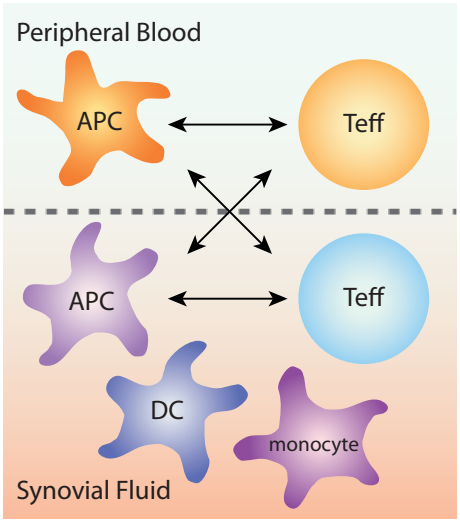
IMMUNOLOGICAL RESEARCH IN JIA

As described above JIA is a very heterogeneous disease consisting of various subclasses that differ in disease severity. The observation that JIA can be self-remitting suggests that regulatory mechanisms are at place that to some extend still control the disease. However, for some reason the balance has shifted from immune regulation and homeostasis towards an over-activated immune response causing autoimmune pathology. Although the introduction of biologicals has improved treatment of JIA, life-long therapy is still required, which is especially unfavorable in pediatric patients. Therefore, the challenge for the future will be to permanently restore immune balance in JIA and accomplish long-lasting, drug-free remission. To achieve this goal more in depth research into what causes immune dysregulation in JIA is required. This research might not only improve JIA patient care, but could also advance the treatment of other autoimmune diseases, since JIA represents a unique model to study autoimmune inflammation.

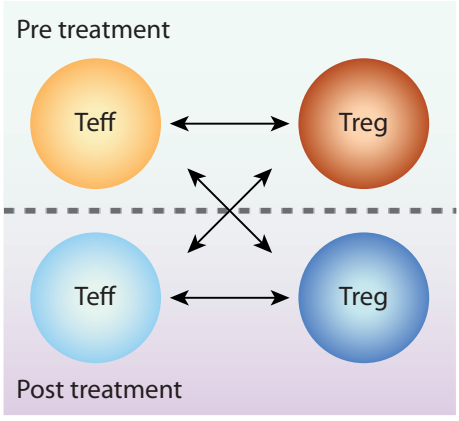
As described above, intra-articular injections with corticosteroids are part of standard care, especially in patients with oligoarticular JIA. At that time synovial fluid (SF) is taken from the inflamed joint to reduce intra-articular pressure and enhance treatment efficacy[20]. Mononuclear cells can be isolated from this SF (SFMC) and used in *in vitro* experiments. Thus, different from many other autoimmune diseases, cells from the site of inflammation are accessible in patients with JIA. Moreover, since inflammation in oligoarticular JIA is restricted to affected joints, mononuclear cells from peripheral blood (PB) (PBMC) can be used as an in-patient control to study specific changes in immune regulation at the site of autoimmune inflammation. Another advantage of using PBMC and SFMC from the same donor is that these cells can be combined *in vitro* without causing an alloreaction that might influence the results[21] (see cross-over experiments described in Figure 1). In this thesis JIA patient material is used to investigate how immune regulation is disturbed in autoimmune inflammation, hopefully bringing the treatment of JIA one step closer to permanent disease remission.



In ***Chapter 4*** Treg and effector cells from peripheral blood and synovial fluid are crossed over to investigate changes in Treg suppressive function and responsiveness of Teff to suppression at the site of inflammation compared to peripheral blood.



In ***Chapters 4 and 6*** APCs and T cells from peripheral blood and synovial fluid are crossed over to study the effect of T cells and different types of APCs from the site of inflammation on Treg-mediated suppression.



In ***Chapter 5*** Treg and effector cells from pre and post treatment are crossed over to investigate changes in Treg suppressive function and responsiveness of Teff to suppression after therapeutic intervention.

Figure 1 | Cross-over experiments used in this thesis to study changes in Treg-mediated suppression at the site of autoimmune inflammation and after therapeutic intervention.

FOXP3$^+$ REGULATORY T CELLS

As already described briefly, regulatory T cells are critical in peripheral tolerance and several subsets of regulatory T cells are known to play a role in controlling unwanted immune activation (reviewed in Chapter 2, Table 1). Given their central role in maintaining self tolerance and preventing autoimmune responses, this thesis specifically focuses on FOXP3$^+$ regulatory T cells, which will be hereafter referred to by the term Treg. The observation that T cells could not only enhance, but also suppress immune responses already dates back from the 1970s, when so called suppressor T cells were described[22;23]. However, by the late 1980s these suppressor T cells fell out of favor because no specific marker for the cells could be identified and the mechanism behind their suppressive function remained unclear. Moreover, some of the initial data describing their suppressive potential could not be reproduced[24;25]. However, due to modern immunological techniques, compelling evidence now confirms the existence of T cells with a suppressive function. Key initial experiments have shown that mice devoid of CD4$^+$ T cells expressing CD25, the alpha-chain of the IL-2 receptor, develop severe autoimmune disease and inoculation with a small number of CD4$^+$CD25$^+$ T cells can prevent development of autoimmunity[26;27]. Thus, regulatory T cells (Treg) reside within the CD4$^+$CD25$^+$ T cell population. In addition, more recently Treg were found to express forkhead box P3 (FOXP3) and this transcription factor is essential for their development, maintenance and function[28-30]. CD4$^+$CD25$^+$FOXP3$^+$ Treg originate from the thymus and are therefore commonly referred to as natural occurring Treg, however, they can also be induced in the periphery from naïve T cells under specific circumstances[31;32]. Both CD25 and FOXP3 are clearly linked with Treg generation and function and deficiencies in these molecules impair Treg mediated control of immune responses[33]. In addition, Treg express cytotoxic T lymphocyte antigen-4 (CTLA-4), which is important for their suppressive function[34]. This is most clearly reflected by the development of a fatal autoimmune disorder in mice with a Treg-specific ablation in CTLA-4[35]. Glucocorticoid-induced TNF receptor (GITR) is also expressed by Treg, but its role in Treg function is less clear[33].

TREG SUPPRESSIVE MECHANISMS

Treg not only control T cell activation and proliferation, but also effector functions such as cytokine production and cytotoxicity. Since their discovery intensive research has focused on how Treg accomplish their suppressive effects. Still, the main suppressive mechanisms used by Treg remain controversial. Over the years different mechanisms of suppression have been described[36]. These include cell contact-dependent mechanisms[37;38], as well as production of suppressive cytokines, such as IL-10[39;40] and TGFβ[41-43]. In addition, Treg can directly target effector T cells (Teff), for instance by inducing cytolysis via production of perforin and/or granzymes[44-46], but can also affect antigen presenting cell (APC) function or alter the cellular environment. For example, IL-2 consumption by Treg has been proposed to lead to cytokine deprivation-mediated apoptosis in Teff[47]. In addition, Treg suppress the expression of costimulatory molecules on APCs[50-55] required for T cell activation, which is at least partially dependent on CTLA-4[35;48;49]. Thus, Treg can exert multiple suppressive functions. Arguably, the

kind of mechanism employed depends on the nature of the immune response, the target cell that needs to be repressed and the location *in vivo*[56]. In addition, it has been proposed that Treg employ different suppressive mechanisms under steady state and inflammatory conditions[57]. Because most experiments concerning Treg function have been performed in mice, the major suppressive mechanisms utilized by human Treg are still incompletely understood.

TREG IN AUTOIMMUNE DISEASE

Over the years accumulating data have been gathered that demonstrate a critical role of Treg in preventing autoimmunity. Perhaps most convincing evidence comes from humans with a mutation in the FOXP3 gene. These patients lack functional Treg and a as a result develop severe autoimmune disease in multiple organs[58;59]. In addition, experiments in animal models of autoimmunity, for instance in experimental arthritis, have shown that Treg control disease development and severity[60-62]. As a consequence, Treg are now considered for the treatment of autoimmune disease including arthritis[63] and several strategies to enhance Treg in patients with autoimmune disease are being explored (reviewed in Chapter 2, Figure 1). In addition, numerous research groups have investigated whether deficiencies in Treg number or function underlie human autoimmune pathology, such as RA and JIA (reviewed in Chapter 2, Table 2). However, the majority of these studies now indicate that Treg numbers are not declined in arthritis patients compared to healthy controls[16;64-66] and although some studies report reduced functionality of Treg in RA patients[16;19;64], others have found no difference[66;67]. Moreover, Treg are enhanced at the actual site of inflammation, in SF obtained from inflamed joints[65-70]. These SF Treg display increased expression of FOXP3 and other functional and activation markers[65;66;69;70]. Most importantly, SF Treg efficiently inhibit Teff and even display enhanced suppressive capacity compared to their PB counterparts[66;69;70]. Thus it appears that numerous and functional Treg are present at the site of inflammation in patients with RA and JIA. However, their suppressive potential might be attenuated by the local proinflammatory environment.

TREG IN A PROINFLAMMATORY ENVIRONMENT

At the site of autoimmune inflammation, in inflamed joints of arthritis patients, specific changes in immune cells and mediators are observed. For instance, T cells show distinct skewing towards certain T cell subsets (reviewed in Chapter 7). In addition, APCs are present in elevated levels and display a highly activated phenotype with enhanced expression of maturation markers; CD40, CD80, CD86 and HLA-DR[71-77]. Finally, as described above, profound levels of proinflammatory cytokines are being produced. This local proinflammatory environment might interfere with Treg function. For instance, strong and prolonged T cell activation via TCR and CD28 signaling can impair Treg mediated suppression[77-79]. Therefore, highly activated APCs at the site of inflammation could reduce Treg function due to their enhanced expression of HLA-DR and costimulatory molecules. In addition APCs might interfere with Treg suppressive potential through production of proinflammatory cytokines. In both an experimental model[80] as well as in patients with systemic lupus erythematosus (SLE)[81] APCs were found to impair Treg mediated control of Teff via production of IL-6 and IFNα, respectively. Other proinflammatory cytokines,

such as IL-1, IL-2, IL-7 and IL-15 might also interfere with Treg function[70;77], or worse facilitate conversion of Treg into pathogenic Teff[82;83]. Interestingly, many studies specifically point towards a direct negative effect of TNFα on human Treg function[19;84] and restoration hereof by anti-TNFα therapy[16;19;85]. Thus, at the site of autoimmune inflammation, proinflammatory cytokines, in particular TNFα, and high levels of activated APCs are thought to interfere with Treg mediated suppression. To gain more insight into the role of Treg in autoimmune pathology and their possible application for the treatment of autoimmune disease, it is therefore essential to study Treg from the site of inflammation in patients with autoimmune disease. Furthermore, when examining Treg function *in vitro* the *in vivo* situation should be mimicked as closely as possible.

TREG FUNCTIONAL ASSAYS

FOXP3 is a less definitive marker for human Treg compared to mice, as it can also be up-regulated in conventional T cells upon activation without conferring a suppressive phenotype[86-90]. To identify Treg in humans the suppressive function of the cells therefore needs to be confirmed using functional assays. Since FOXP3 is an intracellular marker it cannot be used to isolate viable Treg for functional testing. Therefore, Treg are purified based on specific surface marker expression. Human Treg are usually distinguished from $CD4^+$ Teff based on CD25 and low CD127 expression[91;92] These purified Treg are then co-cultured with effector cells and Treg mediated inhibition of T cell activation is analyzed. In these so-called suppression assays, Treg suppressive function is measured by calculating the change in T cell activation (measured by cell proliferation and cytokine production) in the presence of Treg compared to Teff cultured alone. In this thesis we used several *in vitro* suppression assays to not only study Treg function, but also analyze the susceptibility of Teff to suppression, each with their own advantages (summarized in Table 1). Next to performing these different types of suppression assays, cross-over experiments were employed to compare cells from the site of inflammation to PB, or samples taken at different time points (Figure 1).

Table 1 | Suppression assays used in this thesis to study Treg function and Teff responsiveness to suppression

Chapter	Effector cells	Suppression by	Main characteristics
3	$CD4^+CD25^-$ T cells	$CD4^+CD25^+CD127^{low}$ Treg	Clearly defined Teff population: reliable comparison of different types of Treg
4, 5	Total mononuclear cells	$CD4^+CD25^+CD127^{low}$ Treg	Total mononuclear cells: most representative for *in vivo* situation
4, 5	Total mononuclear cells	TGFβ	Treg independent suppression
4	$CD4^+CD25^-CD45RA^+CD45RO^-$ and $CD4^+CD25^-CD45RA^-CD45RO^+$ T cells	$CD4^+CD25^+CD127^{low}$ Treg	Comparison of naive and memory Teff
4, 6	$CD3^+$ T cells and $CD3^-$ APCs	$CD4^+CD25^+CD127^{low}$ Treg	Dissect T cell- and APC-mediated effects on Treg suppression

Abbreviations: Treg, regulatory T cells; TGFβ, transforming growth factor β; APC, antigen presenting cell.

SCOPE AND OUTLINE OF THIS THESIS

In this thesis we study Treg function in patients with JIA to investigate whether deficiencies in Treg play a role in autoimmune pathology and whether these cells may be used to treat autoimmune inflammation. In ***Chapter 2*** we review recent literature on Treg numbers and function in patients with autoimmune arthritis and describe several approaches to target these cells for the treatment of autoimmune disease. In ***Chapter 3*** Treg suppressive mechanisms are investigated and we question whether Treg from healthy donors and from the site of inflammation in patients with JIA suppress T cell activation by inducing apoptosis in Teff. Treg function in inflamed joints of JIA patients is further analyzed in ***Chapter 4*** and we also study the susceptibility of Teff to regulation. In ***Chapter 5*** we investigate whether anti-TNFα therapy enhances responsiveness of Teff to suppression in JIA. In ***Chapter 6*** APCs present at the site of autoimmune inflammation in JIA are characterized and their role in impaired immune regulation is studied. Finally, factors contributing to disturbed T cell regulation in inflamed joints of arthritis patients are reviewed in ***Chapter 7***. In ***Chapter 8*** the findings described in this thesis are discussed in the context of new therapeutic targets to restore immune balance in autoimmune inflammation.

KEY POINTS

- Treg are critical in self tolerance and are considered important targets for the treatment of autoimmune disease
- Accumulating data now indicate that Treg are not deficient in patients with autoimmune disease, however, their suppressive function might be attenuated by the proinflammatory environment
- It is essential to study Treg from the site of inflammation in patients with autoimmune disease and mimic the *in vivo* proinflammatory environment *in vitro*
- JIA represents a unique model to study Treg function at the site of autoimmune inflammation because immune cells from the inflamed joints can be isolated

REFERENCES

1. Prakken B, Albani S, Martini A. Juvenile idiopathic arthritis. Lancet 2011;377:2138-2149.
2. Lin YT, Wang CT, Gershwin ME, Chiang BL. The pathogenesis of oligoarticular/polyarticular vs systemic juvenile idiopathic arthritis. Autoimmun.Rev. 2011;10:482-489.
3. Ravelli A, Martini A. Juvenile idiopathic arthritis. Lancet 2007;369:767-778.
4. Prahalad, S. and Glass, D. N. Is juvenile rheumatoid arthritis/juvenile idopathic arthritis different from rheumatoid arthritis? 4, 303-310. 2012. Arthritis Research.
5. Vastert SJ, Kuis W, Grom AA. Systemic JIA: new developments in the understanding of the pathophysiology and therapy. Best.Pract.Res. Clin.Rheumatol. 2009;23:655-664.
6. Firestein GS. Evolving concepts of rheumatoid arthritis. Nature 2003;423:356-361.
7. Prakken BJ, Albani S. Using biology of disease to understand and guide therapy of JIA. Best. Pract.Res.Clin.Rheumatol. 2009;23:599-608.
8. De Jager W, Hoppenreijs EP, Wulffraat NM et al. Blood and synovial fluid cytokine signatures in patients with juvenile idiopathic arthritis: a cross-sectional study. Ann.Rheum.Dis. 2007;66:589-598.
9. Steiner G, Tohidast-Akrad M, Witzmann G et al. Cytokine production by synovial T cells in rheumatoid arthritis. Rheumatology.(Oxford) 1999;38:202-213.
10. McInnes IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. Nat. Rev.Immunol. 2007;7:429-442.
11. Bulatovic M, Heijstek MW, Verkaaik M et al. High prevalence of methotrexate intolerance in juvenile idiopathic arthritis: development and validation of a methotrexate intolerance severity score. Arthritis Rheum. 2011;63:2007-2013.
12. Feldmann M, Brennan FM, Williams RO, Woody JN, Maini RN. The transfer of a laboratory based hypothesis to a clinically useful therapy: the development of anti-TNF therapy of rheumatoid arthritis. Best.Pract.Res.Clin. Rheumatol. 2004;18:59-80.
13. Goldblatt F, Isenberg DA. New therapies for rheumatoid arthritis. Clin.Exp.Immunol. 2005;140:195-204.
14. Tanaka T, Kishimoto T. Immunotherapeutic implication of IL-6 blockade. Immunotherapy. 2012;4:87-105.
15. McGovern JL, Nguyen DX, Notley CA et al. Th17 cells are restrained by regulatory T cells from patients responding to anti-TNF antibody therapy via inhibition of IL-6. Arthritis Rheum. 2012;64:3129-3138.
16. Ehrenstein MR, Evans JG, Singh A et al. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. J.Exp.Med. 2004;200:277-285.
17. Goldstein I, Ben-Horin S, Koltakov A et al. alpha1beta1 Integrin+ and regulatory Foxp3+ T cells constitute two functionally distinct human CD4+ T cell subsets oppositely modulated by TNFalpha blockade. J.Immunol. 2007;178:201-210.
18. Nadkarni S, Mauri C, Ehrenstein MR. Anti-TNF-alpha therapy induces a distinct regulatory T cell population in patients with rheumatoid arthritis via TGF-beta. J.Exp.Med. 2007;204:33-39.
19. Valencia X, Stephens G, Goldbach-Mansky R et al. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. Blood 2006;108:253-261.
20. Weitoft T, Uddenfeldt P. Importance of synovial fluid aspiration when injecting intra-articular corticosteroids. Ann.Rheum.Dis. 2000;59:233-235.
21. Felix NJ, Allen PM. Specificity of T-cell alloreactivity. Nat.Rev.Immunol. 2007;7:942-953.
22. Gershon RK, Kondo K. Cell interactions in the induction of tolerance: the role of thymic lymphocytes. Immunology 1970;18:723-737.
23. Gershon RK, Kondo K. Infectious immunological tolerance. Immunology 1971;21:903-914.
24. Germain RN. Special regulatory T-cell review: A rose by any other name: from suppressor T cells to Tregs, approbation to unbridled enthusiasm. Immunology 2008;123:20-27.
25. Sakaguchi S. Regulatory T cells: key controllers of immunologic self-tolerance. Cell 2000;101:455-458.
26. Asano M, Toda M, Sakaguchi N, Sakaguchi S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. J.Exp.Med. 1996;184:387-396.
27. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism

of self-tolerance causes various autoimmune diseases. J.Immunol. 1995;155:1151-1164.

28. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat.Immunol. 2003;4:330-336.
29. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299:1057-1061.
30. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. Nat.Immunol. 2003;4:337-342.
31. Apostolou I, von BH. In vivo instruction of suppressor commitment in naive T cells. J.Exp. Med. 2004;199:1401-1408.
32. Chen W, Jin W, Hardegen N et al. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J.Exp. Med. 2003;198:1875-1886.
33. Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. Nat.Immunol. 2005;6:345-352.
34. Wing K, Yamaguchi T, Sakaguchi S. Cell-autonomous and -non-autonomous roles of CTLA-4 in immune regulation. Trends Immunol. 2011;32:428-433.
35. Wing K, Onishi Y, Prieto-Martin P et al. CTLA-4 control over Foxp3+ regulatory T cell function. Science 2008;322:271-275.
36. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. Nat.Rev.Immunol. 2008;8:523-532.
37. Takahashi T, Kuniyasu Y, Toda M et al. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. Int. Immunol. 1998;10:1969-1980.
38. Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J.Exp.Med. 1998;188:287-296.
39. Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. J.Exp.Med. 1999;190:995-1004.
40. Rubtsov YP, Rasmussen JP, Chi EY et al. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity. 2008;28:546-558.
41. Fahlen L, Read S, Gorelik L et al. T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. J.Exp. Med. 2005;201:737-746.
42. Green EA, Gorelik L, McGregor CM, Tran EH, Flavell RA. CD4+CD25+ T regulatory cells control anti-islet CD8+ T cells through TGF-beta-TGF-beta receptor interactions in type 1 diabetes. Proc.Natl.Acad.Sci.U.S.A 2003;100:10878-10883.
43. Li MO, Wan YY, Flavell RA. T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. Immunity. 2007;26:579-591.
44. Cao X, Cai SF, Fehniger TA et al. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. Immunity. 2007;27:635-646.
45. Gondek DC, Lu LF, Quezada SA, Sakaguchi S, Noelle RJ. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. J.Immunol. 2005;174:1783-1786.
46. Grossman WJ, Verbsky JW, Barchet W et al. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. Immunity. 2004;21:589-601.
47. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. CD4(+)CD25(+)Foxp3(+) regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4(+) T cells. Nat. Immunol. 2007;8:1353-1362.
48. Oderup C, Cederbom L, Makowska A, Cilio CM, Ivars F. Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression. Immunology 2006;118:240-249.
49. Onishi Y, Fehervari Z, Yamaguchi T, Sakaguchi S. Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. Proc.Natl. Acad.Sci.U.S.A 2008;105:10113-10118.
50. Cederbom L, Hall H, Ivars F. CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. Eur.J.Immunol. 2000;30:1538-1543.
51. Houot R, Perrot I, Garcia E, Durand I, Lebecque S. Human CD4+CD25high regulatory T cells modulate myeloid but not plasmacytoid dendritic cells activation. J.Immunol. 2006;176:5293-5298.

52. Lewkowich IP, Herman NS, Schleifer KW et al. CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. J.Exp.Med. 2005;202:1549-1561.

53. Misra N, Bayry J, Lacroix-Desmazes S, Kazatchkine MD, Kaveri SV. Cutting edge: human CD4+CD25+ T cells restrain the maturation and antigen-presenting function of dendritic cells. J.Immunol. 2004;172:4676-4680.

54. Taams LS, van Amelsfort JM, Tiemessen MM et al. Modulation of monocyte/macrophage function by human CD4+CD25+ regulatory T cells. Hum.Immunol. 2005;66:222-230.

55. Tiemessen MM, Jagger AL, Evans HG et al. CD4+CD25+Foxp3+ regulatory T cells induce alternative activation of human monocytes/macrophages. Proc.Natl.Acad.Sci.U.S.A 2007;104:19446-19451.

56. Suri-Payer E, Cantor H. Differential cytokine requirements for regulation of autoimmune gastritis and colitis by CD4(+)CD25(+) T cells. J.Autoimmun. 2001;16:115-123.

57. Yamaguchi T, Wing JB, Sakaguchi S. Two modes of immune suppression by Foxp3(+) regulatory T cells under inflammatory or non-inflammatory conditions. Semin.Immunol. 2011;23:424-430.

58. Bennett CL, Christie J, Ramsdell F et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat.Genet. 2001;27:20-21.

59. Wildin RS, Ramsdell F, Peake J et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nat.Genet. 2001;27:18-20.

60. Frey O, Petrow PK, Gajda M et al. The role of regulatory T cells in antigen-induced arthritis: aggravation of arthritis after depletion and amelioration after transfer of CD4+CD25+ T cells. Arthritis Res.Ther. 2005;7:R291-R301.

61. Morgan ME, Sutmuller RP, Witteveen HJ et al. CD25+ cell depletion hastens the onset of severe disease in collagen-induced arthritis. Arthritis Rheum. 2003;48:1452-1460.

62. Morgan ME, Flierman R, van Duivenvoorde LM et al. Effective treatment of collagen-induced arthritis by adoptive transfer of CD25+ regulatory T cells. Arthritis Rheum. 2005;52:2212-2221.

63. Esensten JH, Wofsy D, Bluestone JA. Regulatory T cells as therapeutic targets in rheumatoid arthritis. Nat.Rev.Rheumatol. 2009;5:560-565.

64. Flores-Borja F, Jury EC, Mauri C, Ehrenstein MR. Defects in CTLA-4 are associated with abnormal regulatory T cell function in rheumatoid arthritis. Proc.Natl.Acad. Sci.U.S.A 2008;105:19396-19401.

65. Mottonen M, Heikkinen J, Mustonen L et al. CD4+ CD25+ T cells with the phenotypic and functional characteristics of regulatory T cells are enriched in the synovial fluid of patients with rheumatoid arthritis. Clin.Exp.Immunol. 2005;140:360-367.

66. Van Amelsfort JM, Jacobs KM, Bijlsma JW, Lafeber FP, Taams LS. CD4(+)CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. Arthritis Rheum. 2004;50:2775-2785.

67. Lawson CA, Brown AK, Bejarano V et al. Early rheumatoid arthritis is associated with a deficit in the CD4+CD25high regulatory T cell population in peripheral blood. Rheumatology.(Oxford) 2006;45:1210-1217.

68. Cao D, Van Vollenhoven R, Klareskog L, Trollmo C, Malmstrom V. CD25brightCD4+ regulatory T cells are enriched in inflamed joints of patients with chronic rheumatic disease. Arthritis Res.Ther. 2004;6:R335-R346.

69. De Kleer I, Wedderburn LR, Taams LS et al. CD4+CD25bright regulatory T cells actively regulate inflammation in the joints of patients with the remitting form of juvenile idiopathic arthritis. J.Immunol. 2004;172:6435-6443.

70. Ruprecht CR, Gattorno M, Ferlito F et al. Coexpression of CD25 and CD27 identifies FoxP3+ regulatory T cells in inflamed synovia. J.Exp.Med. 2005;201:1793-1803.

71. Cavanagh LL, Boyce A, Smith L et al. Rheumatoid arthritis synovium contains plasmacytoid dendritic cells. Arthritis Res. Ther. 2005;7:R230-R240.

72. Gattorno M, Chicha L, Gregorio A et al. Distinct expression pattern of IFN-alpha and TNF-alpha in juvenile idiopathic arthritis synovial tissue. Rheumatology.(Oxford) 2007;46:657-665.

73. Lande R, Giacomini E, Serafini B et al. Characterization and recruitment of plasmacytoid dendritic cells in synovial fluid and tissue of patients with chronic inflammatory arthritis. J.Immunol. 2004;173:2815-2824.

74. Thomas R, Quinn C. Functional differentiation of dendritic cells in rheumatoid arthritis: role of CD86 in the synovium. J.Immunol. 1996;156:3074-3086.

75. Evans HG, Gullick NJ, Kelly S et al. In vivo activated monocytes from the site of inflammation in humans specifically promote Th17 responses. Proc.Natl.Acad.Sci.U.S.A 2009;106:6232-6237.

76. Smolewska E, Stanczyk J, Brozik H et al. Distribution and clinical significance of blood dendritic cells (BDC) in children with juvenile idiopathic arthritis. Ann.Rheum.Dis. 2008;67:762-768.

77. Van Amelsfort JM, van Roon JA, Noordegraaf M et al. Proinflammatory mediator-induced reversal of CD4+,CD25+ regulatory T cell-mediated suppression in rheumatoid arthritis. Arthritis Rheum. 2007;56:732-742.

78. Ashley CW, Baecher-Allan C. Cutting Edge: Responder T cells regulate human DR+ effector regulatory T cell activity via granzyme B. J.Immunol. 2009;183:4843-4847.

79. Baecher-Allan C, Viglietta V, Hafler DA. Inhibition of human CD4(+)CD25(+high) regulatory T cell function. J.Immunol. 2002;169:6210-6217.

80. Parietti V, Monneaux F, Decossas M, Muller S. Function of CD4+,CD25+ Treg cells in MRL/lpr mice is compromised by intrinsic defects in antigen-presenting cells and effector T cells. Arthritis Rheum. 2008;58:1751-1761.

81. Yan B, Ye S, Chen G et al. Dysfunctional CD4+,CD25+ regulatory T cells in untreated active systemic lupus erythematosus secondary to interferon-alpha-producing antigen-presenting cells. Arthritis Rheum. 2008;58:801-812.

82. Koenen HJ, Smeets RL, Vink PM et al. Human CD25highFoxp3pos regulatory T cells differentiate into IL-17-producing cells. Blood 2008;112:2340-2352.

83. Xu L, Kitani A, Fuss I, Strober W. Cutting Edge: Regulatory T Cells Induce CD4+CD25-Foxp3- T Cells or Are Self-Induced to Become Th17 Cells in the Absence of Exogenous TGF-beta. J.Immunol. 2007;178:6725-6729.

84. Nagar M, Jacob-Hirsch J, Vernitsky H et al. TNF activates a NF-kappaB-regulated cellular program in human CD45RA$^-$ Regulatory T Cells that Modulates Their Suppressive Function . J.Immunol. 2010;184:3570-3581.

85. Herrath J, Muller M, Amoudruz P et al. The inflammatory milieu in the rheumatic joint reduces regulatory T-cell function. Eur.J.Immunol. 2011;41:2279-2290.

86. Allan SE, Crome SQ, Crellin NK et al. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. Int.Immunol. 2007;19:345-354.

87. Gavin MA, Torgerson TR, Houston E et al. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. Proc. Natl.Acad.Sci.U.S.A 2006;103:6659-6664.

88. Morgan ME, van Bilsen JH, Bakker AM et al. Expression of FOXP3 mRNA is not confined to CD4+CD25+ T regulatory cells in humans. Hum.Immunol. 2005;66:13-20.

89. Wang J, Ioan-Facsinay A, Van der Voort EI, Huizinga TW, Toes RE. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. Eur.J.Immunol. 2007;37:129-138.

90. Tran DQ, Ramsey H, Shevach EM. Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype. Blood 2007;110:2983-2990.

91. Liu W, Putnam AL, Xu-Yu Z et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J.Exp.Med. 2006;203:1701-1711.

92. Seddiki N, Santner-Nanan B, Martinson J et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J.Exp.Med. 2006;203:1693-1700.

2

TREATING ARTHRITIS BY IMMUNOMODULATION: IS THERE A ROLE FOR REGULATORY T CELLS?

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ABSTRACT

The discovery of regulatory T cells (Treg) 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 Treg in patients with autoimmune arthritis. Here, we review recent studies investigating the presence, phenotype and function of Treg in patients with rheumatoid arthritis (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 Treg-enhancing strategies could be most successful for clinical application.

INTRODUCTION

Rheumatoid arthritis (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 TNFα. 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 (IPEX) syndrome, characterized by autoimmune

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
T regulatory 1 (Tr1)	-	Induced in the periphery	IL-10 production
T helper 3 (Th3)	-	Induced in the periphery	TGFβ production

disease in multiple organs[8;9]. More recently, it has been established in both mice and humans that Treg can also be induced in 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 TGFβ[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 synovial fluid (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 proinflammatory environment can interfere with the suppressive function of the cells. High levels of proinflammatory cytokines are present in the inflamed synovium of RA and JIA patients, including IL-6, IL-7, IL-15 and TNFα[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, TNFα can act directly on Treg and, in line with this, it was shown that pre-incubation of Treg with TNFα reduces FOXP3 expression and abrogates suppression[27]. Other proinflammatory 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 costimulatory 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.

Table 2 | Presence, phenotype, and function of Treg in arthritis

Disease	Markers used to identify Treg	Peripheral Blood			Synovial Fluid			Reference
		Presence	Phenotype	Function	Presence	Phenotype	Function	
						FOXP3 mRNA ↑⇑		
JIA	$CD4^+CD25^{high}$	↓	FOXP3 mRNA =	NA	↑⇑	HLA-DR MFI CTLA-4 MFI GITR MFI CD69+ ⇑	⇑	20
JIA	$CD4^+CD25^+$ [a] $CD4^+CD25^+CD27^+$ [b]	NA	NA	NA	⇑	FOXP3 mRNA ⇑	⇑	25
RA	$CD4^+CD25^{high}$	NA	NA	NA	⇑	NA	c	21
RA	$CD4^+CD25^{high}$	=	NA	↓	NA	NA	NA	22
RA	$CD4^+FOXP3^{high}$ [a] $CD4^+CD25^+CD127^{low}$ [b]	=	CTLA-4 MFI ↓	↓	NA	NA	NA	23
RA	$CD4^+CD25^{high}$	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	$CD4^+CD25^{high}$	NA	FOXP3 mRNA ↓ TNFRII+ GITR+ ↑ CD69+ =	↓	NA	NA	NA	27
RA	$CD4^+CD25^+$	=	NA	NA	⇑	CTLA-4+ GITR+ OX40+ CD69+ ↑⇑	c	28

[a] Used for phenotyping. [b] Used for functional assays. [c] Suppressive, 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.

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 responses *in vivo* and be useful in the treatment of autoimmune 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. Furthermore, these cells started to produce effector cytokines, such as IL-2 and IFNγ. Only cells that co-expressed CCR7 and CD62L after expansion showed a stable Treg phenotype and these cells could be generated by selecting the $CD45RA^{+}CD4^{+}CD25^{high}$ 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 autoimmune 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 expansion of Treg with compliance to the Good Manufacturing 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 circumvents the difficulty of obtaining high numbers of natural Treg required for expansion. Treg induction works well in mice in which $CD4^{+}CD25^{-}$ cells activated in the presence of TGFβ 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 expression of FOXP3 in humans does not confirm a regulatory phenotype and can even coincide with IL-2 and IFNγ production[50]. Therefore, *in vitro* induction of Treg is far more complicated in humans compared with mice and it still 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

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]	-

Abbreviations: NOD, non-obese diabetic; PsA, psoriatic arthritis; CIA, collagen induced arthritis; TNBS, trinitrobenzene sulfonic acid; DSS, dextran sodium sulphate.

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 psoriatic arthritis (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 proinflammatory 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 proinflammatory 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 TGFβ 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 TNFα and IFNγ 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 TNFα 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 proinflammatory environment and enhancing responsiveness of effector cells to suppression.

INHIBITION OF PROINFLAMMATORY CYTOKINES

As described above, the *in vivo* proinflammatory 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 proinflammatory 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-TNFα (infliximab) therapy. Both studies reported impaired Treg function before therapy, which was completely restored after infliximab treatment[22;27]. Probably, neutralizing the high TNFα levels in these patients directly reduced the down-regulating effect of TNFα 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-TNFα 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 proinflammatory 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 TNFα. Furthermore, adding both these cytokines to naive 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 TNFα 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 proinflammatory 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 (Figure 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 reduces the down-regulating effect of proinflammatory 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-TNFα therapy in adjuvant arthritis. Both nasal administration of HSP60 peptide as well as a single dose of anti-TNFα (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

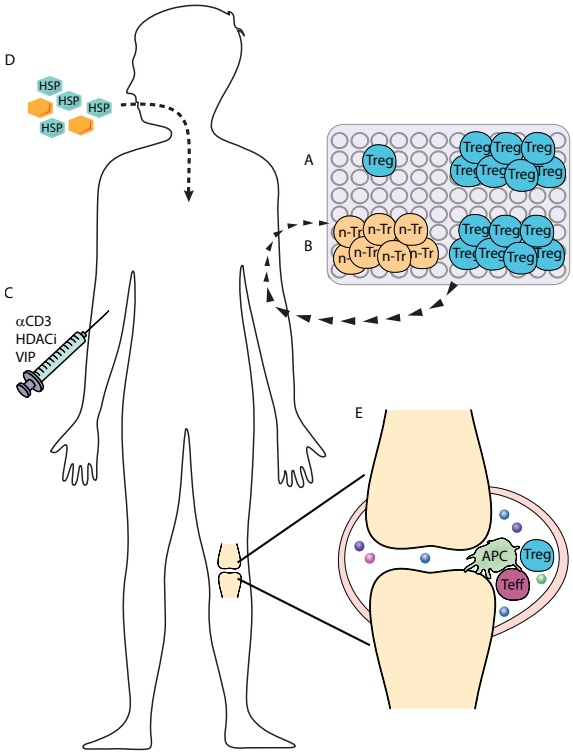

Figure 1 | Methods of enhancing Treg function in arthritis patients. Treg can be enhanced in arthritis patients via different methods: **A**, isolation and *ex vivo* expansion of natural Treg or **B**, *in vitro* induction of Treg from non-Treg (n-Tr), followed by reinfusion into the patient; **C**, *in vivo* induction and expansion of Treg by anti-CD3 antibodies (aCD3), 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 proinflammatory cytokines.

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 proinflammatory environment TGFβ 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 pseudoautologous 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-TNFα 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 proinflammatory 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-TNFα 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.

KEY POINTS

- 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

REFERENCES

1. Strand V, Kimberly R, Isaacs JD. Biologic therapies in rheumatology: lessons learned, future directions. Nat Rev Drug Discov 2007;6:75–92.
2. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995;155:1151–64.
3. Sakaguchi S. Naturally arising FOXP3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. Nat Immunol 2005;6:345–52.
4. Fontenot JD, Gavin MA, Rudensky AY. FOXP3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003;4:330–6.
5. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299:1057–61.
6. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for scurfin in CD4+CD25+ T regulatory cells. Nat Immunol 2003;4:337–42.
7. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. Nat Rev Immunol 2008;8:523–32.
8. Bennett CL, Christie J, Ramsdell F et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet 2001;27:20–1.
9. Wildin RS, Ramsdell F, Peake J et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nat Genet 2001;27:18–20.
10. Apostolou I, von Boehmer H. In vivo instruction of suppressor commitment in naive T cells. J Exp Med 2004;199:1401–8.
11. Chen W, Jin W, Hardegen N et al. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor FOXP3. J Exp Med 2003;198:1875–86.
12. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+ CD25- T cells through Foxp3 induction and down-regulation of Smad 7. J Immunol 2004;172:5149–53.
13. Yamagiwa S, Gray JD, Hashimoto S, Horwitz DA. A role for TGF-beta in the generation and expansion of CD4+CD25+ regulatory T cells from human peripheral blood. J Immunol 2001;166:7282–9.
14. Groux H, O'Garra A, Bigler M et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 1997;389:737–42.
15. Roncarolo MG, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K, Levings MK. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. Immunol Rev 2006;212:28–50.1.
16. Weiner HL. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. Immunol Rev 2001;182:207–14.
17. Morgan ME, Sutmuller RP, Witteveen HJ et al. CD25+ cell depletion hastens the onset of severe disease in collagen-induced arthritis. Arthritis Rheum 2003;48:1452–60.
18. Morgan ME, Flierman R, van Duivenvoorde LM et al. Effective treatment of collagen-induced arthritis by adoptive transfer of CD25+ regulatory T cells. Arthritis Rheum 2005;52:2212–21.
19. Frey O, Petrow PK, Gajda M et al. The role of regulatory T cells in antigen-induced arthritis: aggravation of arthritis after depletion and amelioration after transfer of CD4+CD25+ T cells. Arthritis Res Ther 2005;7:R291–301.
20. De Kleer I, Wedderburn LR, Taams LS et al. CD4+CD25bright regulatory T cells actively regulate inflammation in the joints of patients with the remitting form of juvenile idiopathic arthritis. J Immunol 2004;172:6435–43.
21. Cao D, van Vollenhoven R, Klareskog L, Trollmo C, Malmstrom V. CD25brightCD4+ regulatory T cells are enriched in inflamed joints of patients with chronic rheumatic disease. Arthritis Res Ther 2004;6:R335–46.
22. Ehrenstein MR, Evans JG, Singh A et al. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. J Exp Med 2004;200:277–85.
23. Flores-Borja F, Jury EC, Mauri C, Ehrenstein MR. Defects in CTLA-4 are associated with abnormal regulatory T cell function in rheumatoid arthritis. Proc Natl Acad Sci USA 2008;105:19396–401.

24. Lawson CA, Brown AK, Bejarano V et al. Early rheumatoid arthritis is associated with a deficit in the CD4+CD25 high regulatory T cell population in peripheral blood. Rheumatology 2006;45:1210–7.
25. Ruprecht CR, Gattorno M, Ferlito F et al. Coexpression of CD25 and CD27 identifies FOXP3+ regulatory T cells in inflamed synovia. J Exp Med 2005;201:1793–803.
26. Van Amelsfort JM, Jacobs KM, Bijlsma JW, Lafeber FP, Taams LS. CD4(+)CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. Arthritis Rheum 2004;50:2775–85.
27. Valencia X, Stephens G, Goldbach-Mansky R, Wilson M, Shevach EM, Lipsky PE. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. Blood 2006;108:253–61.
28. Mottonen M, Heikkinen J, Mustonen L, Isomaki P, Luukkainen R, Lassila O. CD4+ CD25+ T cells with the phenotypic and functional characteristics of regulatory T cells are enriched in the synovial fluid of patients with rheumatoid arthritis. Clin Exp Immunol 2005;140:360–7.
29. Allan SE, Crome SQ, Crellin NK et al. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. Int Immunol 2007;19:345–54.
30. De Jager W, Hoppenreijs EP, Wulffraat NM, Wedderburn LR, Kuis W, Prakken BJ. Blood and synovial fluid cytokine signatures in patients with juvenile idiopathic arthritis: a cross-sectional study. Ann Rheum Dis 2007;66:589–98.
31. Harada S, Yamamura M, Okamoto H et al. Production of interleukin-7 and interleukin-15 by fibroblast-like synoviocytes from patients with rheumatoid arthritis. Arthritis Rheum 1999;42:1508–16.
32. Steiner G, Tohidast-Akrad M, Witzmann G et al. Cytokine production by synovial T cells in rheumatoid arthritis. Rheumatology 1999;38:202–13.
33. Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science 2003;299:1033–6.
34. Van Amelsfort JM, Van Roon JA, Noordegraaf M et al. Proinflammatory mediator-induced reversal of CD4+, CD25+ regulatory T cell-mediated suppression in rheumatoid arthritis. Arthritis Rheum 2007;56:732–42.
35. Koenen HJ, Smeets RL, Vink PM, van Rijssen E, Boots AM, Joosten I. Human CD25highFOXP3pos regulatory T cells differentiate into IL-17-producing cells. Blood 2008;112:2340–52.
36. Radhakrishnan S, Cabrera R, Schenk EL et al. Reprogrammed FoxP3+ T regulatory cells become IL-17+ antigen-specific autoimmune effectors in vitro and in vivo. J Immunol 2008;181:3137–47.
37. Xu L, Kitani A, Fuss I, Strober W. Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta. J Immunol 2007;178:6725–9.
38. Smolewska E, Stanczyk J, Brozik H et al. Distribution and clinical significance of blood dendritic cells in children with juvenile idiopathic arthritis. Ann Rheum Dis 2008;67:762–8.
39. Hoffmann P, Eder R, Kunz-Schughart LA, Andreesen R, Edinger M. Large-scale in vitro expansion of polyclonal human CD4(+)CD25high regulatory T cells. Blood 2004;104:895–903.
40. Levings MK, Sangregorio R, Roncarolo MG. Human CD25(+)CD4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. J Exp Med 2001;193:1295–302.
41. Tang Q, Henriksen KJ, Bi M et al. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. J Exp Med 2004;199:1455–65.
42. Scalapino KJ, Tang Q, Bluestone JA, Bonyhadi ML, Daikh DI. Suppression of disease in New Zealand Black/ New Zealand White lupus-prone mice by adoptive transfer of ex vivo expanded regulatory T cells. J Immunol 2006;177:1451–9.
43. Battaglia M, Stabilini A, Roncarolo MG. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. Blood 2005;105:4743–8.
44. Battaglia M, Stabilini A, Migliavacca B, Horejs-Hoeck J, Kaupper T, Roncarolo MG. Rapamycin promotes expansion of functional CD4+CD25+FOXP3+ regulatory T cells of both healthy subjects and type 1 diabetic patients. J Immunol 2006;177:8338–47.
45. Hoffmann P, Eder R, Boeld TJ et al. Only the CD45RA+ subpopulation of CD4+CD25high T cells gives rise to homogeneous regulatory

T-cell lines upon in vitro expansion. Blood 2006;108:4260–7.

46. Tran DQ, Andersson J, Hardwick D, Bebris L, Illei GG, Shevach EM. Selective expression of latency-associated peptide (LAP) and IL-1 receptor type I/II (CD121a/CD121b) on activated human FOXP3+ regulatory T cells allows for their purification from expansion cultures. Blood 2009;113:5125–33.
47. Komatsu N, Mariotti-Ferrandiz ME, Wang Y, Malissen B, Waldmann H, Hori S. Heterogeneity of natural FOXP3+ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. ProcNatl Acad Sci USA 2009;106:1903–8.
48. Zhou X, Bailey-Bucktrout SL, Jeker LT et al. Instability of the transcription factor FOXP3 leads to the generation of pathogenic memory T cells in vivo. Nat Immunol 2009;10:1000–7.
49. Wang J, Ioan-Facsinay A, van der Voort EI, Huizinga TW, Toes RE. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. Eur J Immunol 2007;37:129–38.
50. Tran DQ, Ramsey H, Shevach EM. Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype. Blood 2007;110:2983–90.
51. Chatenoud L. CD3-specific antibody-induced active tolerance: from bench to bedside. Nat Rev Immunol 2003;3:123–32.
52. Herold KC, Hagopian W, Auger JA et al. Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus. N Engl J Med 2002;346:1692–8.
53. Keymeulen B, Vandemeulebroucke E, Ziegler AG et al. Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. N Engl J Med 2005;352:2598–608.
54. Utset TO, Auger JA, Peace D et al. Modified anti-CD3 therapy in psoriatic arthritis: a phase I/II clinical trial. J Rheumatol 2002;29:1907–13.
55. Belghith M, Bluestone JA, Barriot S, Megret J, Bach JF, Chatenoud L. TGF-beta-dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. Nat Med 2003;9:1202–8.
56. Chatenoud L, Primo J, Bach JF. CD3 antibody-induced dominant self tolerance in overtly diabetic NOD mice. J Immunol 1997;158:2947–54.
57. Delgado M, Abad C, Martinez C, Leceta J, Gomariz RP. Vasoactive intestinal peptide prevents experimental arthritis by downregulating both autoimmune and inflammatory components of the disease. Nat Med 2001;7:563–8.
58. Juarranz Y, Abad C, Martinez C et al. Protective effect of vasoactive intestinal peptide on bone destruction in the collagen-induced arthritis model of rheumatoid arthritis. Arthritis Res Ther 2005;7:R1034–45.
59. Delgado M, Chorny A, Gonzalez-Rey E, Ganea D. Vasoactive intestinal peptide generates CD4+CD25+ regulatory T cells in vivo. J Leukoc Biol 2005;78:1327–38.
60. Chorny A, Gonzalez-Rey E, Fernandez-Martin A, Pozo D, Ganea D, Delgado M. Vasoactive intestinal peptide induces regulatory dendritic cells with therapeutic effects on autoimmune disorders. Proc Natl Acad Sci USA 2005;102:13562–7.
61. Delgado M, Gonzalez-Rey E, Ganea D. The neuropeptide vasoactive intestinal peptide generates tolerogenic dendritic cells. J Immunol 2005;175:7311–24.
62. Gonzalez-Rey E, Chorny A, Fernandez-Martin A, Ganea D, Delgado M. Vasoactive intestinal peptide generates human tolerogenic dendritic cells that induce CD4 and CD8 regulatory T cells. Blood 2006;107:3632–8.
63. Gonzalez-Rey E, Fernandez-Martin A, Chorny A, Delgado M. Vasoactive intestinal peptide induces CD4+, CD25+ T regulatory cells with therapeutic effect in collagen-induced arthritis. Arthritis Rheum 2006;54:864–76.
64. Gonzalez-Rey E, Chorny A, Varela N, O'Valle F, Delgado M. Therapeutic effect of urocortin on collagen-induced arthritis by down-regulation of inflammatory and Th1 responses and induction of regulatory T cells. Arthritis Rheum 2007;56:531–43.
65. Elias KM, Laurence A, Davidson TS et al. Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway. Blood 2008;111:1013–20.
66. Mucida D, Park Y, Kim G et al. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. Science 2007;317:256–60.
67. Schambach F, Schupp M, Lazar MA, Reiner SL. Activation of retinoic acid receptor-alpha favours regulatory T cell induction at the expense of IL-17-secreting T helper cell differentiation. Eur J Immunol 2007;37:2396–9.
68. Pino-Lagos K, Benson MJ, Noelle RJ. Retinoic acid in the immune system. Ann N Y Acad Sci 2008;1143:170–87.

69. Nistala K, Wedderburn LR. Th17 and regulatory T cells: rebalancing pro- and anti-inflammatory forces in autoimmune arthritis. Rheumatology 2009;48:602–6.

70. Van YH, Lee WH, Ortiz S, Lee MH, Qin HJ, Liu CP. All-trans retinoic acid inhibits type 1 diabetes by T regulatory (Treg)-dependent suppression of interferon-gamma-producing T-cells without affecting Th17 cells. Diabetes 2009;58:146–55.

71. Bai A, Lu N, Guo Y, Liu Z, Chen J, Peng Z. All-trans retinoic acid down-regulates inflammatory responses by shifting the Treg/Th17 profile in human ulcerative and murine colitis. J Leukoc Biol 2009;86:959–69.

72. Nozaki Y, Yamagata T, Sugiyama M, Ikoma S, Kinoshita K, Funauchi M. Anti-inflammatory effect of all-trans-retinoic acid in inflammatory arthritis. Clin Immunol 2006;119:272–9.

73. Benson MJ, Pino-Lagos K, Rosemblatt M, Noelle RJ. All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. J Exp Med 2007;204:1765–74.

74. Wang J, Huizinga TW, Toes RE. De novo generation and enhanced suppression of human CD4+CD25+ regulatory T cells by retinoic acid. J Immunol 2009;183:4119–26.

75. Lal G, Bromberg JS. Epigenetic mechanisms of regulation of Foxp3 expression. Blood 2009;144:3727–35.

76. Kang SG, Lim HW, Andrisani OM, Broxmeyer HE, Kim CH. Vitamin A metabolites induce gut-homing FoxP3+ regulatory T cells. J Immunol 2007;179: 3724–33.

77. Li B, Samanta A, Song X et al. FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. Proc Natl Acad Sci USA 2007;104:4571–6.

78. Van Loosdregt J, Vercoulen Y, Guichelaar T et al. Regulation of Treg functionality by acetylation-mediated Foxp3 protein stabilization. Blood 2009;115:965–74.

79. Lucas JL, Mirshahpanah P, Haas-Stapleton E, Asadullah K, Zollner TM, Numerof RP. Induction of Foxp3+ regulatory T cells with histone deacetylase inhibitors. Cell Immunol 2009;257:97–104.

80. Tao R, de Zoeten EF, Ozkaynak E et al. Deacetylase inhibition promotes the generation and function of regulatory T cells. Nat Med 2007;13:1299–307.

81. De Zoeten EF, Wang L, Sai H, Dillmann WH, Hancock WW. Expression of HDAC9 by T regulatory cells prevents colitis in mice. Gastroenterology 2009;138:583–94.

82. Reilly CM, Thomas M, Gogal R Jr et al. The histone deacetylase inhibitor trichostatin A upregulates regulatoryT cells and modulates autoimmunity in NZB/W F1 mice. J Autoimmun 2008;31:123–30.

83. Saouaf SJ, Li B, Zhang G et al. Deacetylase inhibition increases regulatory T cell function and decreases incidence and severity of collagen-induced arthritis. Exp Mol Pathol 2009;87:99–104.

84. Samanta A, Li B, Song X et al. TGF-beta and IL-6 signals modulate chromatin binding and promoter occupancy by acetylated FOXP3. Proc Natl Acad Sci USA 2008;105:14023–7.

85. Wang L, de Zoeten EF, Greene MI, Hancock WW. Immunomodulatory effects of deacetylase inhibitors: therapeutic targeting of FOXP3+ regulatory T cells. Nat Rev Drug Discov 2009;8:969–81.

86. Bluestone JA. Regulatory T-cell therapy: is it ready for the clinic? Nat Rev Immunol 2005;5:343–9.

87. Zhang X, Izikson L, Liu L, Weiner HL. Activation of CD25(+)CD4(+) regulatory T cells by oral antigen administration? J Immunol 2001;167:4245–53.

88. Garcia G, Komagata Y, Slavin AJ, Maron R, Weiner HL. Suppression of collagen-induced arthritis by oral or nasal administration of type II collagen. J Autoimmun 1999;13:315–24.

89. Nagler-Anderson C, Bober LA, Robinson ME, Siskind GW, Thorbecke GJ. Suppression of type II collagen-induced arthritis by intragastric administration of soluble type II collagen. Proc Natl Acad Sci USA 1986;83:7443–6.

90. Staines NA, Harper N, Ward FJ, Malmstrom V, Holmdahl R, Bansal S. Mucosal tolerance and suppression of collagen-induced arthritis (CIA) induced by nasal inhalation of synthetic peptide 184–198 of bovine type II collagen (CII) expressing a dominant T cell epitope. Clin Exp Immunol 1996;103:368–75.

91. Min SY, Hwang SY, Park KS et al. Induction of IL-10-producing CD4+CD25+ T cells in animal model of collagen-induced arthritis by oral administration of type II collagen. Arthritis Res Ther 2004;6:R213–9.

92. Broere F, Wieten L, Klein Koerkamp EI et al. Oral or nasal antigen induces regulatory T cells that suppress arthritis and proliferation of arthritogenic T cells in joint draining lymph nodes. J Immunol 2008;181:899–906.

93. Park MJ, Min SY, Park KS et al. Indoleamine 2,3-dioxygenase-expressing dendritic cells

are involved in the generation of CD4+CD25+ regulatory T cells in Peyer's patches in an orally tolerized, collageninduced arthritis mouse model. Arthritis Res Ther 2008;10:R11.

94. Khare SD, Krco CJ, Griffiths MM, Luthra HS, David CS. Oral administration of an immunodominant human collagen peptide modulates collagen-induced arthritis. J Immunol 1995;155:3653–9.
95. Zhang ZY, Lee CS, Lider O, Weiner HL. Suppression of adjuvant arthritis in Lewis rats by oral administration of type II collagen. J Immunol 1990;145:2489–93.
96. Barnett ML, Combitchi D, Trentham DE. A pilot trial of oral type II collagen in the treatment of juvenile rheumatoid arthritis. Arthritis Rheum 1996;39:623–8.
97. Barnett ML, Kremer JM, St Clair EW et al. Treatment of rheumatoid arthritis with oral type II collagen. Results of a multicenter, double-blind, placebo-controlled trial. Arthritis Rheum 1998;41:290–7.
98. Choy EH, Scott DL, Kingsley GH et al. Control of rheumatoid arthritis by oral tolerance. Arthritis Rheum 2001;44:1993–7.
99. Myers LK, Higgins GC, Finkel TH et al. Juvenile arthritis and autoimmunity to type II collagen. Arthritis Rheum 2001;44:1775–81.
100. Sieper J, Kary S, Sorensen H et al. Oral type II collagen treatment in early rheumatoid arthritis. A double-blind, placebo-controlled, randomized trial. Arthritis Rheum 1996;39:41–51.
101. Trentham DE, Dynesius-Trentham RA, Orav EJ et al. Effects of oral administration of type II collagen on rheumatoid arthritis. Science 1993;261:1727–30.
102. Lehmann PV, Forsthuber T, Miller A, Sercarz EE. Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. Nature 1992;358:155–7.
103. Miller A, Lider O, Weiner HL. Antigen-driven bystander suppression after oral administration of antigens. J Exp Med 1991;174:791–8.
104. Yoshino S, Quattrocchi E, Weiner HL. Suppression of antigen-induced arthritis in Lewis rats by oral administration of type II collagen. Arthritis Rheum 1995;38:1092–6.
105. Van Eden W, Van der Zee R, Prakken B. Heat-shock proteins induce T-cell regulation of chronic inflammation. Nat Rev Immunol 2005;5:318–30.
106. Boog CJ, de Graeff-Meeder ER, Lucassen MA et al. Two monoclonal antibodies generated against human hsp60 show reactivity with synovial membranes of patients with juvenile chronic arthritis. J Exp Med 1992;175:1805–10.
107. Albani S, Keystone EC, Nelson JL et al. Positive selection in autoimmunity: abnormal immune responses to a bacterial dnaJ antigenic determinant in patients with early rheumatoid arthritis. Nat Med 1995;1:448–52.
108. Cohen IR. Autoimmunity to chaperonins in the pathogenesis of arthritis and diabetes. Annu Rev Immunol 1991;9:567–89.
109. Munk ME, Schoel B, Modrow S, Karr RW, Young RA, Kaufmann SH. T lymphocytes from healthy individuals with specificity to self-epitopes shared by the mycobacterial and human 65-kilodalton heat shock protein. J Immunol 1989;143:2844–9.
110. Zugel U, Kaufmann SH. Immune response against heat shock proteins in infectious diseases. Immunobiology 1999;201:22–35.
111. De Kleer I, Kamphuis SM, Rijkers GT et al. The spontaneous remission of juvenile idiopathic arthritis is characterized by CD30+ T cells directed to human heat-shock protein 60 capable of producing the regulatory cytokine interleukin-10. Arthritis Rheum 2003;48:2001–10.
112. Kamphuis S, Kuis W, de Jager W et al. Tolerogenic immune responses to novel T-cell epitopes from heat-shock protein 60 in juvenile idiopathic arthritis. Lancet 2005;366:50–6.
113. De Graeff-Meeder ER, van Eden W, Rijkers GT et al. Juvenile chronic arthritis: T cell reactivity to human HSP60 in patients with a favorable course of arthritis. J Clin Invest 1995;95:934–40.
114. Massa M, Passalia M, Manzoni SM et al. Differential recognition of heat-shock protein dnaJ-derived epitopes by effector and Treg cells leads to modulation of inflammation in juvenile idiopathic arthritis. Arthritis Rheum 2007;56:1648–57.
115. Cobelens PM, Heijnen CJ, Nieuwenhuis EE et al. Treatment of adjuvant-induced arthritis by oral administration of mycobacterial Hsp65 during disease. Arthritis Rheum 2000;43:2694–702.
116. Haque MA, Yoshino S, Inada S, Nomaguchi H, Tokunaga O, Kohashi O. Suppression of adjuvant arthritis in rats by induction of oral tolerance to mycobacterial 65-kDa heat shock protein. Eur J Immunol 1996;26:2650–6.
117. Wendling U, Paul L, Van der Zee R, Prakken B, Singh M, Van Eden WA. Conserved mycobacterial heat shock protein (hsp) 70 sequence prevents adjuvant arthritis upon

nasal administration and induces IL-10-producing T cells that cross-react with the mammalian self-hsp70 homologue. J Immunol 2000;164:2711–7.

118. Prakken BJ, Van der Zee R, Anderton SM, Van Kooten PJ, Kuis W, Van Eden W. Peptide-induced nasal tolerance for a mycobacterial heat shock protein 60 T cell epitope in rats suppresses both adjuvant arthritis and nonmicrobially induced experimental arthritis. Proc Natl Acad Sci USA 1997;94:3284–9.
119. Rosenthal M, Bahous I, Ambrosini G. Longterm treatment of rheumatoid arthritis with OM-8980. A retrospective study. J Rheumatol 1991;18:1790–3.
120. Vischer TL. Follow-up with OM-8980 after a double-blind study of OM-8980 and auranofin in rheumatoid arthritis. Clin Rheumatol 1990;9:356–61.
121. Polla BS, Baladi S, Fuller K, Rook G. Presence of hsp65 in bacterial extracts (OM-89): a possible mediator of orally-induced tolerance? Experientia 1995; 51:775–9.
122. Bloemendal A, Van der Zee R., Rutten VP, Van Kooten PJ, Farine JC, Van Eden W. Experimental immunization with anti-rheumatic bacterial extract OM-89 induces T cell responses to heat shock protein (hsp)60 and hsp70; modulation of peripheral immunological tolerance as its possible mode of action in the treatment of rheumatoid arthritis (RA)? Clin Exp Immunol 1997;110:72–8.
123. Prakken BJ, Samodal R, Le TD et al. Epitope-specific immunotherapy induces immune deviation of proinflammatory T cells in rheumatoid arthritis. Proc Natl Acad Sci USA 2004;101:4228–33.
124. Koffeman EC, Genovese M, Amox D et al. Epitope-specific immunotherapy of rheumatoid arthritis: clinical responsiveness occurs with immune deviation and relies on the expression of a cluster of molecules associated with T cell tolerance in a double-blind, placebo-controlled, pilot phase II trial. Arthritis Rheum 2009;60:3207–16.
125. Nadkarni S, Mauri C, Ehrenstein MR. Anti-TNF-alpha therapy induces a distinct regulatory T cell population in patients with rheumatoid arthritis via TGF-beta. J Exp Med 2007;204:33–9.
126. D'Alise AM, Auyeung V, Feuerer M et al. The defect in T-cell regulation in NOD mice is an effect on the T-cell effectors. Proc Natl Acad Sci USA 2008;105:19857–62.
127. Fantini MC, Rizzo A, Fina D et al. Smad7 controls resistance of colitogenic T cells to regulatory T cell mediated suppression. Gastroenterology 2008;136:1308–16.
128. Schneider A, Rieck M, Sanda S, Pihoker C, Greenbaum C, Buckner JH. The effector T cells of diabetic subjects are resistant to regulation via CD4+ FOXP3+ regulatory T cells. J Immunol 2008;181:7350–5.
129. Venigalla RK, Tretter T, Krienke S et al. Reduced CD4+,CD25- T cell sensitivity to the suppressive function of CD4+,CD25high,CD127low/- regulatory T cells in patients with active systemic lupus erythematosus. Arthritis Rheum 2008;58:2120–30.
130. Korn T, Reddy J, Gao W et al. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. Nat Med 2007;13:423–31.
131. Banham AH. Cell-surface IL-7 receptor expression facilitates the purification of FOXP3(+) regulatory T cells. Trends Immunol 2006;27:541–4.
132. Roord ST, Zonneveld-Huijssoon E, Le T et al. Modulation of T cell function by combination of epitope specific and low dose anticytokine therapy controls autoimmune arthritis. PLoS ONE 2006;1:e87.
133. Bresson D, Togher L, Rodrigo E et al. Anti-CD3 and nasal proinsulin combination therapy enhances remission from recent-onset autoimmune diabetes by inducing Tregs. J Clin Invest 2006;116:1371–81.
134. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity 2006;24:179–89.
135. Van Laar JM, Tyndall A. Adult stem cells in the treatment of autoimmune diseases. Rheumatology 2006;45:1187–93.
136. Van Wijk F, Roord ST, Vastert B, De Kleer I, Wulffraat N, Prakken BJ. Regulatory T cells in autologous stem cell transplantation for autoimmune disease. Autoimmunity 2008;41:585–91.
137. De Kleer I, Brinkman DM, Ferster A et al. Autologous stem cell transplantation for refractory juvenile idiopathic arthritis: analysis of clinical effects, mortality, and transplant related morbidity. Ann Rheum Dis 2004;63:1318–26.
138. De Kleer I, Vastert B, Klein M et al. Autologous stem cell transplantation for autoimmunity induces immunologic self-tolerance by reprogramming autoreactive T cells and

restoring the CD4+CD25+ immune regulatory network. Blood 2006;107:1696–702.

139. Herrmann MM, Gaertner S, Stadelmann C et al. Tolerance induction by bone marrow transplantation in a multiple sclerosis model. Blood 2005;106:1875–83.

140. Roord ST, De Jager W, Boon L et al. Autologous bone marrow transplantation in autoimmune arthritis restores immune homeostasis through CD4+CD25+Foxp3+ regulatory T cells. Blood 2008;111:5233–41.

3

HUMAN REGULATORY T CELL SUPPRESSIVE FUNCTION IS INDEPENDENT OF APOPTOSIS INDUCTION IN ACTIVATED EFFECTOR T CELLS

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ABSTRACT

BACKGROUND

$CD4^+CD25^+FOXP3^+$ regulatory T cells (Treg) play a central role in immune balance and prevent autoimmune disease. One outstanding question is how Treg suppress effector immune responses in human. Experiments in mice demonstrated that Treg restrict effector T cell (Teff) responses by deprivation of the growth factor IL-2 through consumption, resulting in apoptosis of Teff.

PRINCIPAL FINDINGS

In this study we investigated the relevance of Teff apoptosis induction for human Treg function. To this end, we studied naturally occurring Treg (nTreg) from peripheral blood of healthy donors, and, to investigate Treg function in inflammation *in vivo*, Treg from synovial fluid (SF) of juvenile idiopathic arthritis (JIA) patients (SF-Treg). Both nTreg and SF-Treg suppress Teff proliferation and cytokine production efficiently as predicted. However, in contrast with murine Treg, neither nTreg nor SF-Treg induce apoptosis in Teff. Furthermore, exogenously supplied IL-2 and IL-7 reverse suppression, but do not influence apoptosis of Teff.

SIGNIFICANCE

Our functional data here support that Treg are excellent clinical targets to counteract autoimmune diseases. For optimal functional outcome in human clinical trials, future work should focus on the ability of Treg to suppress proliferation and cytokine production of Teff, rather than induction of Teff apoptosis

INTRODUCTION

$CD4^+CD25^+FOXP3^+$ regulatory T cells (Treg) are of critical importance for maintenance of immune homeostasis, as numerous experimental mouse models for autoimmune diseases correlate the presence of functional Treg with amelioration of disease severity[1;2]. In humans Treg also play an important role in immune balance, since patients lacking functional Treg, due to loss-of-function mutations in the transcription factor FOXP3, suffer from severe generalized autoimmune disease; immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome[3;4]. In addition, in human autoimmune diseases, like juvenile idiopathic arthritis (JIA), negative correlations are found between the presence of regulatory T cells and disease severity[5]. Therefore, Treg are considered an important therapeutic target for a large range of human immune mediated diseases, and ongoing clinical trials attempt to modulate the population of Treg and thereby restore immune balance. For example, in diabetes mellitus type 1, patients were treated with anti-CD3 antibodies in order to enhance Treg function, which resulted in clinical improvement and increased residual β-cell function[6;7]. Moreover, in a clinical trial applying cord blood transplantation in patients suffering from haematological cancer, infusion of donor-derived Treg is tested to prevent or reduce Graft versus Host Disease (GvHD) (NCT00602693, www.clinicaltrials.gov).

Despite these potentially far-reaching applications of Treg in humans, questions remain with regard to the underlying mechanisms of Treg action, particularly in humans. Treg may suppress effector cells either through cell-cell contact, the production of suppressive cytokines, and/or through the consumption of cytokines and growth factors such as IL-2[8]. It is clear that IL-2 in many aspects is crucial for Treg function[9;10]. For one, it is required for Treg expansion, and regulates FOXP3 expression[11;12], and it is also indispensable for Treg mediated suppression[13]. On the other hand, FOXP3 suppresses IL-2 transcription, by binding to the IL-2 promoter[14;15]. As a result Treg do not produce IL-2, and may even act as a 'sink' for IL-2. Thus, competition for IL-2 between effector T cells (Teff) and Treg, which express a higher level of IL-2Rα chain (CD25) compared to Teff, may counteract proliferation of Teff[16;17]. Accordingly, Pandiyan et al. recently showed that in mice Treg consume IL-2 and thereby induce apoptosis in the Teff population[18;19]. This mechanism of apoptosis through cytokine deprivation was responsible for the suppressive function of Treg. Consistently, IL-2 and other IL-2Rγ-chain binding cytokines, such as IL-7, were able to overcome cell death[18], and, in earlier reports, have been shown to interfere with both murine and human Treg-mediated suppression[17;20].

We aimed to determine whether apoptosis induction via cytokine consumption by Treg is an important mechanism for human Treg-mediated suppression of Teff. As it is not fully understood how human Treg mediate their suppressive action on Teff, we studied the suppressive capacity and induction of apoptosis by naturally occurring Treg from peripheral blood and compared it to, assumedly *in vivo* activated Treg from an inflammatory site, the SF, of JIA patients. Our findings demonstrate that apoptosis induction in Teff is not important for human Treg mediated suppression.

MATERIALS AND METHODS

ETHICS STATEMENT

This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Institutional Review Board of the UMC Utrecht. All patients provided written informed consent for the collection of samples and subsequent analysis.

CELLS, MEDIUM AND REAGENTS

Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood of healthy volunteers and JIA patients and SF mononuclear cells (SFMC) from SF of JIA patients, after informed consent, using Ficoll Isopaque density gradient centrifugation (Amersham Biosciences, NJ, USA). RPMI 1640 containing 10 mM HEPES (Seromed), 2 mM L-glutamine 100 U/ml penicillin-streptomycin and 10% human AB serum was used as culture medium (all Invitrogen, Carlsbad, USA). Where indicated, IL-2 (1000 U/ml (= 60 ng/ml), Chiron, Uxbridge, UK) or IL-7 (10 ng/ml, PeproTech Inc, Rocky Hill, NJ, USA), were added.

SUPPRESSION ASSAY

$CD4^+CD25^-$ effector T cells (Teff), were magnetically isolated from PBMC using a CD4 T Lymphocyte Enrichment Set (BD Biosciences). Subsequently, $CD25^+$ T cells were depleted using CD25 Magnetic Particles (BD Biosciences). All magnetic cell isolations were performed according to the manufacturer's instructions. $CD4^+CD25^-$ T cells were labeled with 3 μM CFSE for 10 min at 37°C and extensively washed. 25.000 Teff (Teff) were plated into anti-CD3-coated wells (OKT3, 1.5 μg/ml), and to control for higher cell numbers in co-cultures (crowdedness) 50.000 Teff were plated (Teff + Teff). $CD4^+CD25^+CD127^-$ T cells were sorted as Treg from PBMC[37;38] (with an average of 58% $FOXP3^+$ cells ± 13% SD) or SFMC[39] (with an average of 24% $FOXP3^+$ cells ± 12% SD) by FACS Aria (BD Biosciences) and added in different ratios to Teff. T cell depleted, irradiated autologous PBMC (3500 Rad) were used as Antigen presenting cells (APCs), 30.000 per well. Cells were cultured for 5 days and proliferation was measured by flow cytometry on a FACS Calibur (BD Biosciences). The levels of $FOXP3^+$ cells in $CD4^+CD25^+CD127^{low}$ T cells directly isolated from PBMC or SFMC were lower than expected. This is due to an underestimation of the percentage of $FOXP3^+$ cells (See Supplementary Figure 4). All data were analyzed using Cellquest software.

FLOW CYTOMETRY STAINING

To determine levels of apoptosis, cells were stained with Annexin V PE and 7-AAD, using a staining kit according to the manufacturer's instructions (all BD Biosciences). $CFSE^+$ cells were gated to determine cell death within the Teff population. For FOXP3 analysis, PBMC were washed twice in FACS buffer (PBS containing 2% FCS and 0.1% sodium azide), adjusted to $0.5\text{-}1x10^6$ cells/ml in FACS buffer and blocked with mouse serum (5 minutes at 4°C). Subsequently, the cells were incubated in 50 ml FACS buffer containing appropriately diluted PE, FITC or PerCP labeled mAbs against human CD4 (clone RPAT4), CD25 (clone 2A3), CD127 (clone hIL-7R-m21), all from BD Biosciences. For intranuclear staining of APC or Pacific Blue FOXP3 (clone PCH101), V450 FOXP3 (clone 259D, BD Bioscience) or

Isotype Control, the cells were first surface stained, then fixed, permeabilized and stained using the FOXP3 staining kit (eBioscience) according to the manufacturer's instructions. Cells were analyzed on a FACS Calibur (BD Biosciences). All data were analyzed using Cellquest software.

ANALYSIS OF CYTOKINE PRODUCTION BY MULTIPLEXED PARTICLE-BASED FLOW CYTOMETRIC ASSAY

Cell culture supernatants were collected, stored at -80°C and processed within 1 month. Cytokine concentrations were measured with the Bio-Plex system in combination with the Bio-Plex Manager software, version 4.0 (Bio-Rad Laboratories, Hercules, CA, USA), which employs the Luminex xMAP technology as previously described[40]. The following cytokines were measured: IL-2, IL-5, IL-7, IL-10, IL-13, IL-17, tumor necrosis factor α (TNFα), and interferon γ (IFNγ).

STATISTICAL ANALYSIS

For statistical analysis of multiple groups One-way ANOVA or nonparametric ANOVA; Kruskal-Wallis test, were used. Bonferroni or Dunn's Multiple Comparison Test post test were used, to compare between 2 selected groups. To compare between two groups, non-parametric T test, Mann Whitney was used. P values below 0.05 were considered significant.

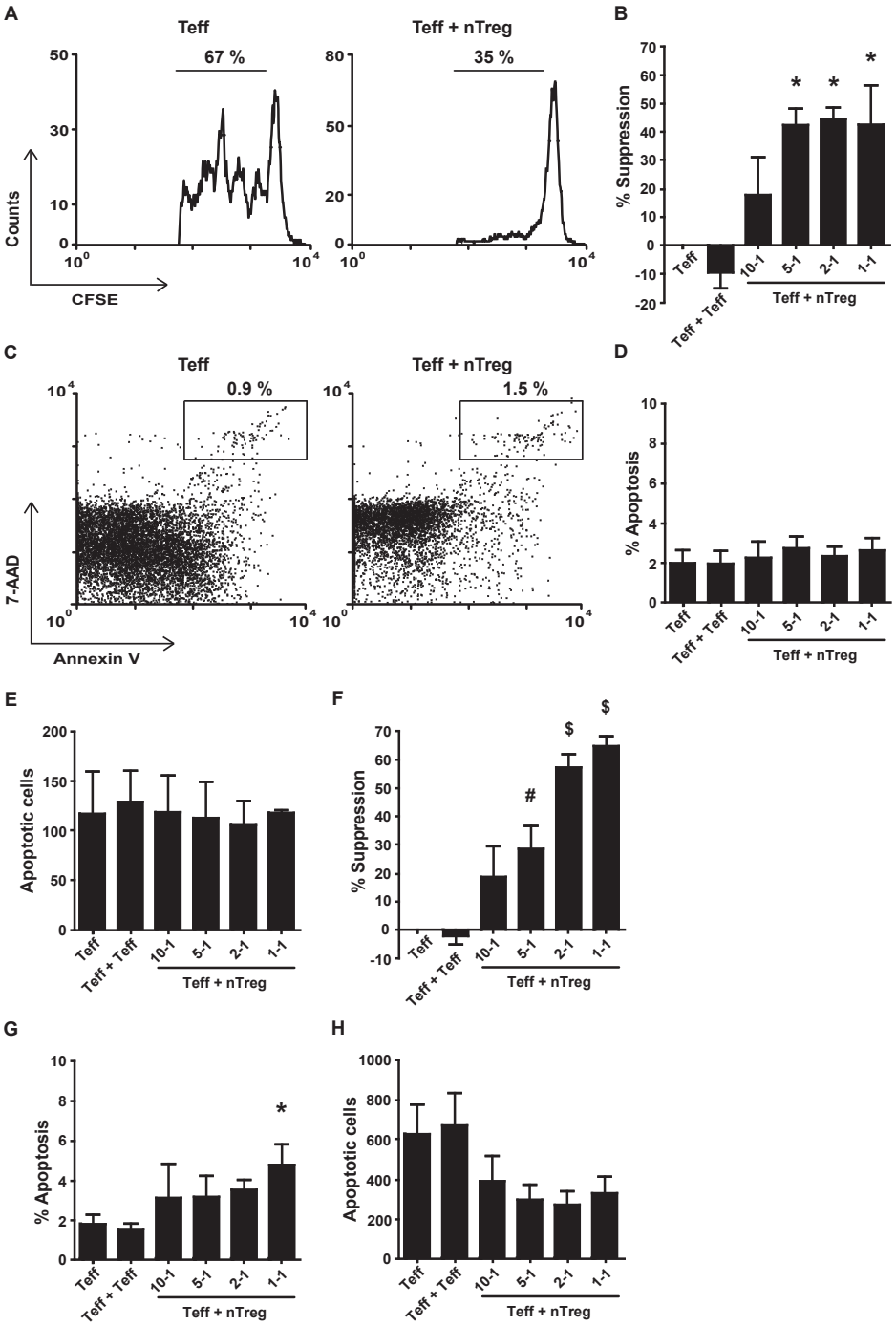
RESULTS

NTREG ARE HIGHLY SUPPRESSIVE WITHOUT INDUCING APOPTOSIS IN TEFF

We first established that human Treg inhibit proliferation of activated Teff. CFSE labeled Teff were co-cultured for 5 days with a graded amount of $CD4^+CD25^+CD127^{low}$ naturally occurring Treg (nTreg), in 200 μl culture medium, and suppression of Teff proliferation and induction of Teff apoptosis were determined. As expected, nTreg inhibited proliferation of Teff, as measured by decreased CFSE dilution in Teff cells (Figure 1 A). This suppression of proliferation increased with titrated amounts of Treg in the culture, in a dose-dependent manner (Figure 1 B).

Next, cells from the same co-cultures were stained with 7-AAD and Annexin V and gated on $CFSE^+$ cells (See Supplementary Figures 1 A and B) to determine apoptosis in Teff. Only few apoptotic cells were found in cultures with Teff only, and the percentage of apoptotic cells did not increase upon the presence of more nTreg (Figures 1 C and D), which was similar for the absolute number of apoptotic cells (Figure 1 E). Thus under normal culture conditions, human nTreg do not induce apoptosis in Teff, while efficiently suppressing Teff proliferation.

We hypothesized that if cytokine consumption by Treg in the vicinity is responsible for apoptosis in Teff, culture of the same number of Teff and Treg in a smaller volume should enhance suppression mediated by apoptosis induction. Therefore, all further cultures were performed in 75 μl instead of 200 μl medium. Under these conditions the level of suppression was higher (up to 65% average at a 1-1 ratio) compared to normal culture conditions (up to 48% average at a 1-1 ratio) (Figure 1 F). Furthermore, a larger number of Teff became apoptotic (up to 750 Annexin V^+7-AAD^+ cells average for Teff + Teff) (Figure 1 H), but in the co-cultures with nTreg the percentage of apoptotic cells only slightly increased (Figure 1 G), and the number of


A
Teff
67 %
Teff + nTreg
35 %
Counts
CFSE
B
% Suppression
Teff
Teff + Teff
10-1
5-1
2-1
1-1
Teff + nTreg
C
Teff
0.9 %
Teff + nTreg
1.5 %
7-AAD
Annexin V
D
% Apoptosis
E
Apoptotic cells
F
% Suppression
G
% Apoptosis
H
Apoptotic cells

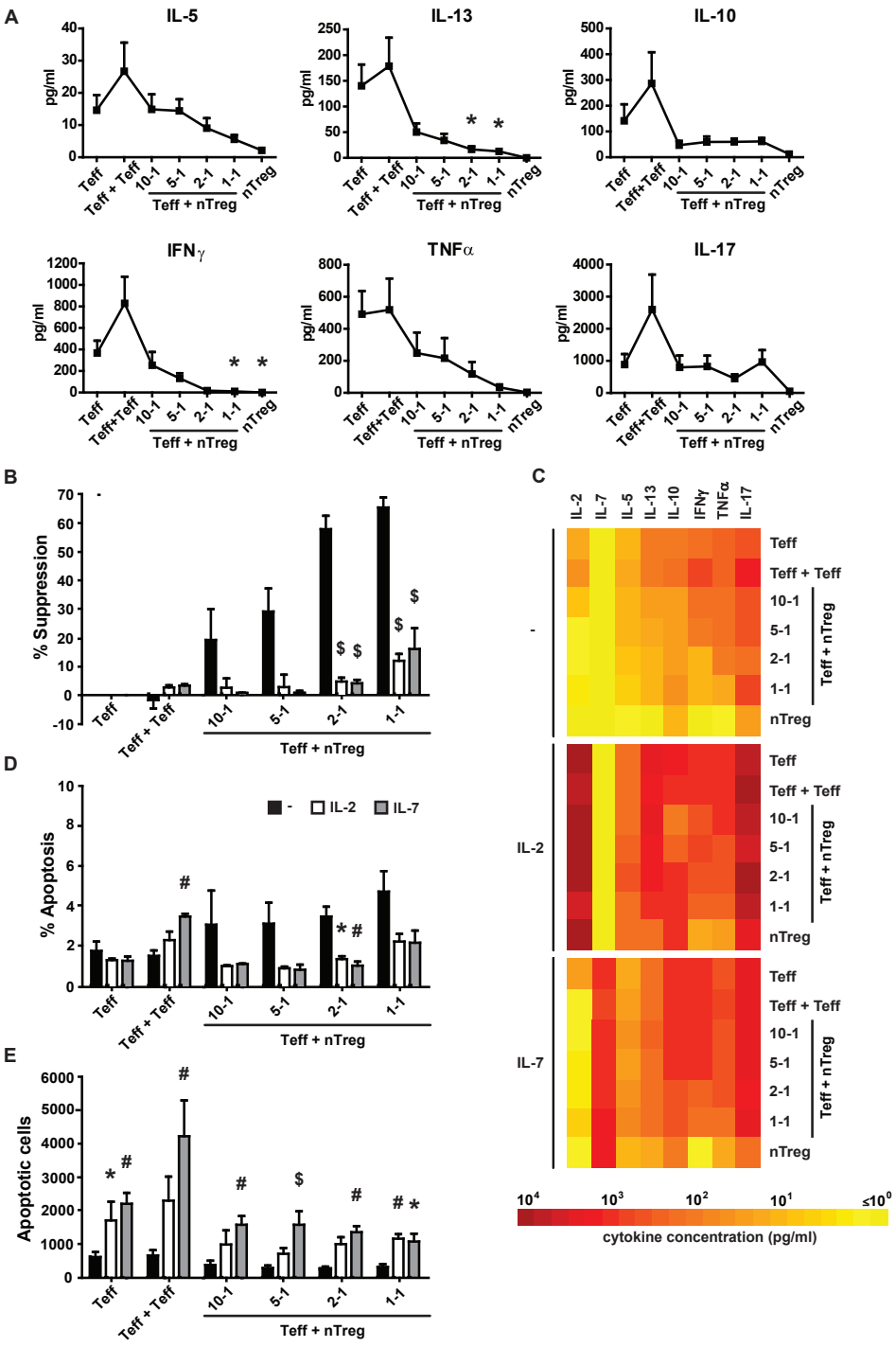
apoptotic Teff even decreased (Figure 1 H). Although we show a low up-regulation of Annexin V on highly activated cells (Supplementary Figure 3 A), the level of apoptosis per cell division was independent of the presence of Treg (Supplementary Figure 5). To establish that day 5 was the appropriate time point to measure apoptosis in our assays, we also measured cell death on day 3 and 4. Consistently, on day 3 and 4 hardly any apoptosis was seen (Supplementary Figures 1 C and D). Furthermore, we show that Teff in our assay are able to go into apoptosis, by titrating Sheath Fluid (BD Biosciences), containing ethanol into cultures with Teff (Supplementary Figures 2 A and B), causing Teff apoptosis in a dose-dependent manner. Thus, apoptosis induction does not occur in Teff + nTreg co-cultures, whereas high levels of suppression are reached. Altogether, these data clearly demonstrate that apoptosis induction is not important for nTreg mediated suppression.

IL-2 AND IL-7 OVERCOME SUPPRESSION, WITHOUT INFLUENCING APOPTOSIS

In mice, cytokine consumption was suggested to be pivotal for Treg-mediated apoptosis in Teff and suppression. Therefore, we investigated whether absence of IL-2Rγ-chain binding cytokines plays a role in the induction of apoptosis in Teff and suppression by human nTreg. In co-cultures of Teff and nTreg we observed a clear decrease in IL-2, as well as other cytokines important for Teff function; IL-5, IL-13, IL-10, IFNγ, TNFα, but not IL-17 (Figures 2 A and C; upper panel). This lack of IL-17 suppression could be due to a resistance of Th17 cells to Treg mediated suppression[21;22]. The decrease of cytokines in the culture medium in the presence of Treg could be due to either a general suppression of Teff cytokine production, or to cytokine consumption.

To investigate this further, we studied whether exogenously added cytokines could affect apoptosis induction of Teff, or suppression of proliferation and cytokine production by Teff. When high concentrations of exogenous IL-2 or IL-7 were added, proliferation of Teff cells increased (data not shown). Furthermore, suppression of Teff proliferation was abrogated in all co-culture ratios (Figure 2 B), which is in line with studies describing abrogation of Treg-mediated suppression by IL-2 and IL-7, by either Teff stimulation, or, in case of IL-2, abrogation of Treg anergy[17;20;23–25]. The high levels of IL-2 or IL-7 abrogated nTreg-mediated suppression of cytokine production by Teff as well (Figure 2 C and Supplementary Table 1). Furthermore, it seems that both IL-2 and IL-7 increase cytokine production of Treg, which may have contributed

◀ **Figure 1 |** Naturally occurring Treg suppress Teff proliferation, but do not induce apoptosis. **A-E**, Cells were cultured in 200 μl medium for 5 days (n = 3). **A**, Proliferation of Teff measured by flow cytometry cultured alone (*left panel*) or in co-culture with naturally occurring Treg (1-1) (*right panel*). 1 representative example is shown. **B**, Level of suppression of Teff proliferation calculated for several ratios of Teff + Treg and Teff + Teff (ratio 1-1), compared to culture of Teff alone (suppression = 0%). **C**, Apoptotic Teff cells ($CFSE^+$) were measured after 7-AAD and Annexin V staining by flow cytometry. Percentage of apoptosis in Teff cultured alone (*left panel*) and in co-culture with Treg (1-1) (*right panel*). 1 representative example is shown. **D**, Average percentage, and **E**, absolute number, corrected for cell input, of apoptotic Teff expressing 7-AAD and Annexin V for several co-culture ratios of Teff + Treg, Teff + Teff (ratio 1-1) and Teff alone. **F-H**, Cells were cultured in 75 μl medium for 5 days (n = 9). **F**, Level of suppression of Teff proliferation calculated for several ratios of Teff + Treg and Teff + Teff (ratio 1-1), compared to culture of Teff alone (suppression = 0%). **G**, Average percentage and **H**, absolute number, corrected for cell input, of apoptotic Teff cells expressing 7-AAD and Annexin V for several co-culture ratios of Teff + Treg, Teff + Teff (ratio 1-1) and Teff alone (n = 9). Error bars represent means ± SEM., *P < .05, #P < .01, $P < .001.


A
IL-5
IL-13
IL-10
IFNγ
TNFα
IL-17
pg/ml
Teff
Teff + Teff
10-1
5-1
2-1
1-1
nTreg
Teff + nTreg
B
% Suppression
C
IL-2
IL-7
IL-5
IL-13
IL-10
IFNγ
TNFα
IL-17
cytokine concentration (pg/ml)
D
% Apoptosis
E
Apoptotic cells

to abrogation of suppression. In contrast, although the percentage of apoptotic cells seems to decrease (Figure 2 D and Supplementary Figure 3 B), IL-2 and IL-7 did not decrease numbers of apoptotic Teff in co-cultures, instead the number of apoptotic cells was even significantly increased (Figure 2 E). Thus, IL-2Rγ-chain binding cytokines prevent suppression of Teff proliferation and cytokine production, but this is not accompanied by a reduction in apoptosis. Although we cannot conclude from these data whether cytokine consumption is involved, this emphasizes that nTreg mediated suppression is independent of apoptosis induction in Teff.

APOPTOSIS INDUCTION IN TEFF IS IRRELEVANT FOR TREG FUNCTION IN INFLAMMATION

Last, we wished to establish the relevance of our finding to human Treg function in ongoing inflammation. Therefore, we studied Treg from within a chronically inflamed environment, the SF of JIA patients (SF-Treg). In SF, Treg are abundantly present and highly activated, due to the chronic inflammation. Furthermore, Teff from SF probably have a different activation state, which may contribute to the ongoing inflammation in JIA. To make a reliable comparison between Treg from the peripheral blood and Treg from the site of inflammation, it is therefore preferable to use the same Teff population in all assays. Therefore, we co-cultured SF-Treg with Teff obtained from peripheral blood of the same patient in 75 μl medium. Probably due a different cellular composition, which may be caused by contaminating activated T cells, suppression of Teff proliferation by SF-Treg was less compared to nTreg (Figures 3 A and B), whereas suppression of Teff cytokine production was similar to nTreg (Figures 3 C and D, see also Supplementary Tables 2 and 3). Still, similar as for nTreg, despite a slight increase in the percentage of apoptotic cells, a decreased number of apoptotic Teff was found in the presence of SF-Treg (Figures 3 E and F). Altogether, even Treg from an inflammatory environment do not induce apoptosis in Teff cells to achieve suppression of Teff.

DISCUSSION

Pivotal studies in mice models have pointed out that Treg are indispensable for the maintenance of peripheral immune tolerance. Also in humans a similar role of Treg is likely, prompting discussions about their clinical applicability. Though comparable in many aspects,

◀ **Figure 2 |** Exogenous IL-2 and IL-7 decrease suppression of Teff proliferation and cytokine production, but do not decrease apoptosis. Cells were cultured in 75 μl medium for 5 days **A**, Levels of cytokines present in culture medium on day 5 of culture in several co-culture ratios of Teff + Treg, Teff + Teff (ratio 1-1) and Teff alone (n = 9). **B**, Level of suppression of $CFSE^+$ Teff proliferation calculated for several ratios of Teff + Treg, Teff + Teff (ratio 1-1) and Teff alone (suppression = 0%) cultured without (*black bars*), or with IL-2 (*white bars*) or IL-7 (*grey bars*). Suppression was calculated by comparing co-cultures with Teff alone with equal cell culture conditions. **C**, Mean levels of cytokines present in culture medium on day 5 of culture in medium (-), with addition of IL-2 (IL-2) or IL-7 (IL-7). A color profile of the means was made to show the differences between culture conditions (see also Supplementary Table 1). **D**, Average percentage and **E**, absolute number, corrected for cell input, of apoptotic $CFSE^+$ Teff cells expressing 7-AAD and Annexin V in several co-culture ratios of Teff + Treg, Teff + Teff (ratio 1-1) and Teff alone cultured without, or with IL-2 or IL-7. (B-E, n = 5). Error bars represent means ± SEM., *P < .05, #P < .01, $P < .001.

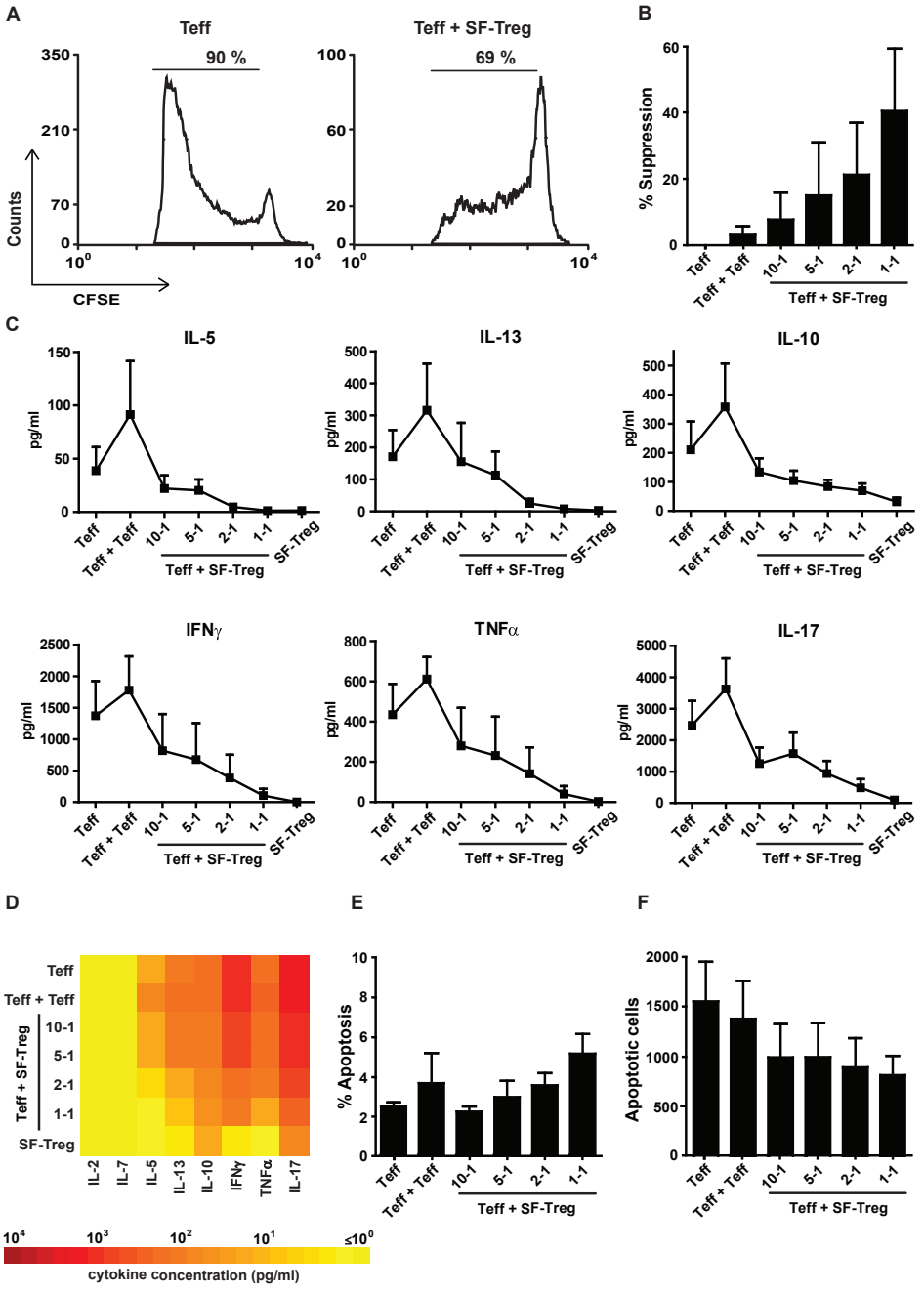


Figure 3 | Synovial fluid-derived Treg suppress Teff proliferation and cytokine production, but do not induce apoptosis. Cells were cultured in 75 µl medium for 5 days. **A**, Proliferation of CFSE^{+} Teff measured by flow cytometry cultured alone (*left panel*), or in co-culture with SF-Treg (1-1) (*right panel*). 1 representative example is shown. **B**, Level of suppression of CFSE^{+} Teff proliferation calculated for several ratios of Teff + SF-Treg and Teff + Teff (ratio 1-1), compared to culture of Teff alone (suppression = 0%). **C**, Levels of ►

several differences between mouse and human Treg phenotype, function and mechanisms of suppression have been identified in the past few years. For instance, the expression of FOXP3 seems to be a more consistent marker for functional Treg in mice, than it is in humans[26–28]. As for mechanisms of suppression, IL-35 production by Treg is important for suppression in mice[29], while IL-35 is not even expressed by human Treg[30]. Since Treg are currently tested for therapeutic applications in humans, it is especially important to determine to what extend results obtained in mice can be translated to human Treg.

Recently, Pandiyan et al.[18] exemplified a new mechanism of action of Treg in mice, namely their capacity to induce apoptosis in Teff, based on specific cytokine consumption as Treg can consume IL-2 produced by Teff. Also, addition of IL-2 to co-cultures of Teff and Treg prevented apoptosis of Teff. Though they did not directly show that addition of IL-2Rγ-chain binding cytokines, which diminished apoptosis, also prevented suppression *in vitro*, *in vivo* they did find that induction of Teff apoptosis is indeed important for Treg function. Furthermore, previous reports show that suppression *in vitro* by murine Treg is prevented by addition of IL-2Rγ-chain binding cytokines[17]. Our current data show some similarities between the mouse and human system, but also reveal an essential difference between mouse and human Treg; human Treg function is not mediated by apoptosis of Teff. Obviously, human experiments such as these are restricted to *in vitro* assays, and only limited numbers of cells are available. However, *in vitro* Treg assays, similar to those used for mice, can be performed with human cells and compared to data obtained in experimental models.

Similar to mice, we show that naturally occurring human Treg very efficiently suppress both proliferation and cytokine production by Teff, which can be reversed by addition of IL-2Rγ-chain binding cytokines. These results are consistent with earlier reports on human and murine Treg which show both inhibition of Teff IL-2 mRNA production, as well as Teff proliferation by Treg, and a decrease of suppression of Teff proliferation by addition of high levels of exogenous IL-2[17;31;32]. Also, Treg derived from a highly inflammatory environment, from inflamed joints of JIA patients, suppress Teff proliferation and cytokine production. Obviously, mouse splenocytes differ in many aspects from human PBMC[33]. Here we show that human Teff seem to be less prone to apoptosis than mouse Teff. When comparing cell death in cultures with only Teff, human Teff show hardly any apoptosis (2%), whereas mouse Teff show a higher level of apoptotic cells (20%)[18]. And, importantly, we show that suppression by human Treg does not involve induction of apoptosis in Teff; the absolute numbers of apoptotic cells decrease in the presence of Treg.

IL-2 is an important cytokine for Treg function, both in mice and humans. However, we do not find a decrease of apoptosis in Teff upon addition of IL-2. This may again be due to the low level of apoptosis in Teff in general. However, it could also be explained by the fact that Teff do not

► cytokines present in culture medium on day 5 of culture in several co-culture ratios of Teff + SF-Treg, Teff + Teff (ratio 1-1) and Teff alone. **D**, Mean levels of cytokines present in culture medium on day 5 of culture. A color profile of the means was made to show the differences between culture conditions (see also Supplementary Table 2). **E**, Average percentage and, **F**, absolute number, corrected for cell input, of apoptotic $CFSE^+$ Teff for several co-culture ratios of Teff + SF-Treg, Teff + Teff (ratio 1-1) and Teff alone (**B-E**, n = 3). Error bars represent means ± SEM.

necessarily require IL-2 to survive or become activated. This is confirmed by recent data obtained by *in vitro* tests on peripheral blood cells from a specific group of IPEX patients. In these patients Teff produce only low levels of IL-2 and, remarkably, the deficit in Treg function can be overcome by addition of IL-2 to cell cultures. Thus, the *in vivo* lack of Treg function could be explained by the decreased production of IL-2 by Teff in these IPEX patients[3;34]. Altogether, this suggests that in humans IL-2 is very important for Treg function, but is not required for Teff survival and function, as these Teff, despite low IL-2 production, are still highly activated and causing disease.

We show here, in line with earlier publications, that addition of IL-2 and IL-7 abrogates suppression of both Teff proliferation and cytokine production. This could be due to higher activation of Teff, as Teff cultured alone proliferate more and produce more cytokines in the presence of IL-2 and IL-7, or, in case of IL-2, to abrogation of Treg anergy. In addition, we do not find a decrease of the added IL-2 in these cultures with Treg present. This suggests that IL-2 is not consumed by the Treg, although we cannot exclude that the level of exogenous IL-2 is simply too high to detect consumption by Treg.

In conclusion, we here point out an important difference between human and murine Treg function: human Treg do not induce apoptosis in Teff to achieve suppression. With these data we emphasize that experimental data from mouse models should be carefully validated in human cells to identify discrepancies, and to ensure that further therapeutic applications are efficient and safe. This does not mean that Treg are less valuable targets for intervention. It could even be argued that if human Treg, instead of eliminating Teff by inducing apoptosis, render Teff either anergic, or even turn them into suppressor cells themselves[35;36], they may be able to exert stronger bystander suppression in an ongoing inflammatory response.

Our functional data here support that Treg are excellent clinical targets to counteract autoimmune diseases. For optimal functional outcome in human clinical trials, future work should focus on the ability of Treg to suppress proliferation and cytokine production of Teff, rather than induction of Teff apoptosis.

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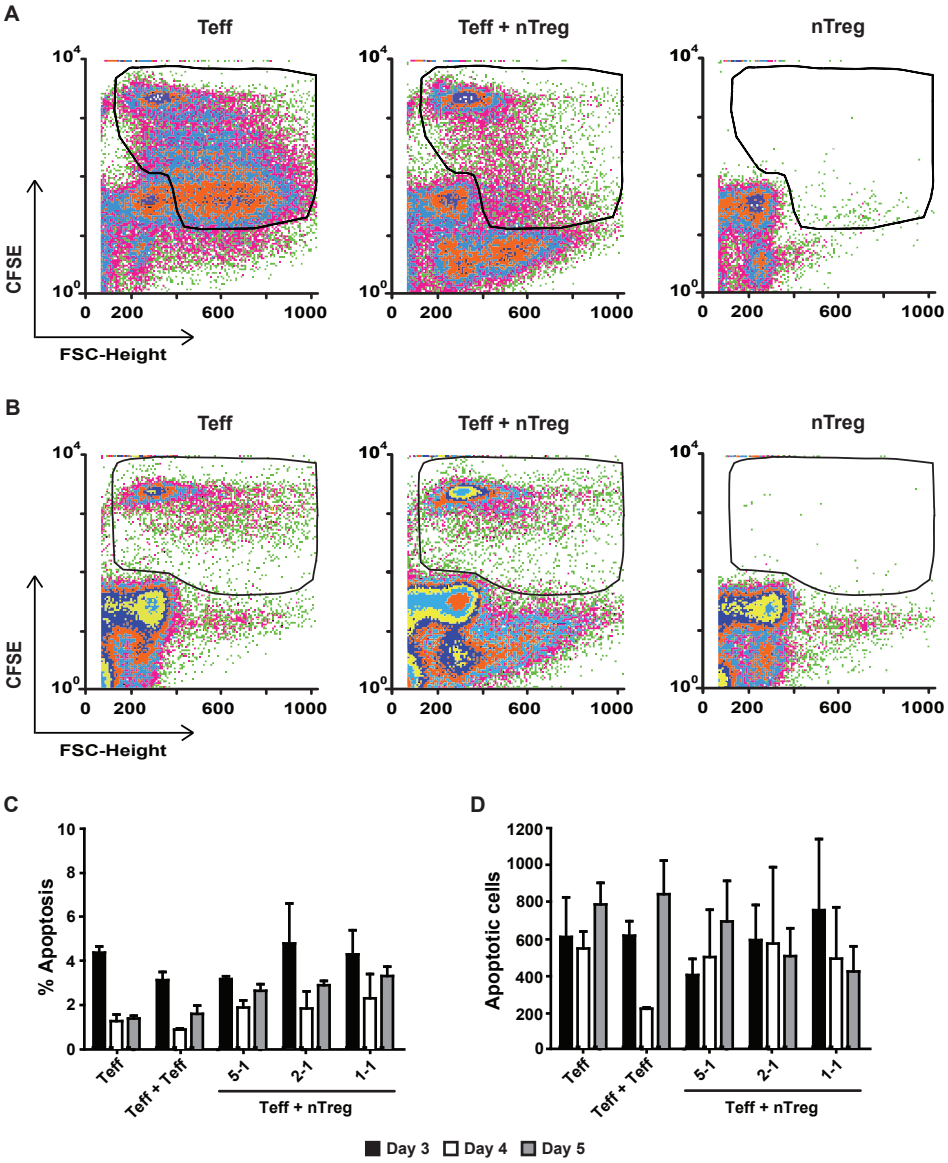
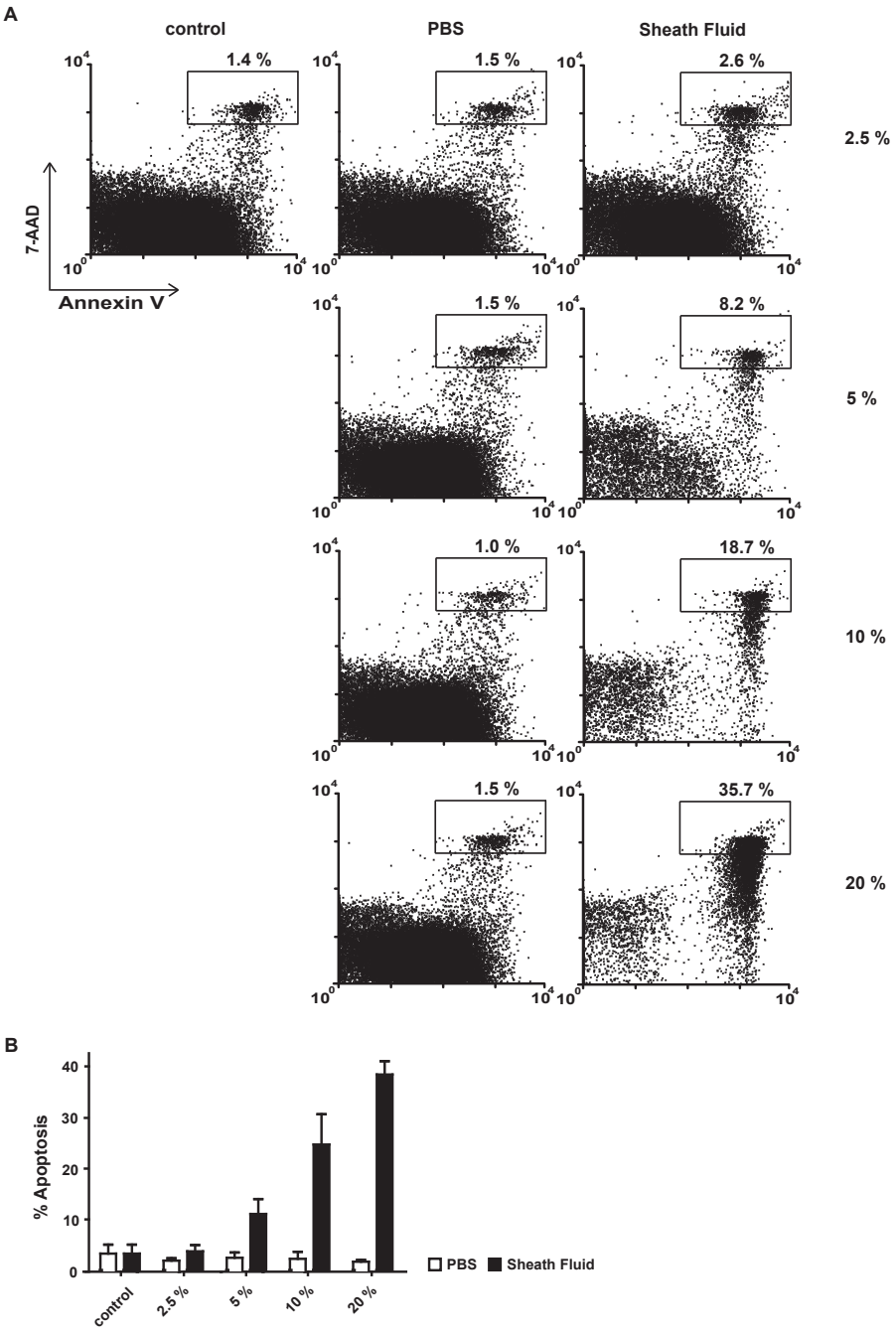
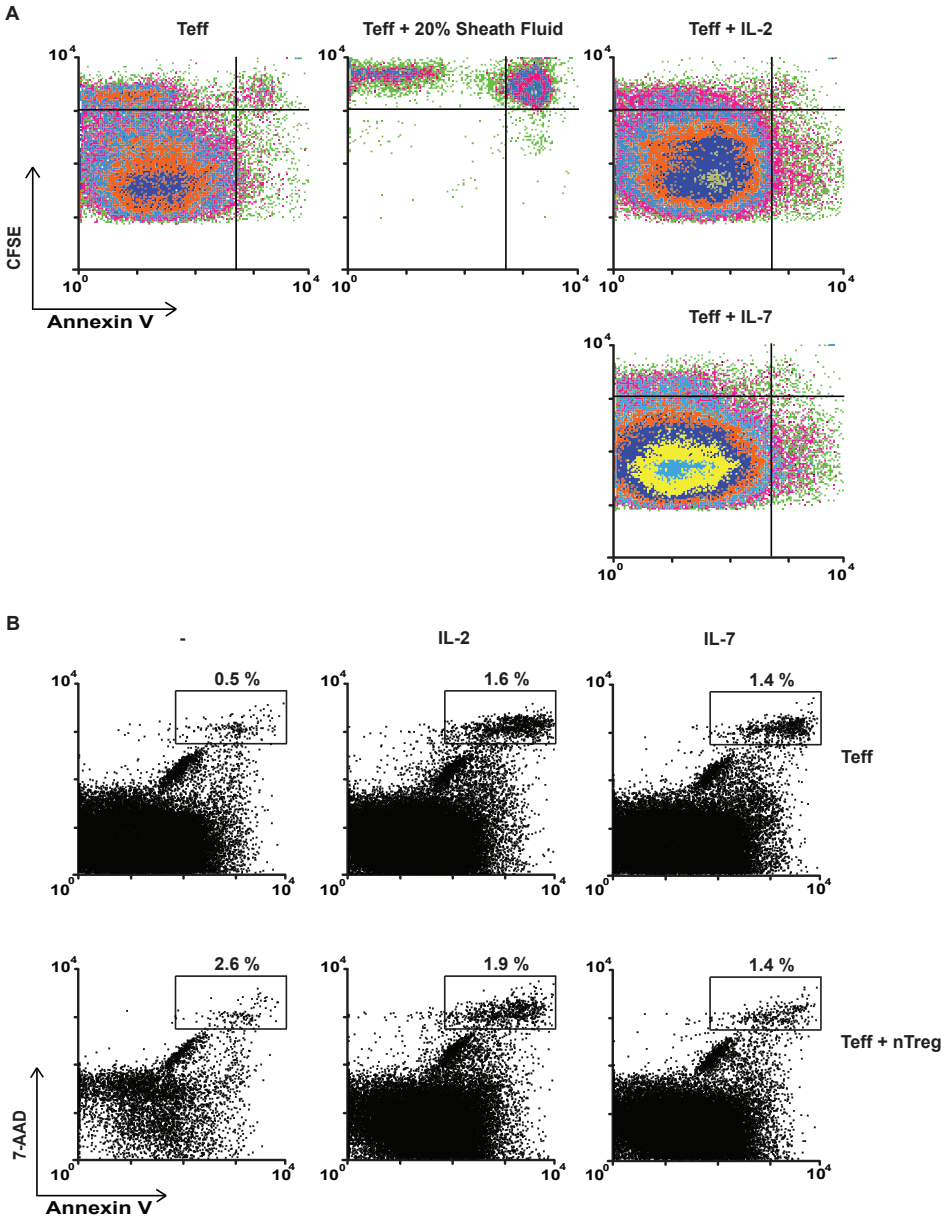
SUPPLEMENTARY INFORMATION



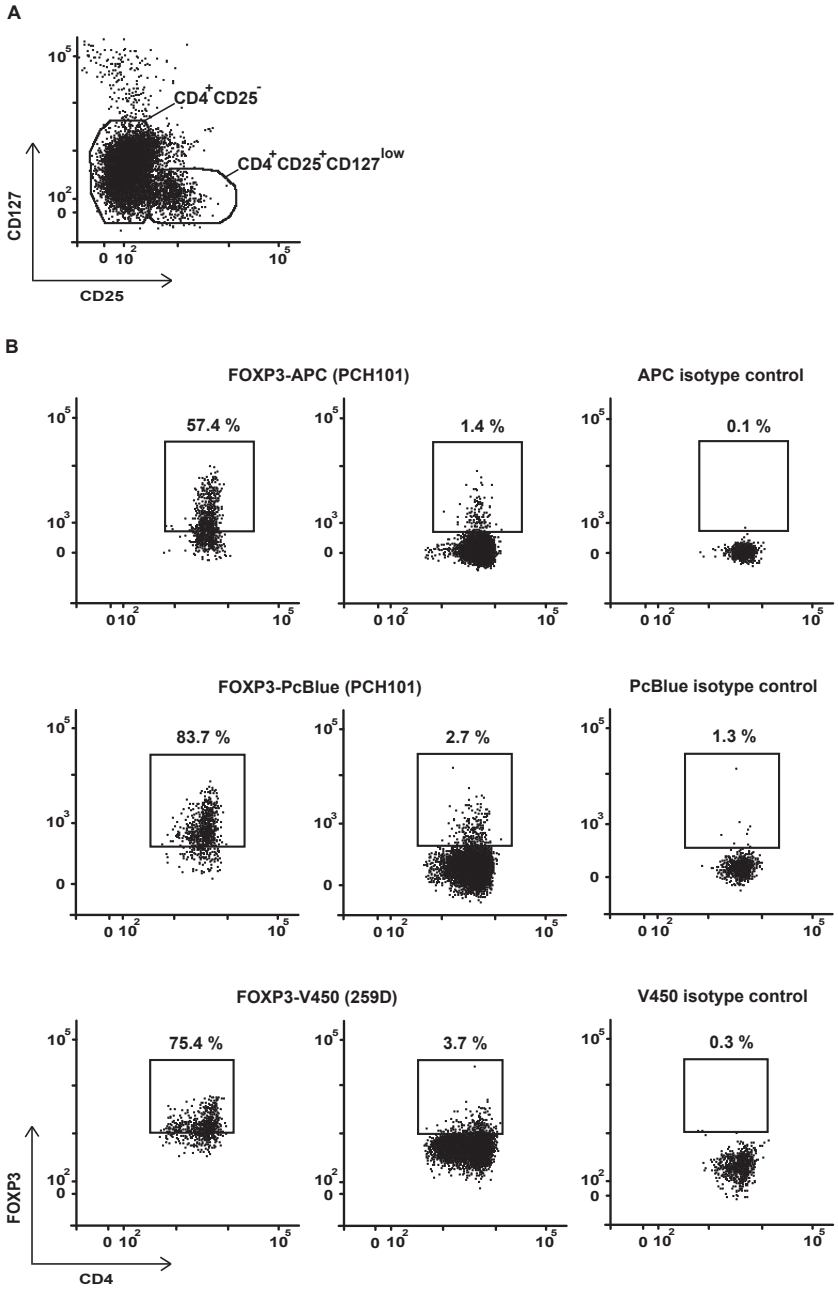
Supplementary Figure 1 | Proliferation and apoptosis of Teff after 3, 4, and 5 days of culture in the presence and absence of Treg. **A**, Gated CFSE^{+} Teff in the presence of APC (*left panel*) or APC + Treg (*middle panel*) after 5 days of culture. For comparison, Treg only + APC are shown as well (*right panel*). 1 representative example of n =9. **B**, Gated CFSE^{+} Teff in the presence of APC (*left panel*) or APC + Treg (*right panel*) after 3 days of culture. For comparison, Treg only + APC are shown as well (*right panel*). 1 representative example of n= 3. **C**, Average percentage and **D**, absolute number, corrected for cell input, of apoptotic Teff expressing 7-AAD and Annexin V for several co-culture ratios of Teff + Treg, Teff + Teff (ratio 1-1) and Teff alone after 3 (*black bars*), 4 (*white bars*) or 5 days (*grey bars*) of culture (n =3). Error bars represent means ± SEM.



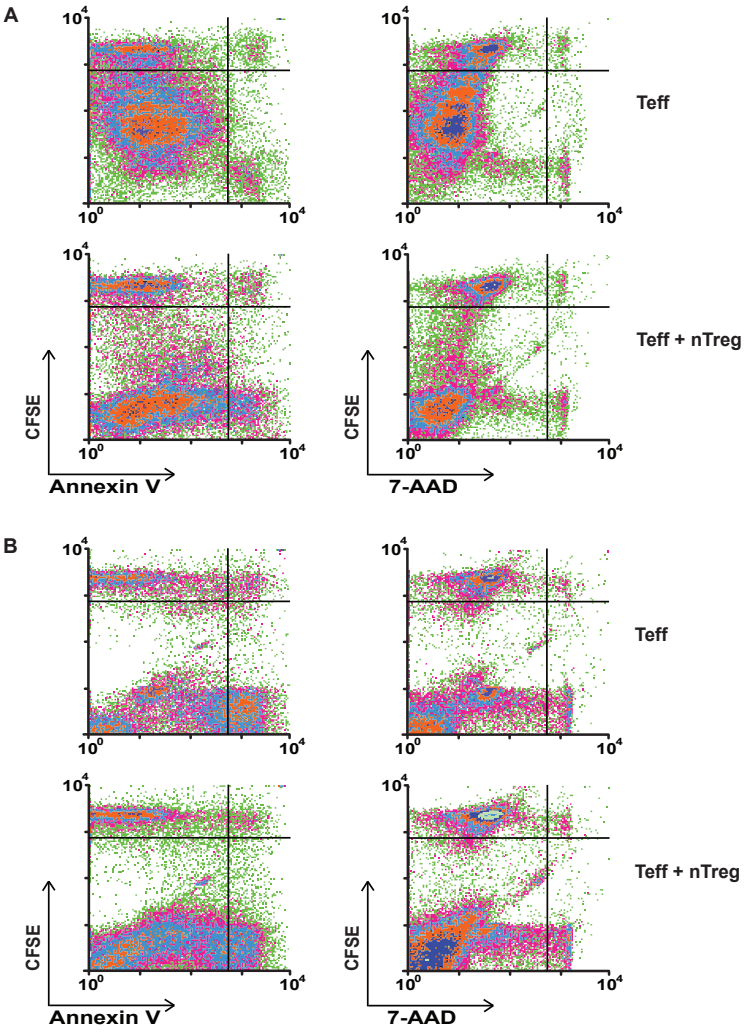
Supplementary Figure 2 | Sheath Fluid, containing ethanol, dose dependently induces apoptosis in Teff. **A**, Percentage of apoptotic Teff expressing 7-AAD and Annexin V after culture for 5 days without (*left panel*), or with increasing amounts of PBS as a control (*middle panel*) or Sheath Fluid to induce apoptotic cells (*right panel*). 1 representative of n= 2. **B**, Average percentage of apoptotic Teff expressing 7-AAD and Annexin V for increasing concentrations of PBS (*white bars*) and Sheath Fluid (*black bars*) (n = 2). Error bars represent means ± SEM.



Supplementary Figure 3 | Annexin V expressing Teff and apoptotic Teff, expressing both Annexin V and 7-AAD, in the presence and absence of IL-2 and IL-7. **A**, Annexin V expression of gated $CFSE^{+}$ Teff cultured for 5 days without additional stimuli (*left panel*), in the presence of 20% Sheath Fluid to induce apoptotic cells (*middle panel*) or in the presence of IL-2 or IL-7 (*right panel*). 1 representative example for each condition is shown. **B**, Percentage of apoptotic Teff expressing 7-AAD and Annexin V alone or in the presence of Treg (1-1), in the absence (*left panel*) or presence of IL-2 (*middle panel*) or IL-7 (*right panel*). 1 representative example of n= 5.



Supplementary Figure 4 | Percentage of FOXP3 expressing cells within the $CD4^+CD25^+CD127^{low}$ Treg population. **A**, CD25 and CD127 expression of gated $CD4^+$ T cells. The gate used for sorting the $CD4^+CD25^+CD127^{low}$ Treg population is indicated. For comparison of FOXP3 expression $CD4^+CD25^-$ cells were gated. 1 representative example is shown. **B**, FOXP3 expression measured by different FOXP3 antibodies within the $CD4^+CD25^+CD127^{low}$ Treg population (*left panel*), within the $CD4^+CD25^-$ cells (*middle panel*) and corresponding isotype controls gated on $CD4^+CD25^+CD127^{low}$ Treg (*right panel*). 1 representative example of n= 4.



Supplementary Figure 5 | Annexin V and 7-AAD expressing cells and apoptotic Teff, expressing both Annexin V and 7-AAD, in the presence and absence of Treg. **A**, Total cells expressing Annexin V (*left panel*) and 7-AAD (*right panel*) in the presence and absence of Treg at day 5 of culture. 1 representative example of n= 9. **B**, Total cells expressing Annexin V (*left panel*) and 7-AAD (*right panel*) in the presence and absence of Treg at day 3 of culture. 1 representative example of n= 3. **C**, Average absolute number of apoptotic Teff expressing 7-AAD and Annexin per cell division (0= undivided cells), in the absence (*black bars*) and presence (*white bars*) of Treg at day 5 of culture. (n= 4) Error bars represent means ± SEM.

Supplementary Table 1 | IL-2 and IL-7 inhibit nTreg mediated suppression of Teff cytokine production, but nTreg do not consume IL-2 or IL-7

	IL-2	IL-	IL-5	IL-13	IL-10	IFNγ	TNFα	IL-17	
Teff	30.7 (45.1)	0.0 (0.0)	14.6 (14.3)	112.3 (99.6)	141.4 (192.2)	367.8 (340.1)	461.2 (403.6)	773.3 (856.9)	
Teff + Teff	78.3 (203.1)	0.0 (0.0)	26.7 (26.8)	142.8 (133.4)	286.4 (363.8)	828.3 (745.2)	486.8 (548.0)	2273.6 (2859.2)	
10-1	7.4 (5.3)	0.0 (0.0)	14.9 (12.3)	40.2 (35.8)	46.7 (44.8)	253.6 (324.4)	232.8 (319.6)	698.6 (847.7)	
5-1	2.9 (2.5)	0.0 (0.0)	14.4 (8.8)	27.2 (25.7)	59.3 (52.1)	131.2 (118.3)	202.6 (290.1)	721.4 (721.7)	medium
2-1	2.1# (1.7)	0.0 (0.0)	9.1 (8.8)	13.3* (13.2)	59.8 (36.2)	17.9 (20.7)	110.5 (198.7)	393.5 (314.9)	
1-1	3.5# (6.7)	0.0 (0.0)	5.6 (4.0)	10.1* (7.7)	61.3 (40.5)	10.9* (13.7)	32.1 (51.3)	844.2 (973.0)	
nTreg	0.6 (1.0)	0.0 (0.0)	2.2 (0.3)	0.0 (0.0)	12.3 (5.3)	0.0* (0.0)	2.7 (3.4)	44.4 (28.4)	
	IL-2#	**IL-7**	**IL-5$**	**IL-13$**	**IL-10***	**IFNγ**	**TNFα#**	**IL-17#**	
Teff	12878.2 (2170.6)	0.0 (0.0)	360.3 (177.5)	4034.3 (1244.5)	2356.2 (2832.7)	1036.7 (513.5)	1270.8 (560.8)	8328.4 (5514.3)	
Teff + Teff	8756.7 (2776.0)	0.0 (0.0)	267.6 (168.8)	2811.4 (1054.6)	1853.1 (594.7)	1478.4 (806.8)	1365.7 (985.3)	12036.4 (4362.6)	
10-1	13720.8 (4420.5)	0.0 (0.0)	298.7 (150.3)	4459.9 (262.8)	860.6 (822.5)	791.3 (122.1)	1022.6 (1262.0)	9417.6 (4929.7)	
5-1	11399.4 (3157.0)	0.0 (0.0)	455.1 (170.7)	3511.6 (1497.2)	885.9 (318.3)	893.5 (353.7)	697.4 (881.1)	6805.1 (5100.3)	IL-2
2-1	11642.7 (6578.4)	0.0 (0.0)	625.5 (364.7)	3082.3 (612.3)	1241.2 (973.0)	663.5 (223.4)	745.1 (419.3)	10086.6 (4898.7)	
1-1	6494.2 (7442.3)	0.0 (0.0)	394.5 (412.2)	1927.7 (1478.0)	2005.5 (1467.8)	513.6 (426.8)	509.0 (442.6)	9473.2 (6655.3)	
nTreg	13213.0 (2457.5)	0.0 (0.0)	225.0 (184.6)	329.7# (159.2)	2214.1 (683.9)	26.0 (4.3)	49.6 (6.4)	5922.7 (1980.1)	
	IL-2	**IL-7$**	**IL-5**	**IL-13**	**IL-10#**	**IFNγ**	**TNFα**	**IL-17**	
Teff	52.0 (94.1)	2245.8 (417.7)	32.6 (11.0)	373.5 (103.5)	2114.2 (1872.5)	1126.4 (1042.2)	778.4 (293.3)	4869.1 (3014.3)	
Teff + Teff	2.5 (0.8)	983.3# (127.1)	43.2 (14.4)	334.7 (176.4)	1854.1 (1233.8)	1486.3 (887.6)	906.4 (345.3)	5021.8 (2421.1)	
10-1	2.2 (0.7)	2048.7 (557.2)	62.9 (36.9)	403.1 (100.4)	1708.6 (1795.6)	1131.3 (827.4)	662.3 (391.0)	4210.7 (2207.8)	
5-1	3.0 (0.2)	2103.1 (257.6)	45.5 (34.0)	331.2 (56.9)	2049.0 (1996.1)	1205.4 (1213.2)	794.1 (286.4)	5026.6 (1879.6)	IL-7
2-1	3.9 (5.3)	2479.4 (242.1)	62.8 (36.1)	282.7 (81.0)	861.4 (507.6)	570.7 (462.5)	621.3 (227.6)	3822.0 (867.8)	
1-1	5.1 (5.4)	3427.8# (655.3)	86.5 (85.1)	354.1 (128.4)	719.3 (430.3)	342.9 (305.5)	375.0 (117.4)	4396.8 (2152.9)	
nTreg	1.2 (0.8)	4590.8$ (77.1)	13.8 (7.4)	38.4 (17.0)	203.3 (53.4)	2.6 (0.6)	20.5* (2.6)	399.5 (152.2)	

Cells were culture in 75 μl medium for 5 days. Mean levels of cytokines present in culture medium on day 5 of culture in medium (n = 9), with addition of IL-2 (n = 5), or with addition of IL-7 (n = 5). All values (mean (SD)) are expressed in pg/ml. When values were compared to Teff only: *P < .05, #P < .01, $P < .001. When cytokine values in cultures with IL-2 or IL-7 were compared to medium: *P < .05, #P < .01, $P < .001 (see upper row of IL-2 and IL-7 tables; significance is depicted per cytokine).

Supplementary Table 2 | SF-Treg suppress Teff cytokine production

	IL-2	IL-7	IL-5	IL-13	IL-10	IFNγ	TNFα	IL-17
Teff	0.8 (0.9)	0.0 (0.0)	38.9 (38.1)	153.9 (130.3)	210.7 (168.3)	1372.5 (952.8)	380.7 (231.1)	2472.5 (1344.4)
Teff + Teff	0.6 (0.5)	0.0 (0.0)	91.5 (86.8)	284.6 (227.2)	357.9 (258.6)	1777.5 (933.6)	535.3 (166.5)	3626.2 (1693.8)
10-1	0.1 (0.1)	0.0 (0.0)	22.0 (21.4)	140.0 (189.4)	134.1 (81.9)	818.1 (1006.0)	244.5 (287.8)	1257.0 (877.1)
5-1	0.3 (0.6)	0.0 (0.0)	20.3 (17.7)	102.1 (115.5)	104.9 (58.8)	676.4 (1001.0)	202.8 (292.7)	1573.5 (1152.0)
2-1	0.1 (0.1)	0.0 (0.0)	4.8 (5.0)	22.8 (22.2)	83.9 (40.7)	383.7 (638.7)	123.5 (198.7)	940.6 (688.7)
1-1	0.0 (0.1)	0.0 (0.0)	1.3 (2.3)	7.3 (9.5)	70.0 (42.8)	105.8 (183.2)	35.0 (59.6)	487.4 (475.1)
nTreg	0.0 (0.0)	0.0 (0.0)	1.5 (2.5)	3.0 (5.1)	32.0 (24.4)	3.4 (5.9)	2.4 (4.2)	96.3 (143.6)

Cells were cultured in 75 µl medium for 5 days (n = 3). Mean levels of cytokines present in culture medium on day 5 of culture. All values (mean (SD)) are expressed in pg/ml.

Supplementary Table 3 | Level of cytokine suppression for nTreg and SF-Treg

	IL-5	IL-13	IL-10	IFNγ	TNFα	IL-17	
Teff	0	0	0	0	0	0	nTreg level of suppression
Teff + Teff	-158,6 (209,8)	-32,3 (62,2)	-5940,3 (16939,9)	-275,1 (416,4)	-132065,5 (396125,5)	-74842,6 (223559,2)	
10-1	-209,8 (346,7)	57,1 (37,2)	-5260,9 (13951,6)	64,1 (36,1)	-2939,6 (7919,8)	-64657,3* (170978,5)	
5-1	-221,8 (258,1)	73,2 (15,7)	-1958,2 (4821,8)	78,9 (18,1)	57,2 (34,4)	-91260,8 (223069,9)	
2-1	-95,6 (224,5)	78,4 (29,5)	-14056,6 (39414,3)	96,2 (4,8)	-2043,2 (6003,1)	-57914,0 (163598,7)	
1-1	10,2* (94,4)	87,2 (11,5)	-14488,1 (43205,0)	96,3 (6,2)	83,0 (32,7)	-80248,3* (240507,0)	
Teff	0	0	0	0	0	0	SF-Treg level of suppression
Teff + Teff	-156.6 (45,2)	-124,5 (71,5)	-111,2 (98,6)	-44,8 (37,1)	-73,4 (90,5)	-59,0 (61,4)	
10-1	42, 6 (75.8)	43,5 (70,3)	23,2 (26,8)	57,4 (36,8)	52,1 (41,7)	52,2* (29,3)	
5-1	52.2 (54.7)	57,2 (43,2)	33,9 (31,1)	68,3 (39,1)	63,3 (45,5)	43,1 (32,5)	
2-1	85.6 (17,1)	90,1 (8,7)	38,5 (46,3)	83,5 (26,3)	78,4 (31,7)	65,4 (22,7)	
1-1	95.5* (7,7)	97,0 (3,5)	50,2 (37,7)	95,6 (7,7)	94,0 (9,7)	81,0* (19,9)	

In this table we show mean + (SD) level of cytokine suppression. We compare per cytokine, per co-culture condition whether there is a significant difference in cytokine suppression between nTreg and SF-Treg by Mann Whitney U test, *P < .05.

REFERENCES

1. You S, Leforban B, Garcia C, Bach JF, Bluestone JA, et al. (2007) Adaptive TGF-beta-dependent regulatory T cells control autoimmune diabetes and are a privileged target of anti-CD3 antibody treatment. Proc Natl Acad Sci USA 104: 6335.
2. Rodriguez-Palmero M, Franch A, Castell M, Pelegri C, Perez-Cano FJ, et al. (2006) Effective treatment of adjuvant arthritis with a stimulatory CD28-specific monoclonal antibody. J Rheumatol 33: 110.
3. Bacchetta R, Passerini L, Gambineri E, Dai M, Allan SE, et al. (2006) Defective regulatory and effector T cell functions in patients with FOXP3 mutations. J Clin Invest 116: 1713.
4. Le BS, Geha RS (2006) IPEX and the role of Foxp3 in the development and function of human Tregs. J Clin Invest 116: 1473.
5. De Kleer IM, Wedderburn LR, Taams LS, Patel A, Varsani H, et al. (2004) CD4+CD25(bright) regulatory T cells actively regulate inflammation in the joints of patients with the remitting form of juvenile idiopathic arthritis. J Immunol 172: 6435.
6. Keymeulen B, Vandemeulebroucke E, Ziegler AG, Mathieu C, Kaufman L, et al. (2005) Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. N Engl J Med 352: 2598–2608.
7. Herold KC, Hagopian W, Auger JA, Poumian-Ruiz E, Taylor L, et al. (2002) Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus. N Engl J Med 346: 1692–1698.
8. Vignali DA, Collison LW, Workman CJ (2008) How regulatory T cells work. Nat Rev Immunol 8: 523–532.
9. Brandenburg S, Takahashi T, de la Rosa M, Janke M, Karsten G, et al. (2008) IL-2 induces in vivo suppression by CD4(+)CD25(+)Foxp3(+) regulatory T cells. Eur J Immunol 38: 1643–1653.
10. Scheffold A, Huhn J, Hofer T (2005) Regulation of CD4+CD25+ regulatory T cell activity: it takes (IL)two to tango. Eur J Immunol 35: 1336–1341.
11. Setoguchi R, Hori S, Takahashi T, Sakaguchi S (2005) Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. J Exp Med 201: 723–735.
12. D'Cruz LM, Klein L (2005) Development and function of agonist-induced CD25+Foxp3+ regulatory T cells in the absence of interleukin 2 signaling. Nat Immunol 6: 1152–1159.
13. Furtado GC, Curotto de Lafaille MA, Kutchukhidze N, Lafaille JJ (2002) Interleukin 2 signaling is required for CD4(+) regulatory T cell function. J Exp Med 196: 851–857.
14. Zorn E, Nelson EA, Mohseni M, Porcheray F, Kim H, et al. (2006) IL-2 regulates FOXP3 expression in human CD4+CD25+ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells in vivo. Blood 108: 1571.
15. Zheng SG, Wang J, Wang P, Gray JD, Horwitz DA (2007) IL-2 is essential for TGF-beta to convert naive CD4+CD25- cells to CD25+Foxp3+ regulatory T cells and for expansion of these cells. J Immunol 178: 2018–2027.
16. Maloy KJ, Powrie F (2005) Fueling regulation: IL-2 keeps CD4+ Treg cells fit. Nat Immunol 6: 1071–1072.
17. De la Rosa M, Rutz S, Dorninger H, Scheffold A (2004) Interleukin-2 is essential for CD4+CD25+ regulatory T cell function. Eur J Immunol 34: 2480–2488.
18. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ (2007) CD4(+)CD25(+)Foxp3(+) regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4(+) T cells. Nat Immunol 8: 1353.
19. Scheffold A, Murphy KM, Hofer T (2007) Competition for cytokines: T(reg) cells take all. Nat Immunol 8: 1285–1287.
20. Van Amelsfort JM, Van Roon JA, Noordegraaf M, Jacobs KM, Bijlsma JW, et al. (2007) Proinflammatory mediator-induced reversal of CD4+,CD25+ regulatory T cell-mediated suppression in rheumatoid arthritis. Arthritis Rheum 56: 732–742.
21. Stummvoll GH, DiPaolo RJ, Huter EN, Davidson TS, Glass D, et al. (2008) Th1, Th2, and Th17 effector T cell-induced autoimmune gastritis differs in pathological pattern and in susceptibility to suppression by regulatory T cells. J Immunol 181: 1908–1916.
22. Chauhan SK, El Annan J, Ecoiffier T, Goyal S, Zhang Q, et al. (2009) Autoimmunity in dry eye is due to resistance of Th17 to Treg suppression. J Immunol 182: 1247–1252.
23. Koenen HJ, Fasse E, Joosten I (2003) IL-15 and cognate antigen successfully expand de novo-induced human antigen-specific regulatory

CD4+ T cells that require antigen-specific activation for suppression. J Immunol 171: 6431–6441.

24. Earle KE, Tang Q, Zhou X, Liu W, Zhu S, et al. (2005) In vitro expanded human CD4+CD25+ regulatory T cells suppress effector T cell proliferation. Clin Immunol 115: 3–9.
25. Hoffmann P, Eder R, Kunz-Schughart LA, Andreesen R, Edinger M (2004) Large-scale in vitro expansion of polyclonal human CD4(+) CD25high regulatory T cells. Blood 104: 895–903.
26. Wang J, Ioan-Facsinay A, van dV, Huizinga TW, Toes RE (2007) Transient expression of FOXP3 in human activated nonregulatory CD4(+) T cells. Eur J Immunol 37: 129.
27. Ziegler SF (2006) FOXP3: of mice and men. Annu Rev Immunol 24: 209–226.
28. Tran DQ, Ramsey H, Shevach EM (2007) Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype. Blood 110: 2983.
29. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, et al. (2007) The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature 450: 566–569.
30. Bardel E, Larousserie F, Charlot-Rabiega P, Coulomb-L'Hermine A, Devergne O (2008) Human CD4+ CD25+ Foxp3+ regulatory T cells do not constitutively express IL-35. J Immunol 181: 6898–6905.
31. Thornton AM, Donovan EE, Piccirillo CA, Shevach EM (2004) Cutting edge: IL-2 is critically required for the in vitro activation of CD4+CD25+ T cell suppressor function. J Immunol 172: 6519–6523.
32. Oberle N, Eberhardt N, Falk CS, Krammer PH, Suri-Payer E (2007) Rapid suppression of cytokine transcription in human CD4+CD25 T cells by CD4+Foxp3+ regulatory T cells: independence of IL-2 consumption, TGFbeta, and various inhibitors of TCR signaling. J Immunol 179: 3578–3587.
33. Mestas J, Hughes CC (2004) Of mice and not men: differences between mouse and human immunology. J Immunol 172: 2731–2738.
34. Roncarolo MG, Gregori S (2008) Is FOXP3 a bona fide marker for human regulatory T cells? Eur J Immunol 38: 925–927.
35. Qiao M, Thornton AM, Shevach EM (2007) CD4+ CD25+ [corrected] regulatory T cells render naive CD4+ CD25- T cells anergic and suppressive. Immunology 120: 447–455.
36. Zanin-Zhorov A, Cahalon L, Tal G, Margalit R, Lider O, et al. (2006) Heat shock protein 60 enhances CD4+ CD25+ regulatory T cell function via innate TLR2 signaling. J Clin Invest 116: 2022.
37. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, et al. (2006) CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med 203: 1701–1711.
38. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, et al. (2006) Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J Exp Med 203: 1693.
39. Raghavan S, Cao D, Widhe M, Roth K, Herrath J, et al. (2008) FOXP3 expression in blood, synovial fluid and synovial tissue during inflammatory arthritis and intra-articular corticosteroid treatment. Ann Rheum Dis 68: 1908–1915.
40. De Jager W, Hoppenreijs EP, Wulffraat NM, Wedderburn LR, Kuis W, et al. (2007) Blood and synovial fluid cytokine signatures in patients with juvenile idiopathic arthritis: a cross-sectional study. Ann Rheum Dis 66: 589–598.

4

FUNCTIONAL HUMAN REGULATORY T CELLS FAIL TO CONTROL AUTOIMMUNE INFLAMMATION DUE TO PKB/C-AKT HYPERACTIVATION IN EFFECTOR CELLS

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ABSTRACT

During the last decade research has focused on the application of FOXP3$^+$ regulatory T cells (Treg) in the treatment of autoimmune disease. However, thorough functional characterization of these cells in patients with chronic autoimmune disease, especially at the site of inflammation, is still missing. Here we studied Treg function in patients with juvenile idiopathic arthritis (JIA) and observed that Treg from the peripheral blood as well as the inflamed joints are fully functional. Nevertheless, Treg-mediated suppression of cell proliferation and cytokine production by effector cells from the site of inflammation was severely impaired, because of resistance to suppression. This resistance to suppression was not caused by a memory phenotype of effector T cells (Teff) or activation status of antigen presenting cells (APCs). Instead, activation of protein kinase B (PKB)/c-akt was enhanced in inflammatory effector cells, at least partially in response to TNFα and IL-6, and inhibition of this kinase restored responsiveness to suppression. We are the first to show that PKB/c-akt hyperactivation causes resistance of effector cells to suppression in human autoimmune disease. Furthermore, these findings suggest that for a Treg enhancing strategy to be successful in the treatment of autoimmune inflammation, resistance because of PKB/c-akt hyperactivation should be targeted as well.

INTRODUCTION

Since their discovery 15 years ago[1], it is now well established that $CD25^+$ regulatory T cells (Treg) are indispensable for immune homeostasis and self-tolerance. Treg suppress the activation, proliferation, and effector functions of a wide range of immune cells via multiple mechanisms[2]. FOXP3 has been identified as a master transcription factor, controlling both Treg development and functionality[3;4]. In addition, human Treg can be identified by high CD25 and low IL-7 receptor (CD127) expression[5;6]. A critical role of Treg in controlling autoimmune responses is demonstrated in various animal models of autoimmune disease[7]. Furthermore, lack of functional Treg leads to severe, systemic autoimmunity in humans[8;9].

Because of their unique function, Treg are considered important for the treatment of autoimmune disease, and several strategies are now being explored to target these cells for therapeutic purposes[10]. However, there is still an ongoing debate whether the numbers and/or function of Treg are changed in patients suffering from chronic autoimmune inflammation[11]. In rheumatoid arthritis (RA) and multiple sclerosis, similar Treg numbers[12;13], or even enhanced numbers in RA[14], were observed in peripheral blood (PB) of patients compared with healthy controls (HC). Thus, it appears that Treg numbers are not reduced in patients suffering from autoimmune inflammation. In addition, it remains unclear whether Treg function is impaired; some studies report reduced functioning of Treg in PB of patients[12;13;15], whereas others have found no difference[14;16].

In addition to these discrepancies concerning Treg numbers and function in the periphery, characterization of Treg functionality at the site of autoimmune inflammation in humans is missing. High levels of Treg have been found at the inflammatory sites in patients with arthritis and inflammatory bowel disease and these cells can suppress $CD4^+CD25^-$ effector cells *in vitro*[17]. Also at the site of inflammation in juvenile idiopathic arthritis (JIA), one of the most common childhood autoimmune diseases, we have previously shown that Treg are present in high numbers and suppress proliferation of $CD4^+CD25^-$ effector cells *in vitro*[18]. However, *in vivo* inflammation persists despite the large numbers of Treg present, suggesting that these cells are defective in their ability to control the ongoing autoimmune response. This may result from the local proinflammatory environment, because *in vitro* experiments have shown that proinflammatory cytokines can affect both Treg function[15;19-21] as well as effector T cell (Teff) responses[22;23]. These data suggest that increasing Treg numbers or enhancing their function for therapeutic purposes might be less effective in a chronic inflammatory environment. However, *ex vivo* data from patients with autoimmune disease are required to clarify the role of Treg at the site of inflammation in humans.

Here, we studied Treg function at the site of inflammation in patients with JIA and compared their inhibitory potential to Treg from PB of both patients and HC. With this approach, we show that Treg from inflamed joints demonstrate efficient suppressive capacity similar to Treg from HC, but control of effector cell proliferation and cytokine production is severely impaired, because of resistance of Teff to suppression. This unresponsiveness to suppression is, at least partially, caused by hyperactivation of protein kinase B (PKB)/c-akt and can be restored by selectively inhibiting PKB/c-akt activation. Taken together, these findings identify resistance of

effector cells to suppression and, more specifically, enhanced PKB/c-akt activation of effector cells as a potential new target in the treatment of autoimmune inflammation.

METHODS

PATIENTS AND HEALTHY CONTROLS

34 patients with oligoarticular and 3 with extended oligoarticular JIA, according to the revised criteria for JIA[24], were included in this study. All patients had active disease and underwent therapeutic joint aspiration at the time of sampling. Patients were between 5 and 18 years of age and were either untreated or treated with nonsteroidal anti-inflammatory drugs (NSAIDs), methotrexate (MTX), or both at the time of inclusion. Informed consent was received from parents/guardians or from participants directly when they were over 12 years of age. 27 volunteers from the laboratory with no history of autoimmune disease were included as HC. The study procedures were approved by the Institutional Review Board of the University Medical Center Utrecht (UMCU) and performed according to the principles expressed in the Helsinki Declaration.

CELL ISOLATION

From JIA patients, synovial fluid (SF) was collected during therapeutic joint aspiration, and, at the same time, blood was drawn via veni puncture or intravenous drip. Blood was collected from HC via veni puncture. SF mononuclear cells (SFMC) and peripheral blood mononuclear cells (PBMC) were isolated using Ficoll Isopaque density gradient centrifugation (GE Healthcare Bio-Sciences AB) and were used either directly, or frozen in FCS (Invitrogen) containing 10% DMSO (Sigma-Aldrich) until further experimentation.

CELL CULTURE CONDITIONS

Cells were cultured in RPMI 1640 supplemented with 2mM L-glutamine, 100 U/ml penicillin-streptomycin, and 10% human AB serum or 10% FCS (all obtained from Invitrogen) in round-bottom 96-well plates (Nunc). Cells were stimulated with 1.5 μg/ml plate-bound anti-CD3 (clone OKT3; eBioscience) and cultured at 37°C and 5% CO2.

SUPPRESSION ASSAYS

Total SFMC or PBMC were used as effector cells and cultured at 200.000 or 100.000 cells per well in 200 or 100 μl culture volume. $CD4^+$ cells were isolated by magnetic cell sorting, using a CD4 T Lymphocyte Enrichment Set (BD Biosciences), according to the manufacturer's instructions. Subsequently, $CD4^+CD25^+CD127^{low}$ Treg were sorted by flow cytometry on FACS Aria (BD Biosciences; Supplementary Figure 1 A). Treg were co-cultured with effector cells at a 1:8 and 1:4 ratio. To control for cell density, effector cells instead of Treg were added at a 1:4 ratio. In some experiments, PKB/c-akt inhibitor VIII (0.1 μM; Calbiochem) was added from the start of culture. At day 4, proliferation of effector cells was analyzed or supernatant was collected to measure cytokine production.

ANALYSIS OF CELL PROLIFERATION

To measure proliferation, effector cells were labeled with 2 μM CFSE (Invitrogen) for 10 minutes at 37°C and extensively washed before use in suppression assays. At day 4, proliferation of effector cells was analyzed by flow cytometry by gating on $CFSE^+$ cells. Proliferation of $CD4^+$ and $CD8^+$ T cells was measured by subsequently gating on $CD3^+$ cells, followed by gating on $CD4^+$ and $CD8^+$ cells, respectively.

DETECTION OF CYTOKINES IN CULTURE SUPERNATANT, PB, AND SF

Supernatant was collected from suppression assays, stored at -80°C and processed within 1 month. Plasma was obtained by centrifugation of PB at 150g and SF at 980g for 10 minutes and stored at -80°C. Cytokine concentrations were measured with the Bio-Plex system in combination with the Bio-Plex Manager Version 4.0 software (Bio-Rad Laboratories), which employs the Luminex technology as previously described[25].

SUPPRESSION ASSAY WITH SORTED MEMORY AND NAIVE TEFF

To study suppression of naive and memory Teff, $CD4^+$ T cells were isolated by magnetic cell sorting, using a CD4 T Lymphocyte Enrichment Set (BD Biosciences, according to the manufacturer's instructions), and subsequently $CD4^+CD25^+CD127^{low}$ Treg, $CD4^+CD25^-$ total Teff, $CD4^+CD25^-CD45RA^+CD45RO^-$ naive Teff, and $CD4^+CD25^+CD45RA^-CD45RO^+$ memory Teff were sorted by flow cytometry on FACS Aria (BD Biosciences). Teff (25.000 cells) were cultured in 75 μl culture volume. Irradiated (3500 rad), autologous PBMC (30.000 cells per well) depleted of $CD3^+$ cells by magnetic cell sorting with anti-human CD3 particles (BD Biosciences, according to the manufacturer's instructions) were used as APCs. Treg were added at a 1:2 ratio to Teff and at day 5 proliferation of Teff was analyzed and supernatant collected to measure cytokine production.

SUPPRESSION ASSAY WITH SORTED APCs AND T CELLS

To study suppression of synovial T cells in the presence of PB- or SF-derived APCs, $CD4^+CD25^+CD127^{low}$ Treg, $CD3^+$ T cells, and $CD3^-$ APCs were sorted by flow cytometry on FACS Aria (BD Biosciences). CFSE labeled $CD3^+$ T cells (100.000 cells per well) were cultured together with 100.000 unlabeled $CD3^-$ APCs in the presence of Treg at a 1:4 and 1:8 ratio. At day 4, proliferation of T cells was analyzed.

TGFβ SUPPRESSION ASSAY

To investigate suppression of SFMC and PBMC by TGFβ, CFSE-labeled SFMC and PBMC were cultured in the presence or absence of 40 ng/ml recombinant human TGFβ1 (Koma Biotech). In some experiments, PKB/c-akt inhibitor VIII (0, 0.01, 0.1, and 1μM; Calbiochem) was added from the start of culture. Cells were cultured for 4 or 5 days and proliferation of $CD4^+$ T cells was analyzed by gating on $CD3^+$ cells and subsequently on $CD4^+$ cells. To study the effect of TNFα and IL-6 on TGFβ-mediated suppression, cells were untreated or pre-treated overnight with TNFα (50 ng/ml), IL-6 (100 ng/ml) or both, CFSE labeled and cultured in the presence or absence TNFα and IL-6 with or without TGFβ.

METHYLATION OF FOXP3 TREG-SPECIFIC DEMETHYLATED REGION (TSDR)

To determine methylation of FOXP3 TSDR, male HC and JIA patients were included. DNA was isolated from sorted $CD4^+CD25^+CD127^{low}$ Treg, using QiaAmp DNA Mini Kit (QIAGEN). Demethylation of the FOXP3 TSDR was determined as previously described[26].

FLOW CYTOMETRY

To detect intracellular cytokine production, cells were stimulated for 4.5 hours with PMA (20 ng/ml; MP Biomedicals) and ionomycin (1 μg/ml; Calbiochem), with Golgistop (1/1500; BD Biosciences) added for the last 4 hours of culture. Before staining, cells were washed twice in FACS buffer (PBS containing 2% FCS (Invitrogen) and 0.1% sodium azide (Sigma-Aldrich)) and subsequently incubated with surface antibodies. After surface staining, cells were washed twice in FACS buffer and acquired directly, or fixed, permeabilized, and intracellularly stained using antihuman FOXP3 staining set (eBioscience, according to the manufacturer's instructions). To stain for phosphorylated PKB/c-akt, cells were fixed, permeabilized and stained using BD Phosflow method according to the manufacturer's instructions. Cells were acquired on FACSCalibur or FACSCanto II and analyzed using CellQuest Version 3.3 or FACS Diva Version 6.13 software, respectively (all BD Biosciences). All antibodies used for flow cytometry are described in the Supplementary Information.

STATISTICAL ANALYSIS

For statistical analysis of multiple groups, 1-way ANOVA, or in case of unequal variances, Kruskal-Wallis test was used. Bonferroni or Dunns posthoc test were used to compare between selected groups and Dunnet posthoc test to compare all groups versus a control group. To analyze paired patient samples, paired T test, or in case of unequal variances, Wilcoxon matched pairs test were used. P values below 0.05 were considered significant. Statistical analysis was performed using GraphPad Prism Version 5.03 (Graphpad Software).

RESULTS

INCOMPLETE SUPPRESSION OF T CELL PROLIFERATION AT THE SITE OF AUTOIMMUNE INFLAMMATION

To study Treg function at the site of autoimmune inflammation, mononuclear cells were isolated from the inflamed synovium of patients with oligoarticular JIA. Consistent with previous reports[18;19] Treg numbers were enriched at the site of inflammation in these patients (Figures 1 A and B), whereas Treg levels in PB did not differ between patients and HC (Figure 1 B). To investigate suppressive capacity, $CD4^+CD25^+CD127^{low}$ Treg were sorted by flow cytometry[5;6] (Supplementary Figure 1 A) and functionally analyzed in *in vitro* suppression assays. FOXP3 analysis consistently revealed a high percentage of $FOXP3^+$ cells within the sorted $CD4^+CD25^+CD127^{low}$ population, which did not differ between SFMC (84% ± 9.1%) and PBMC of both patients (81% ± 3.9%) and HC (82% ± 7.6%). However, when synovial fluid (SF) derived Treg were co-cultured with effector cells

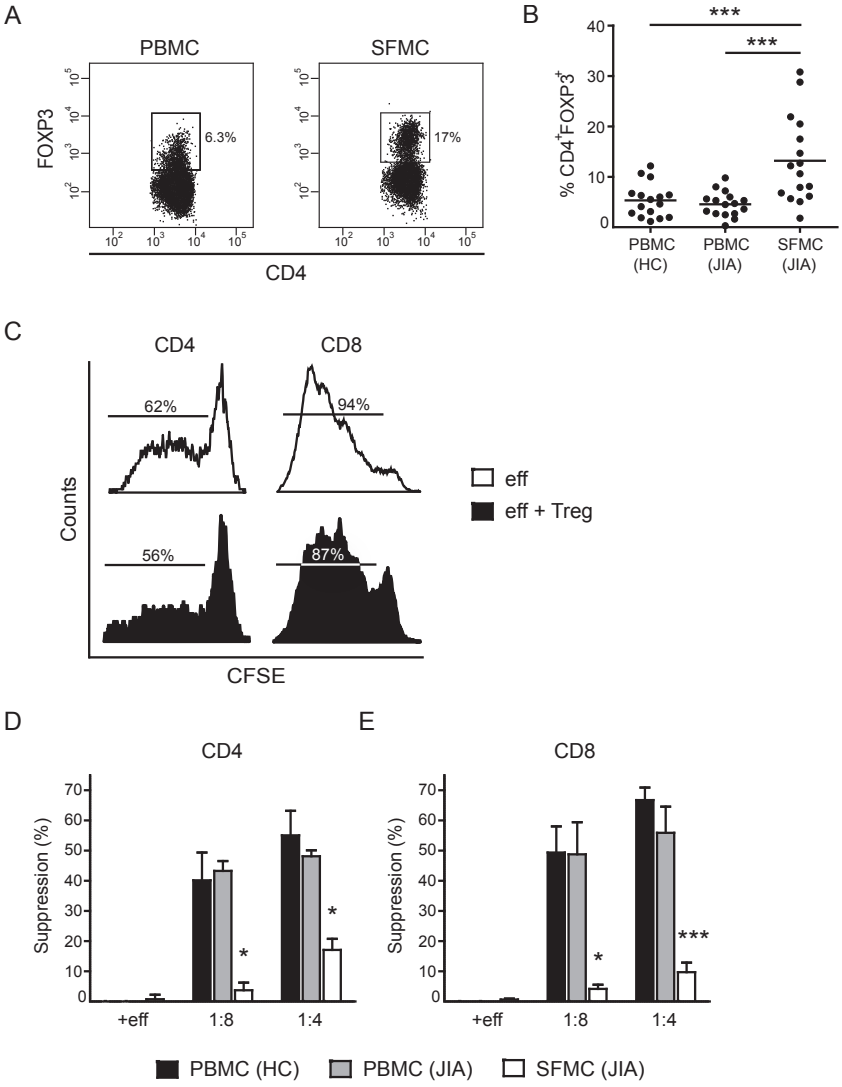


Figure 1 | Treg-mediated suppression of T cell proliferation is impaired at the site of autoimmune inflammation. **A-B**, PBMC and SFMC were stained for CD4 and FOXP3 expression by flow cytometry. **A**, Dotplots showing the percentage of FOXP3$^+$ cells within CD4$^+$ cells in paired PBMC (*left panel*) and SFMC (*right panel*) of JIA patients, 1 representative of n = 16. **B**, Accumulative data of the percentage of CD4$^+$FOXP3$^+$ cells in PBMC of HC and paired PBMC and SFMC from JIA patients (n = 16), ***P < .001. **C-E**, CD4$^+$CD25$^+$CD127^{low} Treg were sorted by flow cytometry and co-cultured with CFSE-labeled effector cells. At day 4, proliferation of CFSE$^+$ effector cells was analyzed. **C**, Proliferation of CD4$^+$ (*left panel*) and CD8$^+$ (*right panel*) SFMC in the absence (*open histograms*) or presence of Treg at 1 to 4 ratio (*filled histograms*). Percentages indicate the percentage of proliferating cells, 1 representative of n = 3. **D-E**, Suppression of CD4$^+$ (***D***) and CD8$^+$ T cell proliferation (***E***) in the presence of Treg at a 1:8 and 1:4 ratio or additional effector cells (+eff) at a 1:4 ratio for PBMC from HC (*black bars*), PBMC from JIA patients (*gray bars*) and SFMC from JIA patients (*white bars*). The results show percentage of suppression in the presence of Treg or additional effector cells relative to effector cells cultured alone. Bars represent mean ± SEM of n = 6 PBMC HC, n = 2 PBMC JIA, and n = 3 SFMC JIA, *P < .05, ***P < .001 compared with PBMC HC.

and proliferation of $CD4^+$ and $CD8^+$ T cells was analyzed, only a minor reduction in the percentage of proliferating cells was observed (Figure 1 C) and this suppression of both $CD4^+$ (Figure 1 D) and $CD8^+$ T cell proliferation (Figure 1 E) was significantly reduced in SFMC (white bars) compared with PBMC of patients (gray bars) and HC (black bars). In contrast, no difference in suppression was observed between PBMC from JIA patients and PBMC from HC. To control for cell density, effector cells instead of Treg were added, which did not result in suppression (Figure 1 D-E; +eff). These data demonstrate that, locally, at the site of autoimmune inflammation, proliferation of both $CD4^+$ and $CD8^+$ T cells is poorly controlled by Treg.

DEFICIENT INHIBITION OF CELL PROLIFERATION IS CAUSED BY RESISTANCE OF EFFECTOR CELLS TO SUPPRESSION

Both reduced functioning of Treg as well as resistance of effector cells to suppression could play a role in the incomplete control of proliferation of effector cells from the site of inflammation[11]. Phenotypical analysis of paired patient samples revealed that FOXP3 content per cell (mean fluorescence intensity (MFI)) was increased in SF derived $CD4^+CD25^+CD127^{low}$ Treg compared with PB Treg (Supplementary Figures 1 B and C). In addition, the percentage of cells with demethylated FOXP3 TSDR was not different for Treg from SF (Supplementary Figure 1 D), suggesting that these cells do not display decreased stability. Other Treg markers, such as, the percentage of CTLA-4 expressing cells (Supplementary Figure 1 E) and both the percentage of GITR expressing cells as well as GITR content per cell (MFI; Supplementary Figure 1 F) were enhanced in SF $CD4^+FOXP3^+$ Treg compared with PB Treg. Thus, SF derived Treg are stable and show enhanced expression of functional and activation markers suggesting that these cells are not deficient in their suppressive capacity. To confirm this, cross-over experiments were performed, in which SF derived Treg (SF-Treg) were co-cultured with PB effector cells, and vice versa. When SF-Treg were co-cultured with PBMC (light gray bars), inhibition of $CD4^+$ (Figure 2 A) and $CD8^+$ T cell proliferation (Figure 2 B) was completely comparable with suppression of PBMC by PB derived Treg (PB-Treg; black bars). Thus, Treg from the site of inflammation show similar suppressive capacity to Treg from PB and, in line with their phenotype, are not impaired in their suppressive function. However, when PB-Treg were co-cultured with SFMC (dark gray bars), the level of suppression of both $CD4^+$ (Figure 2 A) and $CD8^+$ T cell proliferation (Figure 2 B) was markedly reduced compared with PB-Treg cultured with PBMC (black bars). Thus, in the presence of the same PB-Treg, SFMC are less responsive to suppression compared with PBMC. Furthermore, this decrease in suppression was comparable with the decrease in suppression in cultures with both Treg and effector cells from the site of inflammation (SF-Treg + SFMC; white bars). Therefore, the reduced suppression of cell proliferation observed in suppression assays with cells from the site of inflammation is attributable to unresponsiveness of effector cells to suppression.

IMPAIRED CONTROL OF CYTOKINE PRODUCTION BECAUSE OF RESISTANCE OF EFFECTOR CELLS TO SUPPRESSION

Because we observed impaired Treg-mediated suppression of proliferation of Teff from the site of inflammation, we wondered whether suppression of other Teff functions, such

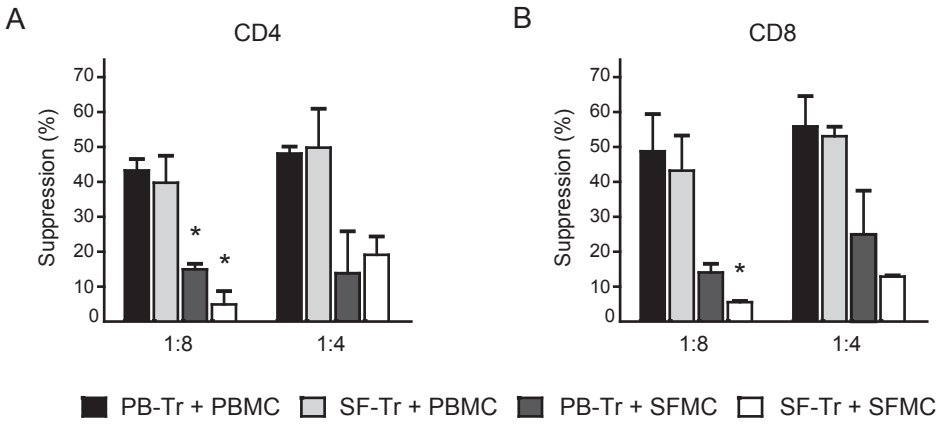
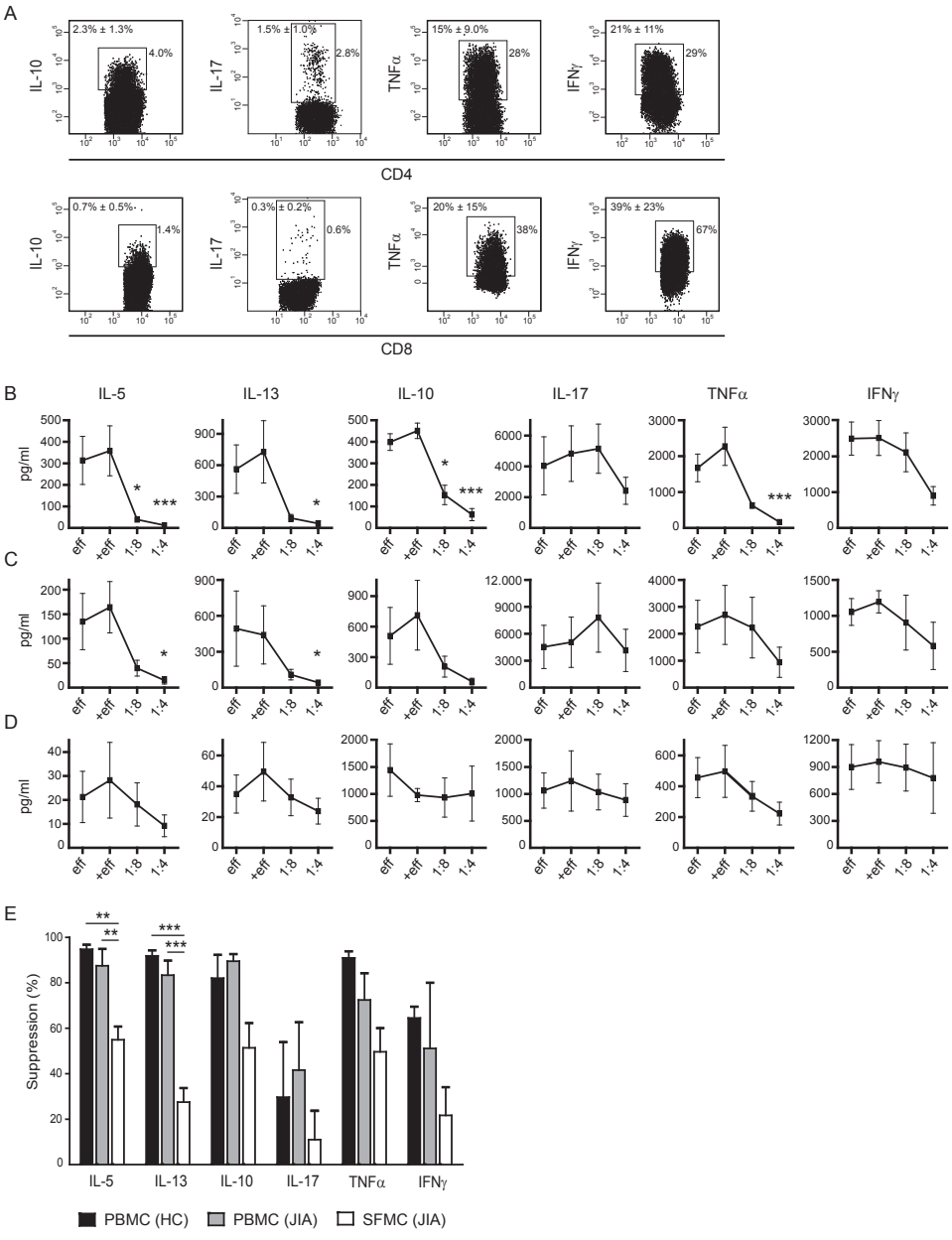


Figure 2 | Normal Treg function at the site of inflammation, but resistance of effector cells to suppression of cell proliferation. PBMC and SFMC were isolated from paired PB and SF samples from JIA patients. $CD4^+CD25^+CD127^{low}$ Treg were sorted from PBMC by flow cytometry and co-cultured with CFSE-labeled PBMC (*black bars*) or SFMC (*dark gray bars*) at 1:8 and 1:4 ratio. Conversely, $CD4^+CD25^+CD127^{low}$ Treg were sorted from SFMC and co-cultured with CFSE-labeled PBMC (*light gray bars*) or SFMC (*white bars*). At day 4, suppression of $CD4^+$ (***A***) and $CD8^+$ T cell proliferation (***B***) was measured. The results show percentage of suppression in the presence of Treg relative to effector cells alone. Bars represent mean ± SEM of n = 2, *P < .05 compared with PB-Tr + PBMC.

as cytokine production, was also impaired. After 4.5 hours PMA and ionomycin stimulation, $CD4^+$ (upper panel) and $CD8^+$ (lower panel) SFMC (gated as shown in Supplementary Figure 2 A) not only express proinflammatory cytokines associated with autoimmune pathology, such as IL-17, TNFα, and IFNγ, but also IL-10 (Figure 3 A) and expression of these cytokines was enhanced compared with paired PBMC (Supplementary Figures 2 B-E). When suppression assays were performed using PBMC from either HC (Figure 3 B) or JIA patients (Figure 3 C), the amount of IL-5, IL-13, IL-10, TNFα, and IFNγ in the culture supernatant generally declined when Treg were added at a 1:8 ratio and further decreased with higher numbers of Treg present (1:4). No clear reduction in IL-17 levels was observed, consistent with previous reports[27;28]. Adding additional effector cells instead of Treg (+eff) did not result in a decrease in cytokine levels and Treg cultured alone did not produce significant amounts of cytokines (data not shown). In contrast to these results with PBMC from patients and HC, cytokine levels in SFMC cultures (Figure 3 D), did not decrease when Treg were added at a 1:8 ratio and only modestly in the presence of Treg at a 1:4 ratio. When the level of suppression at a 1:4 ratio was calculated for each cytokine, suppression was significantly lower in SFMC (white bars) compared with PBMC of both patients (gray bars) and HC (black bars), whereas, again, there was no clear difference between PBMC of JIA patients and PBMC from HC (Figure 3 E). These data demonstrate that a broad range of cytokines produced by effector cells from the site of inflammation are insufficiently controlled by Treg.

To study the role of Treg malfunctioning versus resistance of effector cells to suppression in the incomplete restriction of cytokine production, cross-over experiments were again performed


A
IL-10
IL-17
TNFα
IFNγ
2.3% ± 1.3%
4.0%
1.5% ± 1.0%
2.8%
15% ± 9.0%
28%
21% ± 11%
29%
CD4
0.7% ± 0.5%
1.4%
0.3% ± 0.2%
0.6%
20% ± 15%
38%
39% ± 23%
67%
CD8
B
C
D
IL-5
IL-13
IL-10
IL-17
TNFα
IFNγ
pg/ml
eff
+eff
1:8
1:4
E
Suppression (%)
PBMC (HC)
PBMC (JIA)
SFMC (JIA)

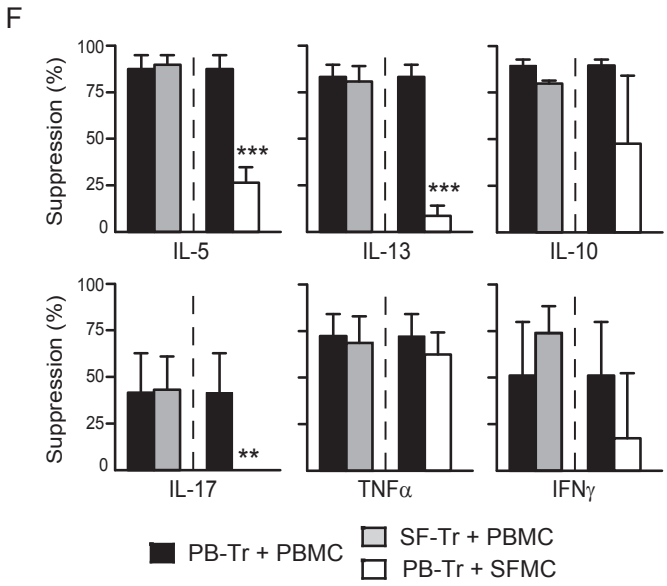


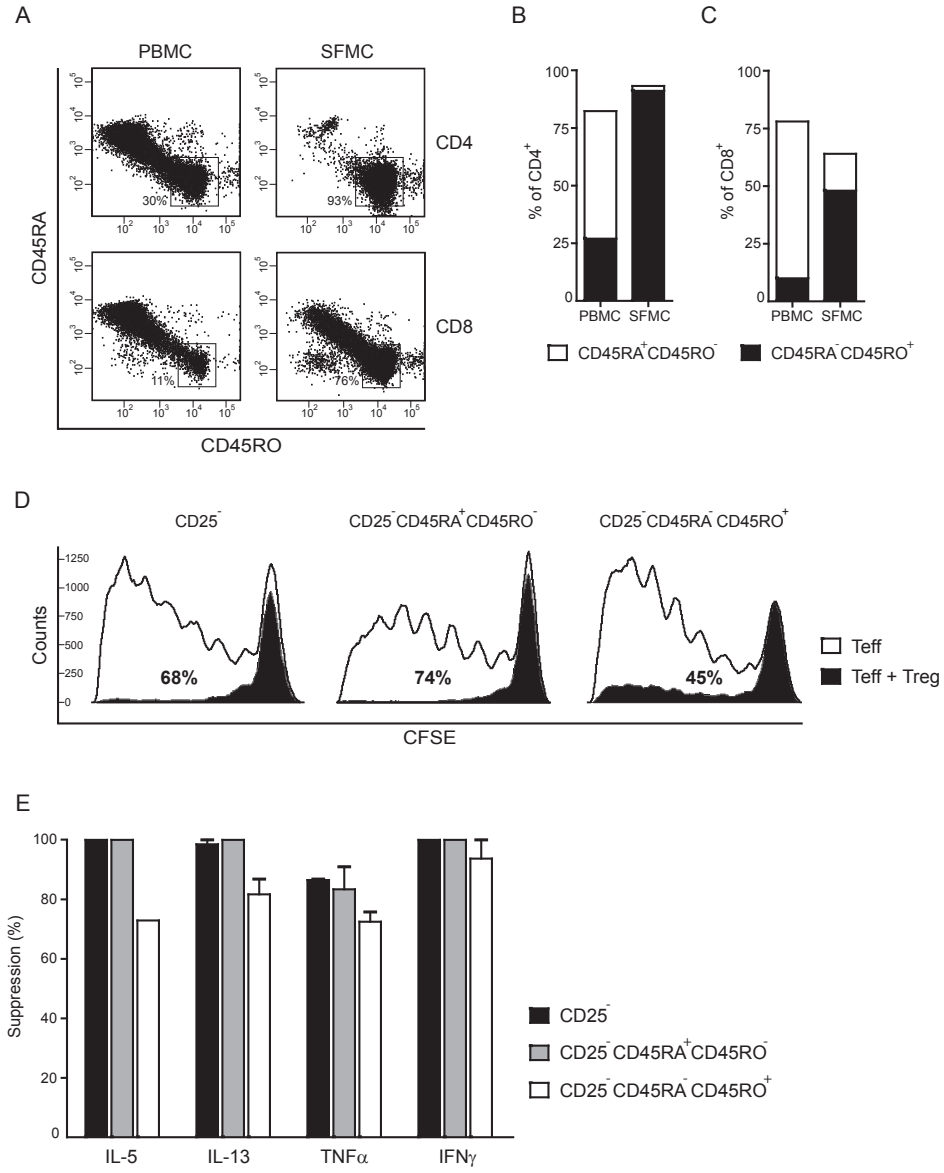
Figure 3 | Effector cytokine production at the site of inflammation is insufficiently controlled, because of resistance of effector cells to suppression. **A**, SFMC were stained for cytokine expression by flow cytometry after 4.5 hours of PMA and Ionomycin stimulation. Dotplots showing the percentage of IL-10, IL-17, TNFα, and IFNγ-positive cells in $CD4^+$ (*upper panel*) and $CD8^+$ cells (*lower panel*), 1 representative of n = 4. Percentages indicate the percentage of positive cells from representative data, or, in upper line, average percentage ± SD from accumulative data of n = 4. **B-F**, $CD4^+CD25^+CD127^{low}$ Treg were sorted by flow cytometry and co-cultured with effector cells. At day 4, cytokine production in the culture supernatant was analyzed. **B-D**, IL-5, IL-13, IL-10, IL-17, TNFα, and IFNγ levels in the absence (eff) or presence of Treg at 1:8 and 1:4 ratio or additional effector cells (+eff) at a 1:4 ratio for PBMC from HC (***B***), PBMC from JIA patients (**C**), and SFMC from JIA patients (***D***). Data represent mean cytokine levels in pg/ml ± SEM of n = 4 PBMC HC, n = 4 PBMC JIA and n = 8 SFMC JIA, *P < .05, ***P < .001 compared with effector cells (eff). **E-F**, Percentage suppression of IL-5, IL-13, IL-10, IL-17, TNFα and IFNγ production in the presence of Treg at a 1:4 ratio relative to effector cells alone. **E**, Percentage suppression in co-cultures of Treg and effector cells from PBMC of HC (*black bars*), PBMC of JIA patients (*gray bars*), and SFMC of JIA patients (*white bars*). Bars represent mean ± SEM of n = 4 PBMC HC, n = 4 PBMC JIA and n = 8 SFMC JIA, **P < .01, ***P < .001. **F**, Percentage suppression in co-cultures of PB derived Treg and PBMC (*black bars*), PB Treg and SFMC (*white bars*), or SF derived Treg and PBMC (*gray bars*). Bars represent mean ± SEM of n = 4, **P < .01, ***P < .001 compared with PB-Tr + PBMC.

(Figure 3 F). In these assays, levels of suppression were clearly reduced when PB-Treg were co-cultured with SFMC (white bars) compared with PB-Treg and PBMC co-cultures (black bars), demonstrating that effector cells from the site of inflammation show resistance to suppression of cytokine production. In contrast, the level of suppression was not affected in SF-Treg and PBMC cultures (gray bars) compared with PB-Treg and PBMC co-cultures, showing that SF-Treg are not impaired in their cytokine suppressive capacity. Together our data show that, despite normal Treg function, suppression of proliferation and cytokine production by cells from the site of autoimmune inflammation is impaired, because of resistance of effector cells to suppression.

RESISTANCE OF AUTOIMMUNE EFFECTOR CELLS TO SUPPRESSION IS NOT CAUSED BY A MEMORY PHENOTYPE

To mimic the *in vivo* situation as closely as possible, total mononuclear cells from the site of inflammation were used as effector cells in our assays. These SFMC differ in cellular composition from PBMC, which could contribute to the reduced responsiveness of these cells to suppression. Therefore, we carefully phenotyped paired SFMC and PBMC *ex vivo* by flow cytometry to gain insight into these differences in cellular constitution. We observed a significant increase in $CD45RA^{-}CD45RO^{+}$ memory cells in SFMC compared with PBMC in both $CD4^{+}$ ($p < .01$) and $CD8^{+}$ T cells ($p < .05$; Figures 4 A-C), which was accompanied by a decrease in the presence of $CD45RA^{+}CD45RO^{-}$ naive cells. In mice, it has been shown that memory effector cells are more resistant to Treg-mediated suppression compared with naive cells[29]. Therefore the high numbers of memory T cells present among effector cells from the site of inflammation might influence the responsiveness of these cells to suppression. To investigate this, we determined whether in our assays memory $CD4^{+}$ T cells sorted from PBMC of HC are less responsive to suppression compared with naive cells (Figures 4 D-E). We found that in the absence of Treg (open histograms) $CD25^{-}CD45RA^{-}CD45RO^{+}$ memory $CD4^{+}$ effector cells (Figure 4 D; right panel) showed enhanced proliferation compared with $CD25^{-}CD45RA^{+}CD45RO^{-}$ naive effector cells (Figure 4 D; middle panel). However, on addition of Treg (filled histograms), T cell proliferation was significantly reduced in both memory and naive cells (Figure 4 D). Similarly, IL-5, IL-13, TNFα, and IFNγ production was suppressed in both memory (white bars) and naive cells (gray bars), albeit to a slightly lower extend in memory cells compared with naive and total cells (Figure 4 E; black bars). In conclusion, consistent with previous results[29] memory cells show reduced responsiveness to suppression, but, the difference in suppression is minimal and not comparable with the highly diminished suppression observed in SFMC. Therefore, the general memory phenotype of cells at the site of autoimmune inflammation cannot explain their resistance to suppression.

Figure 4 | Effector cells from the site of inflammation have a general memory phenotype, which is not the cause of their resistance to suppression. **A-C**, Paired PBMC and SFMC were stained for CD45RA and RO expression by flow cytometry. **A**, Dotplots showing the percentage of $CD45RA^{-}CD45RO^{+}$ memory cells in PBMC (*left panel*) and SFMC (*right panel*) $CD4^{+}$ (*upper panel*) and $CD8^{+}$ T cells (*lower panel*), 1 representative of n = 4. **B-C**, Percentage of $CD45RA^{-}CD45RO^{+}$ memory (*black bars*) and $CD45RA^{+}CD45RO^{-}$ naive cells (*white bars*) in $CD4^{+}$ T cells (***B***) and $CD8^{+}$ T cells (***C***) of paired SFMC and PBMC of n = 4. **D-E**, $CD4^{+}CD25^{+}CD127^{low}$ Treg ►



► were sorted by flow cytometry and co-cultured with CFSE-labeled total, naive, and memory Teff at a 1:2 ratio. At day 5, proliferation of $CFSE^{low}$ Teff (***D***) and cytokine production in the culture supernatant (***E***) was analyzed. **D**, CFSE profile of $CD4^+CD25^-$ total Teff (*left panel*), $CD4^+CD25^-CD45RA^+CD45RO^-$ naive Teff (middle *panel*) and $CD4^+CD25^-CD45RA^-CD45RO^+$ memory Teff (*right panel*) cultured in the absence (*open histograms*) or presence of Treg (*filled histograms*). Percentages indicate the percentage suppression of cell proliferation in the presence Treg relative to effector cells alone, one representative of n = 2. **E**, Percentage suppression of IL-5, IL-13, TNFα and IFNγ production by $CD4^+CD25^-$ total Teff (*black bars*), $CD4^+CD25^-CD45RA^+CD45RO^-$ naive Teff (*gray bars*) and $CD4^+CD25^-CD45RA^-CD45RO^+$ memory Teff (*white bars*) in the presence of Treg relative to effector cells alone. Bars represent mean ± SEM of n = 2.

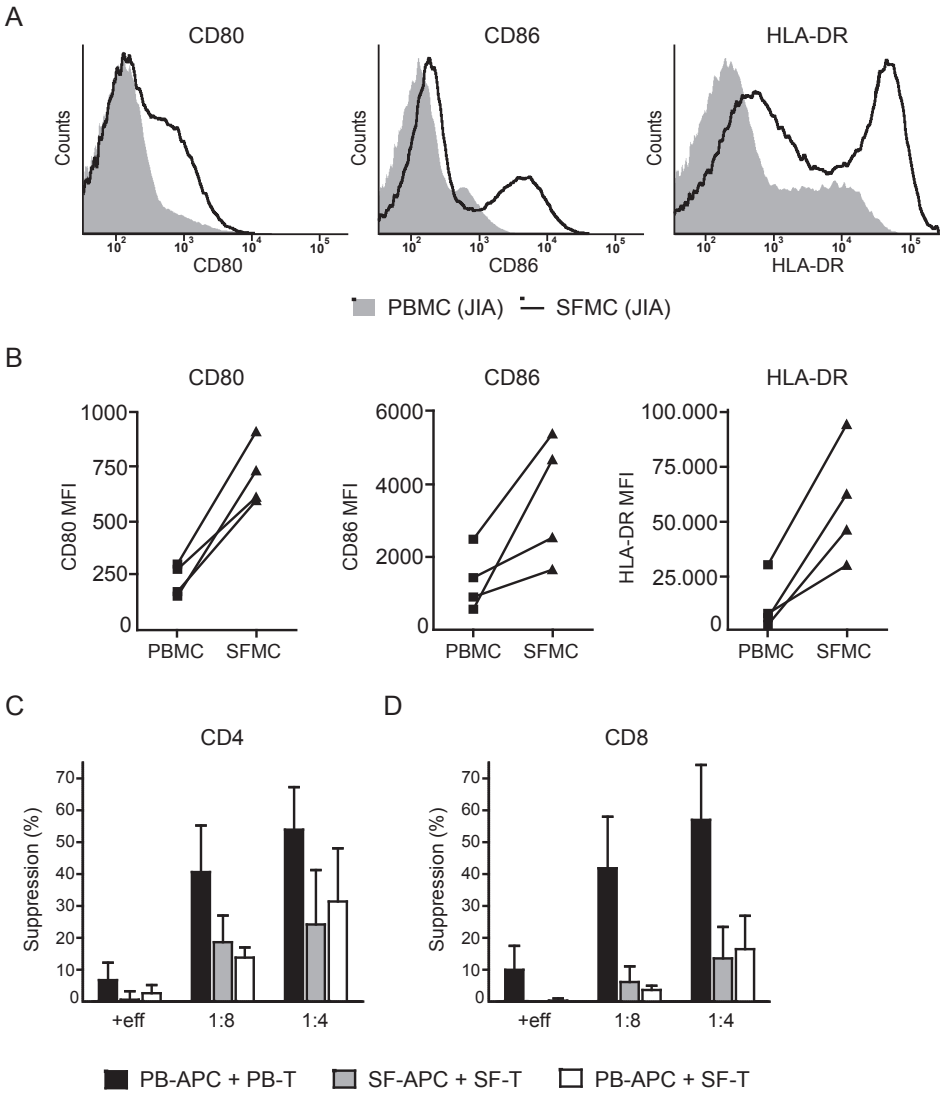


Figure 5 | Resistance of SFMC to suppression is not caused by activation status of APCs. **A-B**, Monocytes from paired PBMC and SFMC of JIA patients were analyzed for CD80, CD86, and HLA-DR expression by flow cytometry. **A**, Histograms showing CD80 (*left panel*), CD86 (*middle panel*), and HLA-DR (*right panel*) fluorescence intensity in paired PBMC (*filled histograms*) and SFMC (*open histograms*), one representative of n = 4. **B**, MFI of CD80 (*left panel*), CD86 (*middle panel*) and HLA-DR (*right panel*) in monocytes from paired PBMC and SFMC of n = 4. **C-D**, $CD3^+$ T cells, $CD3^-$ APCs and $CD4^+CD25^+CD127^{low}$ Treg were sorted by flow cytometry. PB T cells were co-cultured with PB APCs (*black bars*) and SF T cells were co-cultured with SF APCs (*gray bars*) or PB APCs (*white bars*) in the absence or presence of SF Treg at a 1:8 and 1:4 ratio or additional effector cells (+eff) at a 1:4 ratio. Suppression of $CD4^+$ (**C**) and $CD8^+$ T cell proliferation (***D***) was measured. The results show percentage of suppression in the presence of Treg or additional effector cells relative to effector cells alone. Bars represent mean ± SEM of n = 3.

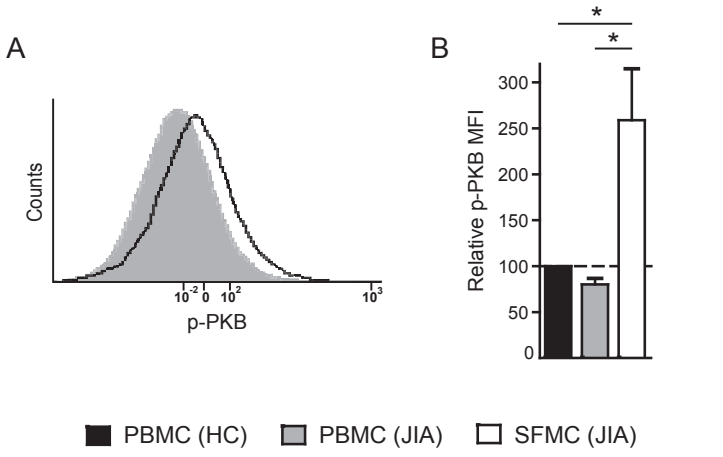
RESISTANCE TO SUPPRESSION NOT CAUSED BY ACTIVATION STATUS OF APCs

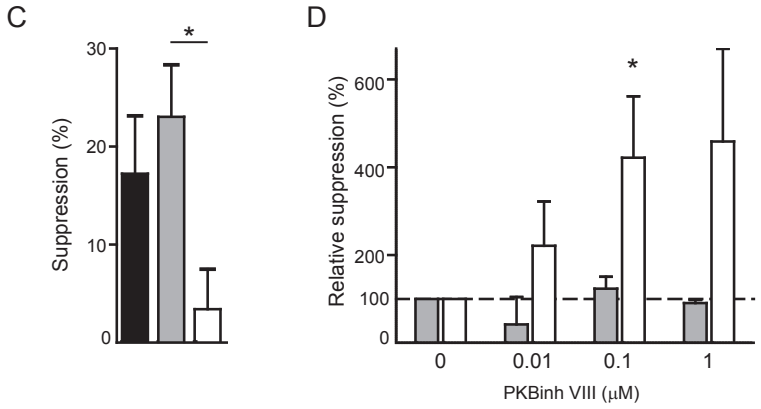
In RA patients it was demonstrated that monocytes from the site of inflammation displayed a more activated phenotype that interfered with Treg-mediated suppression[20]. Because the effector cell population in our experiments contained monocytes, we investigated whether changes in monocyte number and/or activation could explain the resistance of SFMC to suppression. Monocytes were gated on forward side scatter (Supplementary Figure 3) and phenotypically analyzed. No difference in percentage of monocytes between SFMC (24% ± 12%) and PBMC (23% ± 12%) was observed. However, in line with previous reports in RA[20] and JIA[30], monocytes from SF displayed higher expression of CD80, CD86 and HLA-DR (Figures 5 A and B), which could interfere with Treg inhibition. Therefore, we investigated whether in our *in vitro* suppression assays $CD3^-$ APCs contribute to resistance of SFMC to suppression or whether this resistance solely resides within the $CD3^+$ T cell population (Figures 5 C and D). When SF $CD3^-$ APCs were co-cultured with SF $CD3^+$ T cells in the presence of Treg (gray bars), suppression of both $CD4^+$ (Figure 5 C) and $CD8^+$ T cell proliferation (Figure 5 D) was again reduced compared with cultures containing PB-derived APCs and T cells (black bars). However, this reduction in suppression was similar when SF T cells were co-cultured with PB APCs (white bars), clearly demonstrating that the resistance of SFMC to suppression resides within $CD3^+$ T cells and is not caused by enhanced activation of SF APCs.

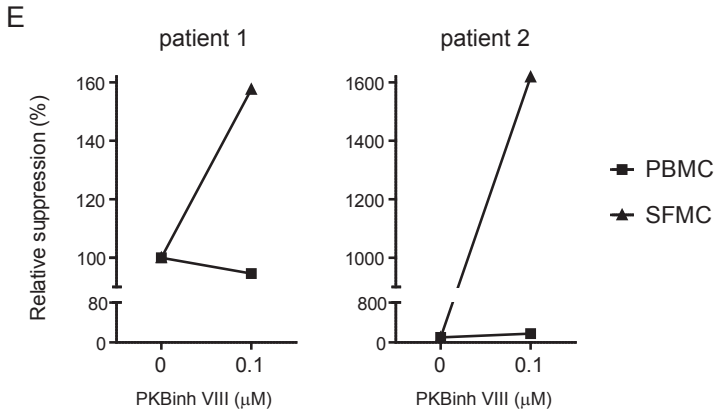
HYPERACTIVATION OF PKB/C-AKT LEADS TO RESISTANCE OF EFFECTOR CELLS TO SUPPRESSION

In addition to a general memory phenotype, $CD4^+$ cells from the site of inflammation expressed higher levels of proliferation and activation markers compared with cells from PB (Supplementary Figure 4), indicating that these cells are in a highly activated state. Because the PI3K-PKB/c-akt module is an important intracellular signaling pathway involved in T cell activation[31] and in mice hyperactivation of this pathway has been shown to induce resistance to suppression[32], we investigated whether hyperactivation of this pathway may be responsible for the resistance of SFMC to suppression. First, we analyzed PKB/c-akt activation in paired SFMC and PBMC *ex vivo* by measuring the level of phosphorylated PKB/c-akt, a measure of activation status, by flow cytometry (Figure 6 A). We found that compared with HC (black bars), the level of phosphorylated PKB/c-akt was unchanged in $CD4^+$ T cells from the PB of JIA patients (gray bars), however, cells from the site of inflammation (white bars) clearly showed enhanced phosphorylated PKB/c-akt levels (Figure 6 B). Thus, Teff at the site of autoimmune inflammation show increased levels of PKB/c-akt activation.

In mice, expression of constitutively activated PKB/c-akt renders effector cells resistant to both Treg- as well as TGFβ-mediated suppression[33], therefore, we investigated whether SFMC, showing enhanced PKB/c-akt activation, are also refractory to TGFβ-mediated suppression. Proliferation of $CD4^+$ T cells from PBMC of HC (black bars) and JIA patients (gray bars) was clearly suppressed by the presence of TGFβ, whereas, only very low levels of suppression were detected in SF $CD4^+$ T cells (white bars; Figure 6C), showing that these cells are resistant to TGFβ-mediated suppression. To investigate whether increased PKB/c-act activation directly leads to resistance of these cells to suppression, we determined whether inhibiting this kinase restored responsiveness to TGFβ.


A
Counts
p-PKB
B
Relative p-PKB MFI
PBMC (HC)
PBMC (JIA)
SFMC (JIA)


C
Suppression (%)
D
Relative suppression (%)
PKBinh VIII (μM)


E
patient 1
patient 2
Relative suppression (%)
PKBinh VIII (μM)
PBMC
SFMC

Culture of SFMC in the presence of a specific PKB/c-akt inhibitor dose-dependently decreased PKB activation in these cells as measured by flow cytometry (Supplementary Figure 5 A). As a result, TGFβ-mediated suppression of SFMC (white bars), but not already responsive PBMC (gray bars), was enhanced in the presence of this inhibitor (Figure 6 D).

In addition, PKB/c-akt inhibition restored responsiveness of SFMC (triangles) to Treg-mediated suppression, without affecting suppression of PBMC (squares; Figure 6 E). Importantly, PKB/c-akt inhibitor treatment did not affect proliferation of SFMC in the absence of TGFβ and Treg (Supplementary Figure 5 B), showing that it specifically targets the unresponsiveness of these cells to suppression, and does not inhibit proliferation in general. Together these data clearly demonstrate that the resistance of effector cells to suppression at the site of inflammation is caused by PKB/c-akt hyperactivation. Furthermore, by pharmacologically targeting this pathway responsiveness of the cells to suppression can be restored.

TNFα AND IL-6 PRESENT AT THE SITE OF INFLAMMATION INDUCE PKB/C-AKT ACTIVATION AND RESISTANCE TO SUPPRESSION

We are the first to show that PKB/c-akt hyperactivation occurs under physiologic conditions at the site of inflammation in human autoimmune disease. It is therefore intriguing to identify the cause of this enhanced PKB/c-akt activation. To investigate whether soluble factors present in the inflammatory environment lead to enhanced PKB/c-akt activation, we first measured the expression of proinflammatory cytokines in PB plasma of HC and paired PB plasma and SF of JIA patients (Figure 7 A). In line with previous reports[25] and regarding effectiveness of TNFα- and IL-6–blocking strategies in arthritis[34], we observed elevated levels of TNFα and IL-6 in SF of JIA patients. Incubation of PBMC from HC with these cytokines resulted in an up-regulation of p-PKB (Figure 7 B) and reduced responsiveness to TGFβ-mediated suppression (Figure 7 C), which was

◀ **Figure 6 |** PKB/c-akt hyperactivation causes resistance of effector cells to suppression. **A-B**, PBMC and SFMC were stained for phosphorylated PKB/c-akt expression by flow cytometry. **A**, Histogram showing phosphorylated PKB/c-akt (p-PKB) fluorescence intensity in $CD4^+$ T cells from paired PBMC (*filled histogram*) and SFMCs (*open histogram*), 1 representative of n = 3. **B**, MFI of phosphorylated PKB/c-akt (p-PKB) in $CD4^+$ T cells from paired PBMC (*gray bar*) and SFMC (*white bar*) of JIA patients relative to PBMC from HC (*black bar*). Bars represent mean ± SEM of n = 3, *P < .05. **C-D**, CFSE-labeled PBMC and SFMC were cultured in the presence or absence of recombinant human TGFβ1 (40 ng/ml) and increasing concentrations of PKB/c-akt inhibitor VIII (PKBinh VIII; 0, 0.01, 0.1, 1μM). At day 5, proliferation of $CD4^+$ T cells was analyzed. **C**, TGFβ-mediated suppression of $CD4^+$ T cell proliferation for PBMC from HC (*black bars*) and PBMC (*gray bars*) and SFMC (*white bars*) from JIA patients. The results show percentage of suppression in the presence of TGFβ relative to cells cultured without TGFβ. Bars represent mean ± SEM of n = 3 PBMC HC, n = 4 PBMC JIA, and n = 5 SFMC JIA, *P < .05. **D**, TGFβ-mediated suppression of $CD4^+$ T cell proliferation for paired PBMC (*gray bars*) and SFMC (white *bars*) in the presence of increasing concentrations of PKB/c-akt inhibitor VIII. The data show the change in TGFβ-mediated suppression for each concentration of PKB/c-akt inhibitor relative to cultures without PKB/c-akt inhibitor. Bars represent mean ± SEM of n = 3, *P < .05 compared with 0 μM PKB/c-akt inhibitor VIII. **E**, $CD4^+CD25^+CD127^{low}$ Treg were sorted from SFMC by flow cytometry and co-cultured with CFSE-labeled PBMC (*squares*) or SFMC (*triangles*) at a 1:4 ratio in the presence or absence of PKB/c-akt inhibitor VIII (PKBinh VIII) (0.1 μM). At day 4, Treg-mediated suppression of $CD4^+$ T cell proliferation was analyzed. The data show the change in Treg-mediated suppression in the presence PKB/c-akt inhibitor relative to cultures without PKB/c-akt inhibitor.

significant when both TNFα and IL-6 were added ($p < .05$). Together, these data identify TNFα and IL-6 as proinflammatory factors that contribute to enhanced PKB/c-akt activation and subsequent resistance to suppression at the site of human autoimmune inflammation.

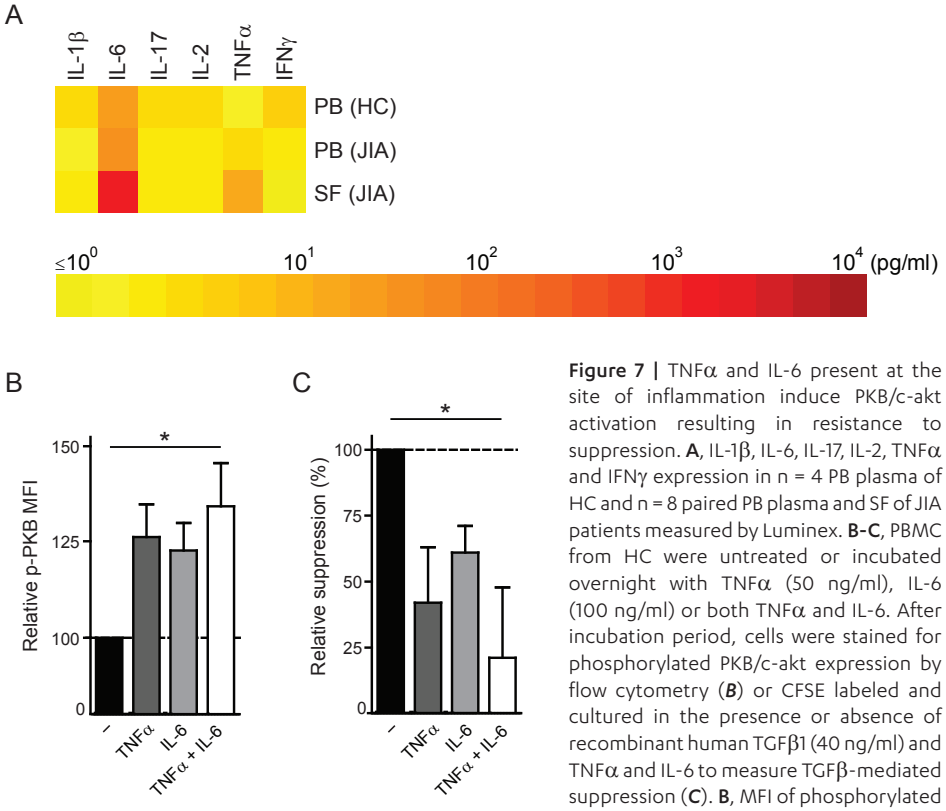


Figure 7 | TNFα and IL-6 present at the site of inflammation induce PKB/c-akt activation resulting in resistance to suppression. **A**, IL-1β, IL-6, IL-17, IL-2, TNFα and IFNγ expression in n = 4 PB plasma of HC and n = 8 paired PB plasma and SF of JIA patients measured by Luminex. **B-C**, PBMC from HC were untreated or incubated overnight with TNFα (50 ng/ml), IL-6 (100 ng/ml) or both TNFα and IL-6. After incubation period, cells were stained for phosphorylated PKB/c-akt expression by flow cytometry (***B***) or CFSE labeled and cultured in the presence or absence of recombinant human TGFβ1 (40 ng/ml) and TNFα and IL-6 to measure TGFβ-mediated suppression (**C**). **B**, MFI of phosphorylated PKB/c-akt (p-PKB) in $CD4^+$ T cells in the presence of TNFα (*dark gray bars*), IL-6 (*light gray bars*) or both (*white bars*) relative to cultures without cytokines added (*black bars*). Bars represent mean ± SEM of n = 5, *P < .05. **C**, TGFβ-mediated suppression of $CD4^+$ T cell proliferation in the absence (*black bars*) or presence of TNFα (*dark gray bars*), IL-6 (*light gray bars*) or both (*white bars*). The data show the change in TGFβ-mediated suppression in the presence of cytokines compared with cultures without cytokines added. Bars represent mean ± SEM of n = 5, *P < .05.

DISCUSSION

After 15 years of research into Treg biology the main question is whether these cells can be used in treatment of autoimmune disease. To resolve this issue more information on Treg function at the site of autoimmune inflammation in humans is required. Here we studied Treg function in patients with JIA and show that both in the periphery and at the site of inflammation, Treg are not deficient in number and function. Nevertheless, effector cells from the site of inflammation are poorly controlled, because these cells are resistant to suppression. We further demonstrate that

this unresponsiveness to suppression is, at least partially, caused by PKB/c-akt hyperactivation and can be restored by specific PKB/c-akt inhibition. These data therefore identify PKB/c-akt as a potential novel target for the treatment of autoimmune disease.

In PB of JIA patients, no change in the numbers of Treg was observed and these cells suppressed both cell proliferation and cytokine production similar to Treg from HC. In contrast, defective Treg function has previously been described in peripheral blood of RA patients[12;15]. Perhaps differences in Treg function in the periphery are less pronounced in oligoarticular JIA, because of its relative mild and local pathology. Alternatively, because CD127 has become available as an additional marker to isolate Treg[5;6], the differences in results could also be explained by differences in Treg purity. Other studies are, however, in agreement with our data and report no difference in Treg function in patients with established arthritis[14;16;35].

At the site of inflammation in patients with JIA, increased numbers of Treg were present and these cells showed normal suppressive capacity. Thus, we and others[14;16;18;19;36] have observed that Treg from the site of inflammation are fully functional. However, by mimicking the *in vivo* situation as closely as possible and using total mononuclear cells as effector cells in our assays, we were able to demonstrate that these Treg still failed to control effector cells from the site of inflammation. This was caused by reduced responsiveness of these effector cells to suppression. In other experimental models of autoimmune disease and some patient studies, most profoundly systemic lupus erythematosus and type 1 diabetes, resistance of effector cells to suppression has also been described[22;37-42]. However, in oligoarticular JIA unresponsiveness of effector cells to suppression occurs locally, at the site of inflammation, and not systemically. In genetically prone mice effector cells become resistant to suppression before clinical overt disease[37;39], suggesting that this phenomenon acts early in disease pathology and is therefore an important target in controlling autoimmune inflammation. However, to target this resistance of effector cells effectively, the underlying mechanism needs to be clarified.

Here, we show that autoimmune inflammatory effector cells are resistant to both TGFβ- as well as Treg-mediated suppression and that this resistance does not result from a general memory phenotype of the cells or activation status of APCs. Instead, hyperactivation of PKB/c-akt is responsible for the unresponsiveness to suppression, as $CD4^+$ Teff from the site of inflammation showed increased PKB/c-akt phosphorylation and selective inhibition of this kinase restored responsiveness of cells to suppression. We are the first to show that PKB/c-akt is involved in resistance to suppression in human autoimmune disease, consistent with findings in mice[33;43].

Phosphorylation and activation of PKB/c-akt is regulated by the generation of lipid products by phosphoinositide 3 kinases (PI3Ks) and PI3Ks become activated in lymphocytes upon binding of antigens, costimulatory molecules, cytokines, and chemokines[32;44]. PI3K-PKB activation by chemokines is, however, both rapid and transient[44], therefore, enhanced PKB/c-akt activation in cells at the site of inflammation is likely to result from either TCR, CD28, or cytokine signaling. We show that TNFα and IL-6 are elevated in synovial fluid of JIA patients and induce PKB/c-akt activation and resistance to suppression, in line with previous reports showing that these cytokines can confer resistance to suppression in mice[22;23]. Other cytokines and CD28 signaling can activate the PI3K-PKB pathway as well[45], therefore it is likely

that a combination of environmental factors contributes to resistance to suppression at the site of human autoimmune inflammation. However, given the effectiveness of TNFα and IL-6 blockade in the treatment of arthritis[34], it is intriguing to speculate that TNFα and IL-6 are critical in inducing resistance to suppression and that the effectiveness of these strategies can partially be explained by inhibiting PKB/c-akt activation.

Independent of the initial cause of PKB/c-akt activation, we show that selective PKB/c-akt inhibition is sufficient to restore responsiveness of effector cells to suppression, making this kinase an attractive target for therapeutic intervention. Clinical efficacy of PI3K inhibition, upstream of PKB/c-akt, has already been demonstrated in experimental models of arthritis[46] and systemic lupus erythematosus[47]. Targeting this pathway might therefore be beneficial in a wide range of autoimmune inflammatory conditions. However, for a PKB/c-akt targeted approach to be successful negative effects on Treg function must be prevented. In human PB-derived Treg PKB/c-akt was hypoactivated and this hypoactivation was essential for their suppressive function[48]. Therefore, PKB/c-akt inhibition might not negatively affect Treg function and could even enhance de novo generation of Treg[49;50]. As a result, selective PKB/c-akt inhibition might be especially effective when combined with a Treg enhancing strategy, ensuring responsiveness of effector cells to suppression and simultaneously creating an environment suited for Treg induction.

In conclusion, the data presented in this study provide new insights in the pathology of autoimmune disease and raise important therapeutic implications. Our findings argue for a Teff instead of Treg-targeted approach to control autoimmune inflammation. More specifically, responsiveness of effector cells to suppression should be restored by selectively inhibiting PKB/c-akt activation.

ACKNOWLEDGMENTS

The authors thank Mariska van Dijk for technical assistance in Luminex assays and Nico Wulffraat for patient inclusion.

SUPPLEMENTARY INFORMATION

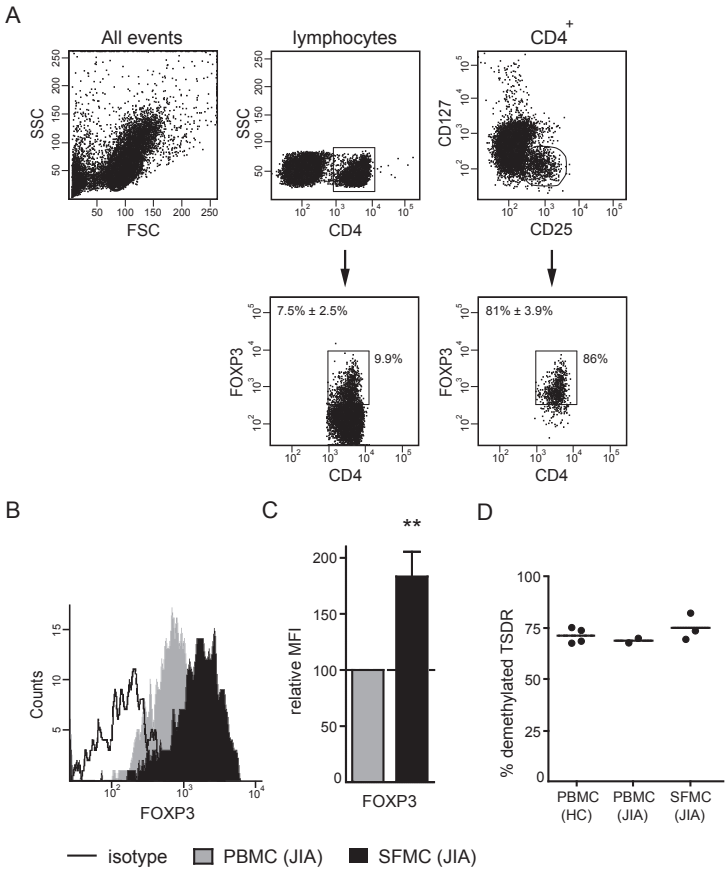
ANTIBODIES USED FOR FLOW CYTOMETRY.

To stain cells for sorting of Treg, Teff and APCs, the following monoclonal antibodies were used: anti-CD3 PerCp/Cy5.5 (UCHT1, Biolegend), anti-CD4-FITC, anti-CD4-APC or anti-CD4-PcBlue (all clone RPA-T4, eBioscience), anti-CD25-APC, anti-CD25-PE/Cy7 (both clone M-A251, BD Biosciences) or anti-CD25-PerCp/Cy5.5 (BD96, Biolegend), anti-CD45RA-FITC (JS-83, eBioscience), anti-CD45RO PcBlue (UCHL-1, Biolegend), and anti-CD127-PE (hIL-7R-M21, BD Biosciences). To check for FOXP3 expression in sorted $CD4^+CD25^+CD127^{low}$ Treg, anti-CD4-PercP (SK3, BD Biosciences) or anti-CD4 PerCP/Cy5.5 (RPA-T4, Biolegend), anti-CD25-FITC (MA251), anti-CD127-PE (hIL-7R-M21, both BD Biosciences) and anti-FOXP3-PcBlue or anti-FOXP3-eFluor450 (both clone PCH101, eBioscience) were used. Anti-CD3-PerCp (SK7, BD Biosciences) or anti-CD3 PerCp/Cy5.5 (UCHT1), anti-CD4-PcBlue (RPA-T4, both from Biolegend) and anti-

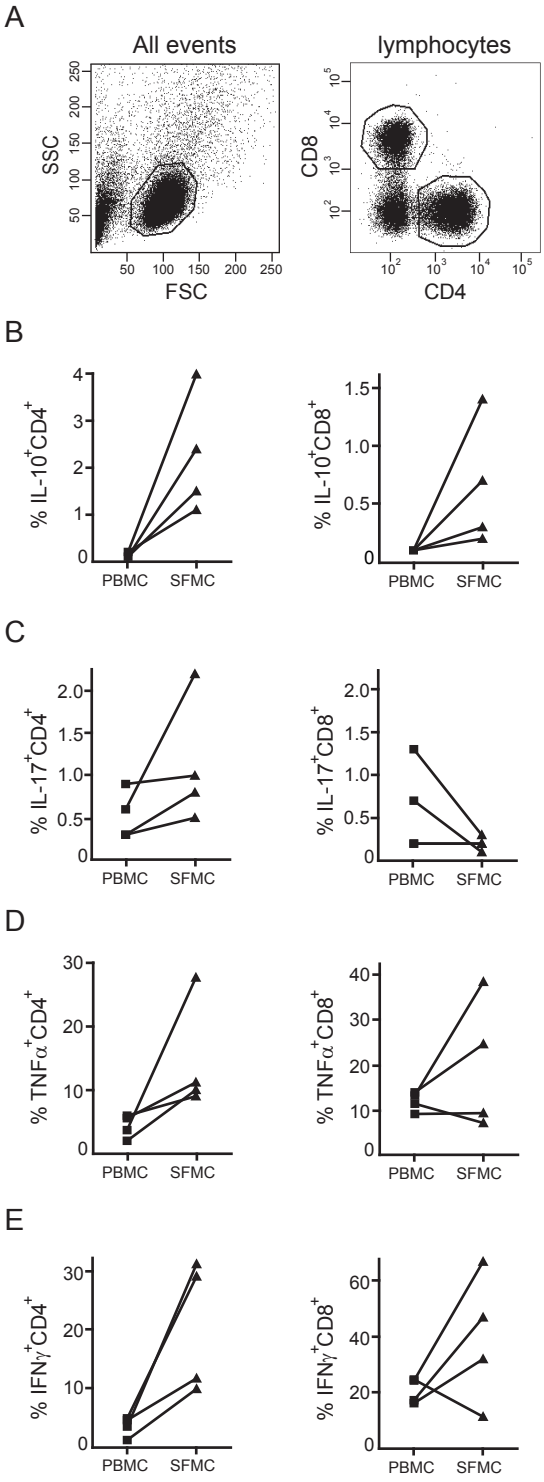
CD8-APC (SK1, BD Biosciences) were used to distinguish between $CD4^+$ and $CD8^+$ T cells within CFSE-labeled effector cells. For phenotyping, paired SFMC and PBMC were stained with the following antibodies: anti-CD4-PE (RPA-T4), anti-CD4-PerCp (SK3, both from BD Biosciences) or anti-CD4-PcBlue (RPA-T4, Biolegend), anti-CD8-PerCp (SK1), anti-CD25-PE/Cy7 (M-A251), anti-CD62L-FITC (Dreg 56), anti-CD69-APC (FN50), anti-CD80-FITC or anti CD80-PE (both L307.4), anti-CD86-FITC or anti-CD86-PE (both IT2.2), anti HLA-DR-PE/Cy7 (L243), anti-Ki67-FITC (B56, all obtained from BD Biosciences), anti-CD45RA-FITC (JS-83, eBioscience), anti-CD45RO-PcBlue (UCHL-1, Biolegend), anti-FOXP3-APC or anti-FOXP3-PcBlue (both clone PCH101, eBioscience), anti-IL-10-PE (JES3-19F1, BD Biosciences), anti-IL-17-PE (eBio64DEC17, eBioscience), anti-TNFα-APC (Mab11, Biolegend), anti-IFNγ-FITC (25723,11), anti-CD152(CTLA-4)-APC (BNI3, both BD Biosciences), and anti-GITR-FITC (110416, R&D Systems). To measure phosphorylated PKB/c-akt in $CD4^+$ T cells, cells were stained with anti-CD3-PE/Cy7 (UCHT1), anti-CD4-PcBlue (RPA-T4, both Biolegend), and anti-akt (PKB)-PE (pS473) (BD Biosciences).

SUPPLEMENTARY FIGURES

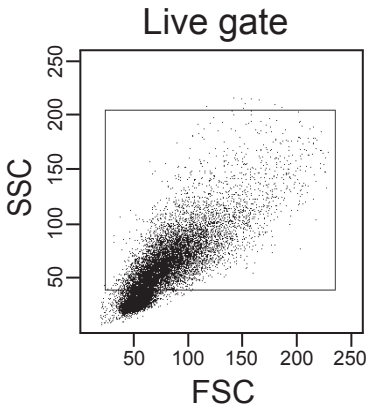
Supplementary Figure 1 shows the purity of sorted Treg and *ex vivo* phenotype of SF and PB derived Treg. Supplementary Figure 2 represents intracellular cytokine production in paired PBMC and SFMC. Supplementary Figure 3 depicts gating strategy applied to analyze monocytes by flow cytometry. Supplementary Figure 4 shows *ex vivo* expression of proliferation and activation markers in $CD4^+$ T cells of paired SFMC and PBMC. Supplementary Figure 5 represents p-PKB MFI and proliferation of $CD4^+$ T cells upon treatment with PKB/c-akt inhibitor.



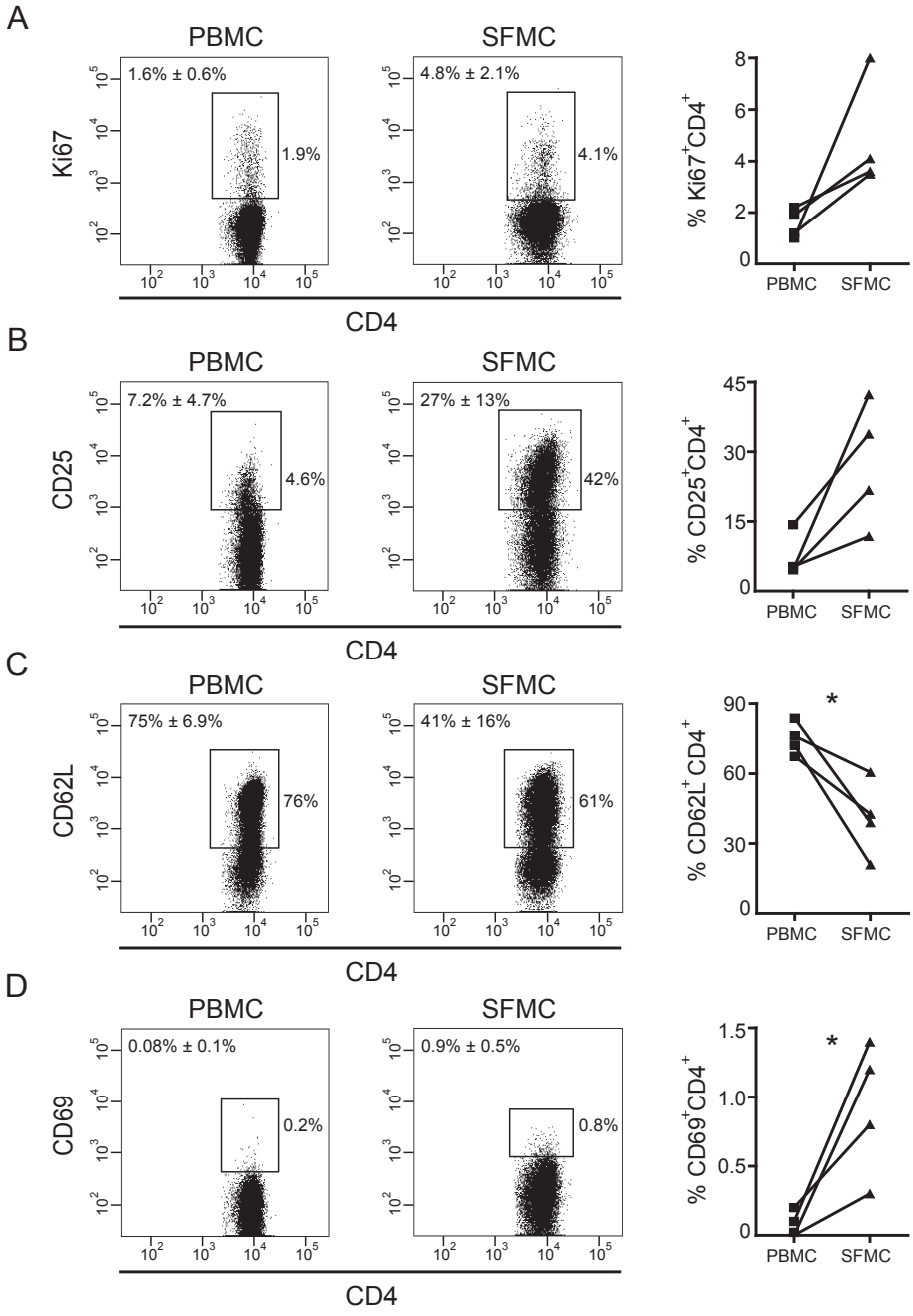
Supplementary Figure 1 | Purity of sorted Treg and *ex vivo* phenotype of SF and PB derived Treg, showing enhanced FOXP3 content per cell in SF $CD4^+CD25^+CD127^{low}$Treg. **A**, Sorting strategy applied to isolate $CD4^+CD25^+CD127^{low}$ Treg by flow cytometry as described in Methods. The areas depicted represent the gates used to sort the cells or analyze the percentage of $FOXP3^+$ cells. **B**, Histogram showing FOXP3 fluorescence intensity in sorted SF derived $CD4^+CD25^+CD127^{low}$ Treg (*filled black histogram*), paired PB derived $CD4^+CD25^+CD127^{low}$ Treg (*filled gray histogram*) and isotype control (*open histogram*), one representative of n=14. **C**, FOXP3 MFI in sorted SF derived $CD4^+CD25^+CD127^{low}$ Treg (*black bar*) relative to paired PB derived $CD4^+CD25^+CD127^{low}$ Treg (*gray bar*). Bars represent means ± SEM of n = 14, $^{**}P < .01$. **D**, Percentage of cells with demethylated FOXP3 TSDR in sorted $CD4^+CD25^+CD127^{low}$ Treg from n = 4 PBMC of HC, n = 3 PBMC of JIA patients (2 patients pooled) and n = 3 SFMC of JIA patients. **E**, Percentage of CTLA-4 expressing cells (*left panel*) and CTLA-4 content per cell (*right panel*) in paired $CD4^+FOXP3^+$ PBMC (*squares*) and SFMC (*triangles*) of n = 3. **F**, Percentage of GITR expressing cells (*left panel*) and GITR content per cell (*right panel*) in paired $CD4^+FOXP3^+$ PBMC (*squares*) and SFMC (*triangles*) of n = 3.



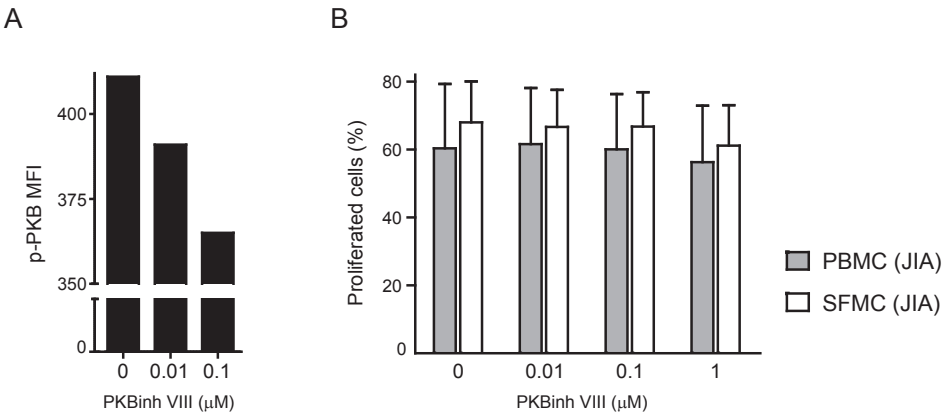
Supplementary Figure 2 | Cytokine production in paired PBMC and SFMC after short PMA and Ionomycin stimulation. **A**, Gating strategy applied to analyze intracellular cytokine production in $CD4^+$ and $CD8^+$ cells by flow cytometry. **B-E**, Percentage of IL-10 (***B***), IL-17 (***C***), TNFα (***D***) and IFNγ (***E***) positive cells in $CD4^+$ cells (*left panels*) and $CD8^+$ cells (*right panels*) in paired PBMC (*squares*) and SFMC (*triangles*) of n=4 JIA patients.



Supplementary Figure 3 | Gating strategy applied to analyze phenotype of SF and PB derived monocytes by flow cytometry. After exclusion of dead cells, monocytes were gated on forward side scatter using the depicted area.



Supplementary Figure 4 | $CD4^+$ cells from SFMC show enhanced expression of proliferation and activation markers *ex vivo* compared to PBMC. Percentage of Ki67 (***A***), CD25 (***B***), CD62L (***C***) and CD69 (***D***) positive cells in $CD4^+$ cells from paired PBMC and SFMC from JIA patients measured *ex vivo* by flow cytometry. Representative dotplots showing the percentage of positive cells within $CD4^+$ cells (*left panels*) and accumulative data (*right panels*) of n = 4, *P < .05.



Supplementary Figure 5 | Treatment with PKB/c-akt inhibitor dose-dependently reduces PKB phosphorylation, but does not change cell proliferation. **A-B**, CFSE-labeled PBMC and SFMC were cultured in the presence of increasing concentrations of PKB/c-akt inhibitor VIII (PKBinh VIII) (0, 0.01, 0.1, 1 μM). At day 5, expression of phosphorylated PKB/c-akt (***A***) and proliferation of $CD4^+$ T cells (***B***) was analyzed by flow cytometry. **A**, MFI of phosphorylated PKB/c-akt (p-PKB) in $CD4^+$ SFMC, n=1 in duplicate. **B**, Proliferation of $CD4^+$ T cells from paired PBMC (*gray bars*) and SFMC (*white bars*) of JIA patients, bars represent means ± SEM of n = 3.

REFERENCES

1. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol*. 1995;155(3):1151-1164.
2. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol*. 2008;8(7):523-532.
3. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol*. 2003;4(4):330-336.
4. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. 2003;299(5609):1057-1061.
5. Liu W, Putnam AL, Xu-Yu Z, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med*. 2006;203(7):1701-1711.
6. Seddiki N, Santner-Nanan B, Martinson J, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med*. 2006;203(7):1693-1700.
7. Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol*. 2005;6(4):345-352.
8. Bennett CL, Christie J, Ramsdell F, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet*. 2001;27(1):20-21.
9. Wildin RS, Ramsdell F, Peake J, et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet*. 2001;27(1):18-20.
10. Bluestone JA. Regulatory T-cell therapy: is it ready for the clinic? *Nat Rev Immunol*. 2005;5(4):343-349.
11. Buckner JH. Mechanisms of impaired regulation by CD4(+)CD25(+)FOXP3(+) regulatory T cells in human autoimmune diseases. *Nat Rev Immunol*. 2010;10(12):849-859.
12. Ehrenstein MR, Evans JG, Singh A, et al. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. *J Exp Med*. 2004;200(3):277-285.
13. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J Exp Med*. 2004;199(7):971-979.
14. Van Amelsfort JM, Jacobs KM, Bijlsma JW, Lafeber FP, Taams LS. CD4(+)CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. *Arthritis Rheum*. 2004;50(9):2775-2785.
15. Valencia X, Stephens G, Goldbach-Mansky R, et al. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. *Blood*. 2006;108(1):253-261.
16. Mottonen M, Heikkinen J, Mustonen L et al. CD4+ CD25+ T cells with the phenotypic and functional characteristics of regulatory T cells are enriched in the synovial fluid of patients with rheumatoid arthritis. *Clin Exp Immunol*. 2005;140(2):360-367.
17. Brusko TM, Putnam AL, Bluestone JA. Human regulatory T cells: role in autoimmune disease and therapeutic opportunities. *Immunol Rev*. 2008;223:371-390.
18. De Kleer IM, Wedderburn LR, Taams LS, et al. CD4+CD25bright regulatory T cells actively regulate inflammation in the joints of patients with the remitting form of juvenile idiopathic arthritis. *J Immunol*. 2004;172(10):6435-6443.
19. Ruprecht CR, Gattorno M, Ferlito F, et al. Coexpression of CD25 and CD27 identifies FoxP3+ regulatory T cells in inflamed synovia. *J Exp Med*. 2005;201(11):1793-1803.
20. Van Amelsfort JM, Van Roon JA, Noordegraaf M, et al. Proinflammatory mediator-induced reversal of CD4+,CD25+ regulatory T cell-mediated suppression in rheumatoid arthritis. *Arthritis Rheum*. 2007;56(3):732-742.
21. Koenen HJ, Smeets RL, Vink PM, et al. Human CD25highFoxp3pos regulatory T cells differentiate into IL-17-producing cells. *Blood*. 2008; 112(6):2340-2352.
22. Korn T, Reddy J, Gao W, et al. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. *Nat Med*. 2007;13(4):423-431.
23. Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science*. 2003;299(5609):1033-1036.

24. Petty RE, Southwood TR, Baum J, et al. Revision of the proposed classification criteria for juvenile idiopathic arthritis: Durban, 1997. *J Rheumatol*. 1998;25(10):1991-1994.

25. De Jager W, Hoppenreijs EP, Wulffraat NM, et al. Blood and synovial fluid cytokine signatures in patients with juvenile idiopathic arthritis: a cross-sectional study. *Ann Rheum Dis*. 2007;66(5):589-598.

26. Baron U, Floess S, Wieczorek G, et al. DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. *Eur J Immunol*. 2007;37(9):2378-2389.

27. Chauhan SK, El AJ, Ecoiffier T, et al. Autoimmunity in dry eye is due to resistance of Th17 to Treg suppression. *J Immunol*. 2009;182(3):1247-1252.

28. Stummvoll GH, DiPaolo RJ, Huter EN, et al. Th1, Th2, and Th17 effector T cell-induced autoimmune gastritis differs in pathological pattern and in susceptibility to suppression by regulatory T cells. *J Immunol*. 2008;181(3):1908-1916.

29. Yang J, Brook MO, Carvalho-Gaspar M, et al. Allograft rejection mediated by memory T cells is resistant to regulation. *Proc Natl Acad Sci U S A*. 2007;104(50):19954-19959.

30. Smolewska E, Stanczyk J, Brozik H, et al. Distribution and clinical significance of blood dendritic cells (BDC) in children with juvenile idiopathic arthritis. *Ann Rheum Dis*. 2008;67(6):762-768.

31. Ward SG, Cantrell DA. Phosphoinositide 3-kinases in T lymphocyte activation. *Curr Opin Immunol*. 2001;13(3):332-338.

32. Wohlfert EA, Clark RB. 'Vive la Resistance!'– the PI3K-Akt pathway can determine target sensitivity to regulatory T cell suppression. *Trends Immunol*. 2007;28(4):154-160.

33. Pierau M, Engelmann S, Reinhold D, et al. Protein kinase B/Akt signals impair Th17 differentiation and support natural regulatory T cell function and induced regulatory T cell formation. *J Immunol*. 2009;183(10):6124-6134.

34. Prakken B, Albani S, Martini A. Juvenile idiopathic arthritis. *Lancet*. 2011;377(9783):2138-2149.

35. Lawson CA, Brown AK, Bejarano V, et al. Early rheumatoid arthritis is associated with a deficit in the CD4+CD25high regulatory T cell population in peripheral blood. *Rheumatology (Oxford)*. 2006;45(10):1210-1217.

36. Makita S, Kanai T, Oshima S, et al. CD4+CD25bright T cells in human intestinal lamina propria as regulatory cells. *J Immunol*. 2004;173(5):3119-3130.

37. D'Alise AM, Auyeung V, Feuerer M, et al. The defect in T-cell regulation in NOD mice is an effect on the T-cell effectors. *Proc Natl Acad Sci U S A*. 2008;105(50):19857-19862.

38. Fantini MC, Rizzo A, Fina D, et al. Smad7 controls resistance of colitogenic T cells to regulatory T cell mediated suppression. *Gastroenterology*. 2009;136(4):1308-1316.

39. Monk CR, Spachidou M, Rovis F, et al. MRL/Mp CD4+,CD25- T cells show reduced sensitivity to suppression by CD4+,CD25+ regulatory T cells in vitro: a novel defect of T cell regulation in systemic lupus erythematosus. *Arthritis Rheum*. 2005;52(4):1180-1184.

40. Schneider A, Rieck M, Sanda S, et al. The effector T cells of diabetic subjects are resistant to regulation via CD4+ FOXP3+ regulatory T cells. *J Immunol*. 2008;181(10):7350-7355.

41. Venigalla RK, Tretter T, Krienke S, et al. Reduced CD4+,CD25- T cell sensitivity to the suppressive function of CD4+,CD25high,CD127low/- regulatory T cells in patients with active systemic lupus erythematosus. *Arthritis Rheum*. 2008;58(7):2120-2130.

42. You S, Belghith M, Cobbold S, et al. Autoimmune diabetes onset results from qualitative rather than quantitative age-dependent changes in pathogenic T-cells. *Diabetes*. 2005;54(5):1415-1422.

43. Wohlfert EA, Callahan MK, Clark RB. Resistance to CD4+CD25+ regulatory T cells and TGF-beta in Cbl-b-/- mice. *J Immunol*. 2004;173(2):1059-1065.

44. Cantrell D. Protein kinase B (Akt) regulation and function in T lymphocytes. *Semin Immunol*. 2002;14(1):19-26.

45. Walker LS. Regulatory T cells overturned: the effectors fight back. *Immunology*. 2009;126(4):466-474.

46. Camps M, Ruckle T, Ji H, et al. Blockade of PI3Kgamma suppresses joint inflammation and damage in mouse models of rheumatoid arthritis. *Nat Med*. 2005;11(9):936-943.

47. Barber DF, Bartolome A, Hernandez C, et al. PI3Kgamma inhibition blocks glomerulonephritis and extends lifespan in a mouse model of systemic lupus. *Nat Med*. 2005;11(9):933-935.

48. Crellin NK, Garcia RV, Levings MK. Altered activation of AKT is required for the suppressive function of human CD4+CD25+ T regulatory cells. *Blood*. 2007;109(5):2014-2022.

49. Haxhinasto S, Mathis D, Benoist C. The AKTmTOR axis regulates de novo differentiation of CD4+Foxp3+ cells. *J Exp Med*. 2008;205(3):565-574.

50. Sauer S, Bruno L, Hertweck A, et al. T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. *Proc Natl Acad Sci U S A*. 2008;105(22):7797-7802.

5

ANTI-TNFα THERAPY TARGETS PKB/C-AKT INDUCED RESISTANCE OF EFFECTOR CELLS TO SUPPRESSION IN JUVENILE IDIOPATHIC ARTHRITIS

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Submitted

ABSTRACT

OBJECTIVE

Resistance of effector T cells (Teff) to suppression contributes to disturbed immune regulation in autoimmune disease. Targeting this unresponsiveness to suppression might therefore have beneficial effects in autoimmune inflammation. In juvenile idiopathic arthritis (JIA) we have recently shown that Teff from inflamed joints are refractory to suppression, which was associated with enhanced PKB/c-akt activation in these cells. Here we investigated whether anti-IL-6 and anti-TNFα target unresponsiveness of Teff to suppression in patients with JIA.

METHODS

Resistant Teff from the inflamed joints of JIA patients were cultured in the presence of etanercept or anti-IL-6 *in vitro* and PKB/c-akt activation and responsiveness to suppression was measured. In addition, *in vivo* effects of TNFα blockade were investigated using peripheral blood samples of patient before and after start of etanercept therapy.

RESULTS

In vitro treatment of synovial fluid Teff with anti-IL-6 led to improved Treg-mediated suppression of cell proliferation in some, but not all patients. Blocking TNFα with etanercept however clearly enhanced suppression in all samples analyzed. In the presence of etanercept PKB/c-akt activation of Teff was reduced and Teff became more susceptible to TGFβ-mediated suppression, indicating that anti-TNFα directly targets resistant Teff. This was confirmed by *ex vivo* data from patients treated with etanercept demonstrating enhanced responsiveness of effector cells to suppression after therapy.

CONCLUSION

This study is the first to show resistance of Teff to suppression as a target of anti-TNFα therapy in arthritis, resulting in improved regulation of inflammatory effector cells.

INTRODUCTION

Both TNFα and IL-6 are involved in perpetuation of the inflammatory response associated with autoimmune arthritis[1] and monoclonal antibodies against TNFα, such as infliximab and adalimumab or the soluble receptor, etanercept, are now widely used in the treatment of rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA)[1;2]. In addition, efficacy of a humanized anti-IL-6 receptor antibody, tocilizumab, has recently been verified in clinical trials and is now approved for application in RA and systemic JIA[3]. Anti-TNFα and anti-IL-6 clearly have direct anti-inflammatory effects on the innate immune system. In addition, these biologicals can target the adaptive immune response, for instance by improving regulatory T cell (Treg) function[4;5] or by inducing[6;7] or expanding Treg[8].

We[9] and others[10] have recently shown that effector T cells (Teff) from the inflamed synovium of patients with JIA are refractory to suppression, resulting in impaired Treg- and TGFβ-mediated suppression of cell proliferation and cytokine production. This resistance to suppression has been described in RA and other human autoimmune diseases as well[11]. In the inflamed joints of patients with JIA, resistance to suppression correlated with the activation status of the cells[10]. More specifically, we demonstrated that the unresponsiveness to suppression resulted at least partially from PKB/c-akt hyperactivation in inflammatory Teff[9]. TNFα and IL-6 are likely candidates to induce this local resistance to suppression, since these cytokines are highly present at the site of inflammation and incubation of peripheral blood mononuclear cells (PBMC) from healthy donors with TNFα and IL-6 led to reduced responsiveness of these cells to suppression[9]. Also in a mouse model of multiple sclerosis (MS), resistance of Teff to suppression was detected locally in the inflamed central nervous system and was found to be associated with high TNFα and IL-6 production[12]. Therefore our results in JIA patients and these data from mice suggest that anti-TNFα and anti-IL-6 may directly target auto-aggressive T cells by enhancing the responsiveness of these cells to suppression.

Here, we investigated whether blocking TNFα and IL-6 influences PKB/c-akt hyperactivation and subsequent resistance of Teff to suppression in human autoimmune inflammation. We studied this not only *ex vivo* using resistant Teff from inflamed joints of JIA patients, but also in patients treated with etanercept.

PATIENTS AND METHODS

STUDY POPULATION

5 patients with oligoarticular JIA, 2 patients with extended oligoarticular JIA and 11 patients with polyarticular JIA, according to the revised criteria for JIA[13] with an average age of 11 ± 5.5 years were included in this study. Informed consent was obtained from all patients either directly or from parents/guardians when they were under 12 years of age. The study was approved by the local ethics committee. Synovial fluid (SF) was obtained from 10 patients with active disease undergoing therapeutic joint aspiration. These patients were either untreated (n = 6), or treated with non-steroidal anti-inflammatory drugs (NSAIDs) (n = 3) or methotrexate (MTX) (n = 1) at the time of inclusion. In addition, peripheral blood was drawn from 6 rheumatoid factor negative polyarticular

and 2 extended oligoarticular JIA patients before and shortly after (3.4 ± 2.3 months) start of etanercept therapy. These patients were treated with etanercept 0.8mg/kg body weight once a week. They additionally received MTX (n = 7), NSAIDs (n = 4), or low dose steroids (<0.3 mg/kg, n = 1).

CELL ISOLATION

SF Mononuclear Cells (SFMC) and PBMC were isolated using Ficoll Isopaque density gradient centrifugation (GE Healthcare Bio-Sciences, AB) and frozen in foetal calf serum (FCS) (Invitrogen) containing 10% DMSO (Sigma-Aldrich) until further experimentation.

CELL CULTURE AND REAGENTS

Mononuclear cells (2 x 10^5/200 μl or 1 x 10^5/100 μl) were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, and 10% human AB serum (all obtained from Invitrogen) at 37°C and 5% CO_2 in round-bottom 96-well plates (Nunc). Cells were stimulated with 1.5 μg/ml plate-bound anti-CD3 (clone OKT3, eBioscience). In some conditions etanercept, anti-human IL-6 (eBioscience) or tocilizumab was added at 1 μg/ml.

SUPPRESSION ASSAYS

Total SFMC or PBMC were used as effector cells. To measure proliferation cells were stained with a cell tracer violet cell proliferation kit (Invitrogen). $CD4^+CD25^+CD127^{low}$ Treg were sorted by flow cytometry on FACS Aria (BD Biosciences) and co-cultured with effector cells at a 1 to 8 ratio. In some experiments mononuclear cells were enriched for $CD4^+$ T cells before sorting using a CD4 T Lymphocyte Enrichment Set (BD Biosciences). To study TGFβ–mediated suppression, 40 ng/ml recombinant human TGFβ (Koma Biotech) was added. At day 4, proliferation of Teff was analyzed by flow cytometry and/or supernatant was collected to measure cytokine production.

PKB/C-AKT ACTIVATION ASSAY

SFMC were cultured for 3 days and the amount of phosphorylated PKB/c-akt was measured by flow cytometry.

CYTOKINE MEASUREMENT

Supernatant was collected from suppression assays and cytokine concentrations were measured by Luminex technology.

FLOW CYTOMETRY

Cells were stained by flow cytometry as previously described[9].

STATISTICAL ANALYSIS

For statistical comparison between two groups Mann Whitney test was used. To analyze paired patient samples, paired T test, or in case of unequal variances, Wilcoxon matched pairs test were used. P values below 0.05 were considered significant. Statistical analysis was performed using GraphPad Prism (GraphPad Software).

RESULTS

IN VITRO EXPOSURE TO ETANERCEPT IMPROVES TREG-MEDIATED SUPPRESSION OF SYNOVIAL FLUID TEFF

We[9] and others[10] have shown that Teff from the inflamed joints of JIA patients are resistant to Treg mediated suppression of cell proliferation and cytokine production. Furthermore, we established that TNFα and IL-6, highly present at the site of inflammation, contribute to this resistance to suppression[9]. Therefore, we questioned whether blocking TNFα and IL-6 can reduce resistance to suppression and enhance inhibition of autoimmune inflammatory effector cells. To investigate this we cultured SFMC from the inflamed joints of JIA patients and blocked TNFα or IL-6 *in vitro*. Blocking TNFα by etanercept did not alter Teff proliferation in the absence of Treg (data not shown). However, suppression of both $CD4^+$ and $CD8^+$ T cell proliferation by $CD4^+CD25^+CD127^{low}$ Treg was significantly increased (Figures 1 A and B). The short term *in vitro* TNFα blockade was not sufficient to completely alleviate resistance of inflammatory effector cells to suppression, since there was no increase in suppression of a broad range of cytokines, including IL-13, IL-17, TNFα and IFNγ in these cultures (data not shown). Blocking IL-6 also increased Treg-mediated suppression of synovial fluid Teff (Figure 1 C), but only in 6 out of 9 patients (Figure 1 D). Since blocking TNFα resulted in a profound and significant increase in Treg-mediated suppression of cell proliferation we further investigated the mechanism behind this improvement of Teff inhibition in our next set of experiments.

ETANERCEPT TARGETS PKB/C-AKT INDUCED RESISTANCE OF SYNOVIAL FLUID TEFF TO SUPPRESSION

We have previously shown that impaired control of synovial fluid Teff by Treg resulted from resistance of Teff to suppression and this unresponsiveness to suppression was at least partially caused by PKB/c-akt hyperactivation[9]. To establish that the improved suppression in the presence of etanercept resulted from reduced PKB/c-akt induced resistance to suppression, we therefore measured phosphorylated PKB/c-akt (p-PKB) as a measure of activated PKB/c-akt by flow cytometry. When SFMC were cultured in the presence of etanercept p-PKB levels in both $CD4^+$ and $CD8^+$ T cells were significantly reduced (Figures 2 A and B), indicating that *in vitro* etanercept treatment targets PKB/c-akt mediated resistance of these cells to suppression. To further confirm a direct effect on Teff we cultured SFMCs in the presence of TGFβ to measure Treg independent inhibition of cell proliferation. In correspondence with reduced p-PKB levels, an increase in TGFβ-mediated suppression of cell proliferation in the presence of etanercept was observed (Figure 2 C). In line with a significantly stronger reduction in PKB activation in $CD8^+$ T cells (0.78 ± 0.08 compared to 0.89 ± 0.05 in $CD4^+$ T cells, $p = 0.03$; figures 2 A and B), the increase in TGFβ-mediated suppression was more pronounced in $CD8^+$ T cells for which a significant increase in suppression was observed in all patients analyzed (Figure 2 C; right panel). In contrast, TGFβ-mediated suppression of $CD4^+$ T cell proliferation was increased in 5 out of 10 patients (Figure 2 C; left panel). These data indicate that TNFα blockade more effectively targets resistance of $CD8^+$ T cells to suppression; possibly because $CD8^+$ T cells contain more

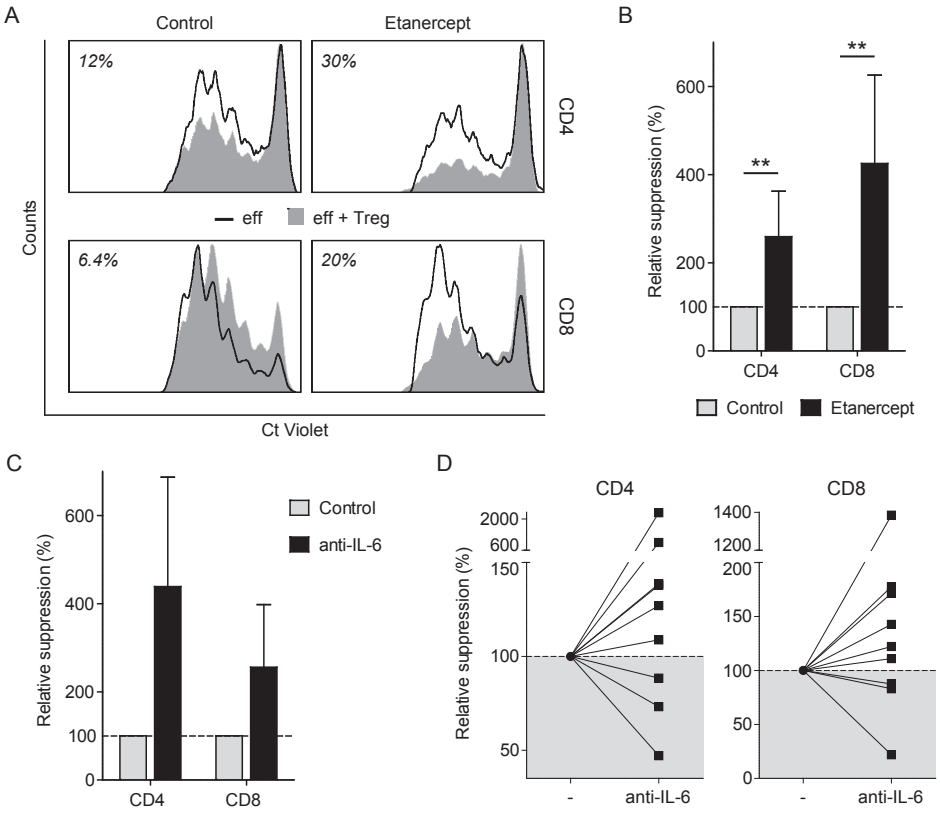

Figure 1 | *In vitro* blocking of TNFα by etanercept enhances Treg mediated suppression of synovial fluid Teff. **A-D**, SFMC were labeled with cell trace violet to measure cell proliferation and stimulated with anti-CD3 in the absence or presence of etanercept (***A*** *and* ***B***) or anti-IL-6 (***C*** *and* ***D***) for 4 days. To measure suppression of cell proliferation $CD4^+CD25^+CD127^{low}$ Treg were added. **A**, Proliferation of $CD4^+$ (*upper panel*) and $CD8^+$ T cells *(lower panel*) in the absence (*control*) or presence of etanercept with (*filled histogram*) or without Treg (*open histogram*). Percentages indicate the percentage of suppression in the presence of Treg, one representative of n = 10. **B**, Treg mediated suppression of $CD4^+$ and $CD8^+$ T cell proliferation in the presence of etanercept (*black bars*) relative to control *(gray bars*), mean ± SEM of n = 10. **C**, Treg mediated suppression of $CD4^+$ and $CD8^+$ T cell proliferation in the presence of anti-IL-6 (*black bars*) relative to control (*gray bars*), mean ± SEM of n = 9. **D**, Relative suppression $CD4^+$ (*left panel*) and $CD8^+$ T cell proliferation (*right panel*) in the presence of anti-IL-6, individual data of n = 9. **P < .01.

TNFα producing cells when analyzed directly *ex vivo* (Figure 2 D). In conclusion, *in vitro* etanercept directly targets resistance of Teff to suppression as shown by reduced PKB/c-akt activation and enhanced TGFβ-mediated suppression of these cells. We next investigated the long-term *in vivo* effects of TNFα blocking in JIA patients receiving etanercept therapy.

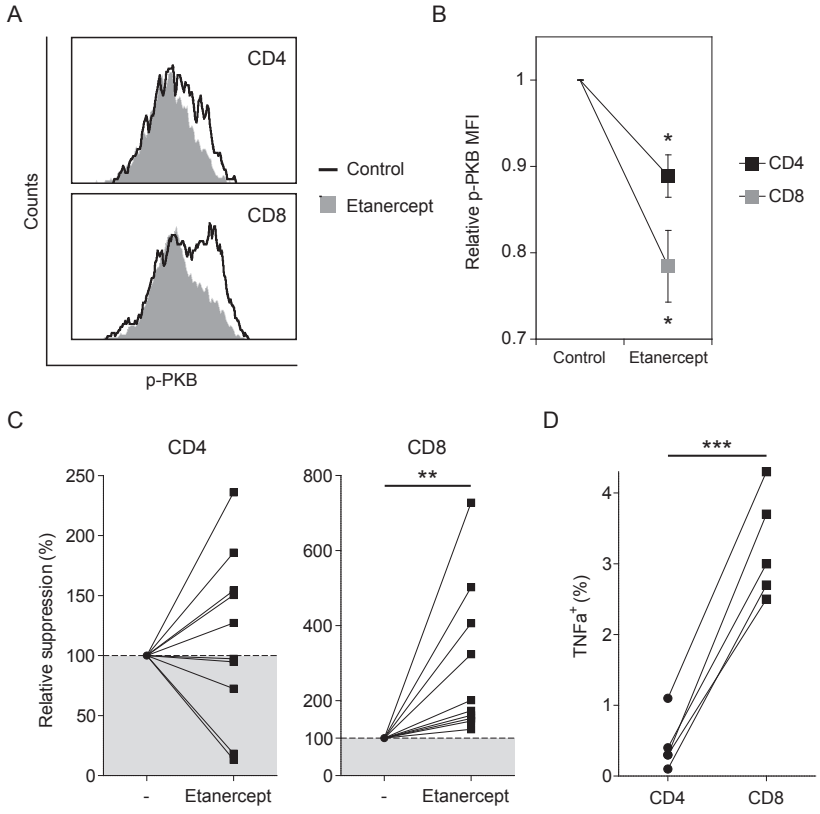

Figure 2 | Etanercept targets PKB/c-akt induced resistance to suppression *in vitro*. **A** and **B**, SFMC were stimulated with anti-CD3 in the absence or presence of etanercept for 3 days. Phosphorylated PKB/c-akt (p-PKB) was measured by flow cytometry. **A**, p-PKB staining in $CD4^+$ (*upper panel*) and $CD8^+$ T cells (*lower panel*) in the absence (*open histogram*) or presence of etanercept (*filled histogram*), one representative of n = 6. **B**, p-PKB fluorescence intensity (MFI) in $CD4^+$ (*black square*) and $CD8^+$ T cells (*gray square*) in the presence of etanercept relative to control, mean ± SEM of n = 6. **C**, SFMC were labeled with cell trace violet to measure cell proliferation and stimulated with anti-CD3 in the absence or presence of etanercept for 4 days. To measure suppression of cell proliferation TGFβ was added. Suppression of $CD4^+$ (*left panel*) and $CD8^+$ T cell proliferation (*right panel*) in the presence of etanercept relative to control, individual data of n = 10. **D**, Percentage of $TNF\alpha^+$ cells in $CD4^+$ and $CD8^+$ T cells analyzed *ex vivo* by flow cytometry, individual data of n = 5. *P < .05; **P < .01; ***P < .001.

IN VIVO ETANERCEPT THERAPY DOES NOT ALTER TREG NUMBERS OR FUNCTION, BUT TARGETS RESISTANCE OF TEFF TO SUPPRESSION

Although we have previously shown local impairment of T cell regulation in oligoarticular JIA patients, occurring only in inflamed joints[9], we hypothesized that in patients with extended oligoarticular or polyarticular JIA changes in immune regulation might be detected systemically due to a more severe and less localized pathology. Therefore, we used peripheral blood samples of extended oligoarticular and polyarticular JIA patients before and after start of etanercept

therapy to study the long-term effects of *in vivo* TNFα blockade. In line with McGovern et al.[6] we observed no change in the percentages of $CD4^+FOXP3^+$ Treg upon *in vivo* etanercept therapy (Figure 3 A; left panel). In addition, the percentage of $CD4^+FOXP3^+$ Treg expressing CTLA-4 and GITR (Figure 3 A; middle and right panel) and FOXP3, CTLA-4 and GITR expression levels per cell (MFI) (data not shown) were not different before and after treatment. In line with these results Treg function was not altered upon etanercept therapy: Treg mediated suppression of cytokine production was similar when Treg from before (Treg pre) or after treatment (Treg post) were tested in *in vitro* suppression assays (Figure 3 B). However when effector cells from before (eff pre) and after therapy (eff post) were compared, significant higher suppression of IL-13, TNFα and IFNγ was observed after treatment (Figure 3 C), clearly demonstrating enhanced responsiveness of Teff to suppression upon etanercept therapy. Thus, *in vivo* etanercept administration effectively targets resistance of Teff to suppression, resulting in enhanced suppression of cytokine production. In line with this decreased resistance of Teff to suppression, p-PKB levels were reduced in both $CD4^+$ and $CD8^+$ T cells from patients after therapy (Figure 3 D). All together, these data demonstrate that anti-TNFα therapy in JIA patients does not target Treg numbers or function, but reduces PKB mediated resistance of Teff to suppression.

DISCUSSION

We[9] and others[10] have recently shown that resistance of Teff to suppression contributes to disturbed T cell regulation in patients with JIA. In addition, this unresponsiveness of Teff to suppression has now been described in patients with RA and other autoimmune diseases as well[11], indicating that it is a general mechanism underlying autoimmune pathology. Therefore, effectively targeting this resistance to suppression could restore T cell regulation and reduce ongoing autoimmune inflammation. Here, we show that anti-IL-6, and especially anti-TNFα therapy, target resistance of Teff to suppression and thereby improve Treg mediated control of both $CD4^+$ and $CD8^+$ T cells.

Using resistant synovial fluid mononuclear cells from the inflamed joints of JIA patients we established that blocking TNFα with etanercept *in vitro* resulted in improved Treg mediated suppression of cell proliferation. Another TNFα blocking agent, adalimumab, was also shown to enhance Treg mediated suppression of SFMC proliferation *in vitro*[14]. However, we were able to show that etanercept directly targets Teff and not Treg, as TGFβ-mediated suppression of cell proliferation, independent of Treg was enhanced as well. In addition PKB/c-akt activation, previously shown to cause resistance of synovial fluid Teff to suppression[9], was reduced upon exposure to etanercept. Anti-IL-6 treatment also resulted in enhanced Treg-mediated suppression of synovial fluid Teff in line with Herrath et al.[14], but less profound effects compared to TNFα blocking were observed. This might result from a strong positive feedback loop between TNFα and PKB/c-akt signaling. Very recently it has been shown that PKB/c-akt activation positively regulates pro-inflammatory cytokine production, which was more pronounced for TNFα than for IL-6[15]. In addition, we have shown that TNFα induces PKB/c-akt activation[9]. Thus, a positive feedback loop exists in which PKB/c-act induces TNFα

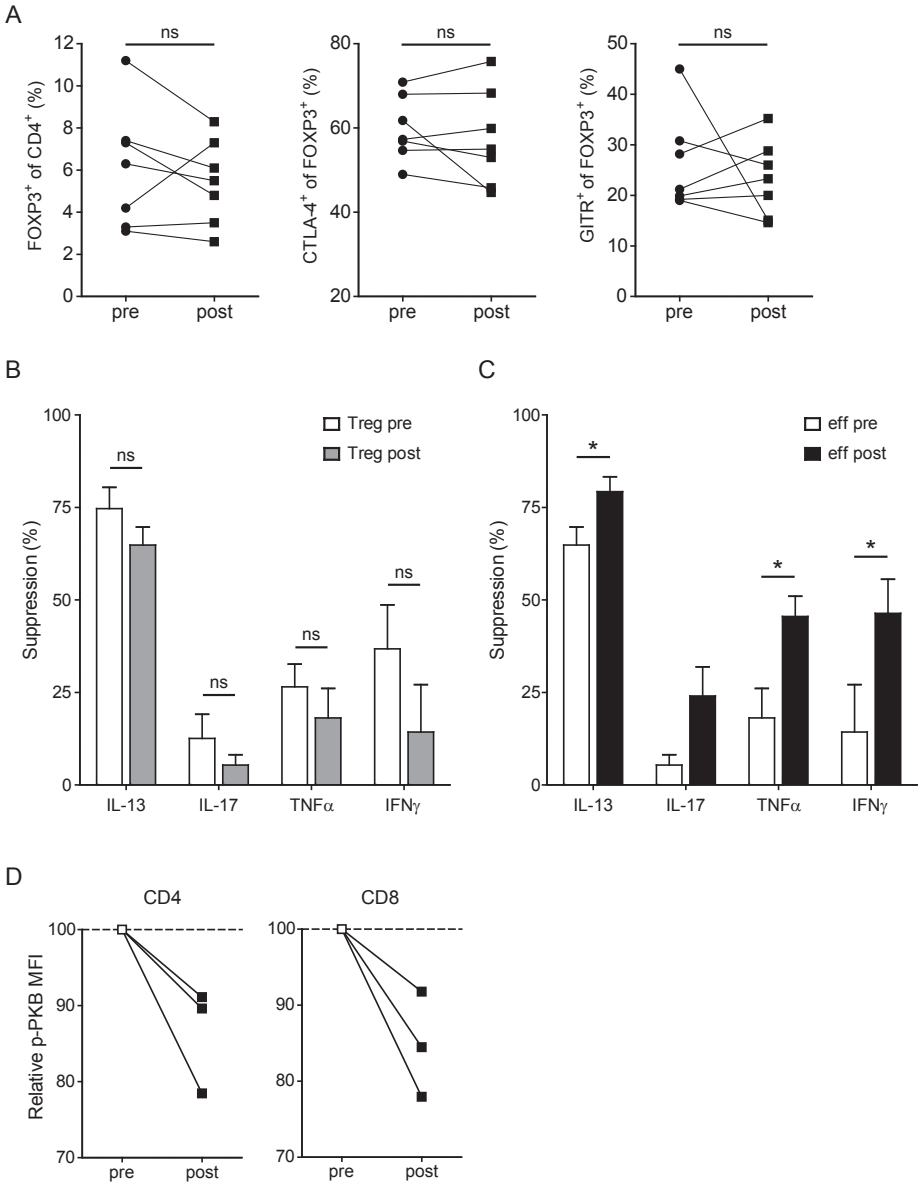

Figure 3 | *In vivo* etanercept treatment targets resistance of Teff to suppression. **A-D**, PBMC were isolated from JIA patients before and after start of etanercept therapy. **A**, Percentage FOXP3$^+$ cells within CD4$^+$ T cells (*left panel*); CTLA-4$^+$ (*middle panel*) and GITR$^+$ cells (*right panel*) within CD4$^+$FOXP3$^+$ T cells measured *ex vivo* by flow cytometry, individual data of n = 7. **B-C**, CD4$^+$CD25$^+$CD127^{low} Treg were added to PBMC effector cells stimulated with anti-CD3 and at day 4 cytokine production was measured. **B**, Suppression of cytokines by Treg isolated from before (*Treg pre; white bars*, n = 6) or after therapy *(Treg post; gray bars,* n =3), mean ± SEM. **C**, Suppression of cytokines in the presence of effector cells from before (*eff pre; white bars,* n = 3) or after therapy (*eff post; black bars,* n = 6), mean ± SEM. **D**, Relative p-PKB MFI after (post) compared to before therapy (pre) in CD4$^+$ (*left panel*) and CD8$^+$ T cells (*right panel*) measured *ex vivo* by flow cytometry, individual data of n = 3. *P <. 05.

and vice versa. Blocking TNFα will target and interrupt this positive feedback loop, explaining the profound effect of etanercept on resistance to suppression.

Interestingly, etanercept treatment appeared to more effectively targeting resistance of $CD8^+$ T cells to suppression compared to $CD4^+$ T cells. This was not likely due to increased responsiveness of $CD8^+$ T cells to TNFα, since TNF receptor (TNFR)II expression was higher on $CD4^+$ T cells and TNFRI expression was low on both $CD4^+$ and $CD8^+$ T cells (data not shown). However, a higher number of TNFα producing cells was detected within the $CD8^+$ T cell population, which might explain the more extensive effects of etanercept on these cells.

We were able to confirm our *in vitro* results with data from patients receiving etanercept therapy *in vivo*. We show that upon etanercept treatment Treg numbers and function do not change, in line with a recent publication by McGovern et al.[6]. However, we extended these findings by showing that etanercept reduces resistance of Teff to suppression and thereby improves T cell regulation without targeting Treg.

In conclusion, our data identify a novel mechanism of action of anti-TNFα, etanercept, therapy, namely restoring responsiveness of Teff to suppression by inhibiting their PKB/c-akt activation status. This is probably not a long lasting effect, since patients relapse after discontinuation of therapy. Therefore, more research into what determines long-lasting remission is required and the data described in this study might aid to that search. In addition, our results provide new insight in the pathology of autoimmune disease as they again stress the importance of hyperactivated Teff in disturbed immune regulation during autoimmune inflammation.

REFERENCES

1. Prakken B, Albani S, Martini A. Juvenile idiopathic arthritis. Lancet 2011;377:2138-49.
2. Chimenti MS, Graceffa D, Perricone R. Anti-TNFalpha discontinuation in rheumatoid and psoriatic arthritis: is it possible after disease remission? Autoimmun Rev 2011;10:636-40.
3. Tanaka T, Kishimoto T. Immunotherapeutic implication of IL-6 blockade. Immunotherapy 2012;4:87-105.
4. Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA et al. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. J Exp Med 2004;200:277-85.
5. Valencia X, Stephens G, Goldbach-Mansky R, Wilson M, Shevach EM, Lipsky PE. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. Blood 2006;108:253-61.
6. McGovern JL, Nguyen DX, Notley CA, Mauri C, Isenberg DA, Ehrenstein MR. Th17 cells are restrained by regulatory T cells from patients responding to anti-TNF antibody therapy via inhibition of IL-6. Arthritis Rheum 2012;64:3129-38.
7. Nadkarni S, Mauri C, Ehrenstein MR. Anti-TNF-alpha therapy induces a distinct regulatory T cell population in patients with rheumatoid arthritis via TGF-beta. J Exp Med 2007;204:33-39.
8. Goldstein I, Ben-Horin S, Koltakov A, Chermoshnuk H, Polevoy V, Berkun Y et al. Alpha1beta1 Integrin+ and regulatory Foxp3+ T cells constitute two functionally distinct human CD4+ T cell subsets oppositely modulated by TNFalpha blockade. J Immunol 2007;178:201-10.
9. Wehrens EJ, Mijnheer G, Duurland CL, Klein M, Meerding J, van Loosdregt, J et al. Functional human regulatory T cells fail to control autoimmune inflammation due to PKB/c-akt hyperactivation in effector cells. Blood 2011;118:3538-48.
10. Haufe S, Schepp C, Kuemmerle-Deschner J, Hansmann S, Rieber N, Tzaribachev N et al. Impaired suppression of synovial fluid CD4(+) CD25(-) T cells from patients with juvenile idiopathic arthritis by CD4(+) CD25(+) regulatory T cells. Arthritis Rheum 2011;63:3153-62.
11. Wehrens EJ, Prakken BJ, Van Wijk F. T cells out of control-impaired immune regulation in the inflamed joint. Nat Rev Rheumatol 2012;18:34-42.
12. Korn T, Reddy J, Gao W, Bettelli E, Awasthi A, Petersen TR et al. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. Nat Med 2007;13:423-31.
13. Petty RE, Southwood TR, Baum J, Bhettay E, Glass DN, Manners P et al. Revision of the proposed classification criteria for juvenile idiopathic arthritis: Durban, 1997. J Rheumatol 1998;25:1991-94.
14. Herrath J, Muller M, Amoudruz P, Janson P, Michaelsson J, Larsson PT et al. The inflammatory milieu in the rheumatic joint reduces regulatory T-cell function. Eur J Immunol 2011;41:2279-90.
15. Cheng J, Phong B, Wilson DC, Hirsch R, Kane LP. Akt fine-tunes NF-kappaB-dependent gene expression during T cell activation. J Biol Chem 2011;286:36076-85.

6

INFLAMMATORY MONOCYTES INDUCE RESISTANCE OF EFFECTOR T CELLS TO SUPPRESSION

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ABSTRACT

Ever since their discovery research has focused on whether deficiencies in FOXP3$^+$ regulatory T cells (Treg) underlie human autoimmune pathology. Recently also Treg extrinsic factors have been identified as a cause of regulatory defects in autoimmune disease. More specifically, resistance of effector T cells (Teff) to suppression has been shown to contribute to disturbed immune regulation in autoimmune inflammation. Therefore, targeting this unresponsiveness to suppression could be a promising treatment option for patients with autoimmune disease. To effectively target resistance of Teff to suppression, however, more information on how this resistance is induced and maintained is required. Recently, different subpopulations of human antigen presenting cells (APCs) have been described, but their presence and function at the site of autoimmune inflammation remains unclear. Here, we characterized APCs present at the site of inflammation in patients with juvenile idiopathic arthritis (JIA) and investigated their role in inducing Teff resistance to suppression. We observed a clear difference in the composition of APCs in synovial fluid (SF) obtained from inflamed joints compared to peripheral blood (PB). Moreover, SF monocytes displayed strong proinflammatory characteristics with especially high TNFα and IL-6 production directly *ex vivo*. Upon co-culture with Teff, these SF monocytes and not SF dendritic cells (DCs) induced unresponsiveness of Teff to suppression, resulting in impaired Treg mediated control of cell proliferation and cytokine production. These data shed new light on the role of monocytes in autoimmune pathology, indicating that monocytes actively contribute to the ongoing inflammation by interfering with T cell regulation. Moreover, our results identify inflammatory monocytes and their ability to induce resistance to suppression as a new target to treat autoimmune inflammation.

INTRODUCTION

Given their central role in maintaining self tolerance intensive research over the years has focused on whether deficiencies in FOXP3$^+$ regulatory T cells (Treg) underlie human autoimmune pathology. However, Treg numbers and function are often not declined in patients with autoimmune disease, but extrinsic factors at the site of inflammation can limit their suppressive outcome[1]. Specifically data from mouse models and patients with autoimmune disease now point towards resistance of effector T cells (Teff) to suppression, instead of malfunctioning of Treg, as an important mechanism of immune dysregulation in autoimmunity[2]. Also in patients with juvenile idiopathic arthritis (JIA) we[3] and others[4] have shown that Teff from inflamed joints are resistant to Treg mediated suppression. This resistance to suppression is T cells intrinsic, as it is maintained *ex vivo* without the presence of antigen presenting cells (APCs) from the site of inflammation. Furthermore, we showed that specific PKB/c-akt hyperactivation in inflammatory Teff caused their unresponsiveness to suppression. However, induction of resistance to suppression is still incompletely understood and might involve local APCs present at the site of autoimmune inflammation.

In both RA[5;6] and JIA[3;7] monocytes and dendritic cells (DCs) from inflamed joints have a more activated phenotype compared to their peripheral blood (PB) counterparts. In addition, *in vitro* experiments have indicated that highly activated APCs can interfere with T cell regulation[5;8]. However, *ex vivo* data on the role of inflammatory monocytes and DCs in the disturbed Treg/Teff balance in patients with autoimmune disease are still missing. Recently, human PB monocyte and DC subpopulations have been thoroughly characterized by use of cell surface markers and genetic profiling[9;10]. Although APC subset nomenclature is still evolving, at present 3 subsets of human monocytes, as well as 2 types of conventional DCs (cDCs) and plasmacytoid DCs (pDCs) are being recognized[11]. The presence and function of these newly characterized APCs at the site of inflammation in patients with autoimmune disease has yet to be determined.

Here, we not only characterized the recently described subsets of APCs in inflamed joints of JIA patients, but also *ex vivo* analyzed the role of these cells in inducing Teff resistance to suppression. We observed clear differences in the composition of monocytes and DCs in synovial fluid (SF) compared to PB. Importantly, we show that monocytes from the site of autoimmune inflammation, and not DCs, induce T cell resistance to suppression, leading to impaired Treg-mediated control of Teff.

METHODS

PATIENTS

15 patients with JIA (10 with oligoarticular JIA, 2 with extended oligoarticular JIA and 3 with polyarticular JIA) according to the revised criteria for JIA[12], were included in this study. All patients had active disease and underwent therapeutic joint aspiration at the time of inclusion. Patients were between 2 and 18 years of age and were either untreated or treated

with non steroidal anti inflammatory drugs (NSAIDs), methotrexate (MTX) or both. Informed consent was received from parents/guardians or from participants directly when they were over 12 years of age. The study procedures were approved by the Institutional Review Board of the University Medical Center Utrecht (UMCU) and performed according to the principles expressed in the Helsinki Declaration.

CELL ISOLATION

Synovial fluid (SF) was collected during therapeutic joint aspiration and at the same time blood was drawn via veni puncture. Synovial fluid mononuclear cells (SFMC) and PB mononuclear cells (PBMC) were isolated using Ficoll Isopaque density gradient centrifugation (GE Healthcare Bio-Sciences, AB) and were used either directly, or frozen in FCS (Invitrogen) containing 10% DMSO (Sigma-Aldrich) until further experimentation.

CELL CULTURE CONDITIONS

Cells were cultured in RPMI 1640 supplemented with 2mM L-glutamine, 100 U/ml penicillin-streptomycin, and 10% human AB serum (all obtained from Invitrogen) in round-bottom 96-well plates (Nunc). Cells were stimulated with 1.5 μg/ml plate-bound anti-CD3 (clone OKT3 (eBioscience)) and cultured at 37°C and 5% CO2.

SUPPRESSION ASSAYS WITH SF AND PB APCs

To study suppression of PB T cells in the presence of PB- or SF-derived APCs, $CD4^+CD25^+CD127^{low}$ Treg, $CD3^+$ T cells, and $CD3^-$ APCs were sorted by flow cytometry on FACS Aria (BD Biosciences). PB $CD3^+$ T cells were labeled with 2μM CFSE (Invitrogen) for 7 minutes at 37°C and extensively washed before used in suppression assays. 100.000 $CD3^+$ T cells were co-cultured with 100.000 $CD3^-$ APCs in the presence of SF Treg at a 1 to 4 and 1 to 8 ratio in 200 μl culture volume. To control for cell density Teff instead of Treg were added at a 1 to 4 ratio. At day 4, proliferation of T cells was analyzed by flow cytometry.

SUPPRESSION ASSAYS WITH SF MONOCYTES AND DCs

To study suppression of PB T cells in the presence of SF-derived monocytes and DCs, $CD4^+CD25^+CD127^{low}$ Treg, $CD3^+$ T cells, $CD11c^+CD14^+$ monocytes and $CD11c^+CD14^-$ DCs were sorted by flow cytometry on FACS Aria (BD Biosciences). PB $CD3^+$ T cells were labeled with 2μM cell trace (Invitrogen) for 7 minutes at 37°C and extensively washed before used in suppression assays. 70.000 $CD3^+$ T cells were co-cultured with 30.000 SF-derived monocytes or DCs in the presence of SF Treg at a 1 to 4 and 1 to 8 ratio in 100 μl culture volume. To control for cell density Teff instead of Treg were added at a 1 to 4 ratio. At day 4, proliferation of T cells was analyzed by flow cytometry and supernatant was collected to measure cytokine production.

CYTOKINE MEASUREMENT

To measure cytokine production by SF-derived monocytes and dendritic cells, $CD11c^+CD14^+$ monocytes and $CD11c^+CD14^-$ DCs were sorted by flow cytometry and 90.000 cells were cultured

in 100 µl culture volume. Cells were either untreated or stimulated with LPS (100 ng/ml (Sigma-Aldrich)) or Poly(I:C) (30 µg/ml (Sigma-Aldrich)). After overnight culture supernatant was collected and stored at -80°C until analysis. To measure Treg mediated suppression of cytokine production, supernatant was collected from suppression assays and stored at -80°C until analysis. Cytokine concentrations were measured by Luminex technology as previously described[13].

FLOW CYTOMETRY

To detect intracellular cytokine production, cells were cultured in the absence or presence of LPS (1 µg/ml (Sigma-Aldrich)) for 4 hours with Golgistop (1/1500 (BD Biosciences)) added for the last 3 hours of culture. Before staining, cells were washed twice in FACS buffer (PBS containing 2% FCS (Invitrogen) and 0.1% sodium azide (Sigma-Aldrich)). To prevent aspecific binding to Fc receptors cells were incubated with purified human FcR binding inhibitor (eBioscience). After this blocking step cells were washed twice in FACS buffer and incubated with surface antibodies. Upon surface staining cells were washed again in FACS buffer and acquired directly, or fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences) followed by intracellular cytokine staining. Cells were acquired on FACSCanto II and analyzed using FACS Diva Version 6.13 software (all BD Biosciences).

STATISTICAL ANALYSIS

For statistical comparison between two groups T test, or in case of unequal variances, Mann Whitney test was used. To analyze paired patient samples, paired T test, or in case of unequal variances, Wilcoxon matched pairs test were used. P values below 0.05 were considered significant. Statistical analysis was performed using GraphPad Prism Version 5.03 (GraphPad Software).

RESULTS

APCs FROM THE SITE OF AUTOIMMUNE INFLAMMATION INDUCE RESISTANCE TO SUPPRESSION

We have previously shown that T cells isolated from SF of patients with JIA are resistant to suppression and that APCs from inflamed joints are not required to maintain this resistant phenotype *ex vivo*[3]. However, we hypothesized that local APCs present in inflamed joints may still play a role in the induction of T cell resistance to regulation. To investigate this, paired patients samples were used and PB T cells were cultured in the presence of PB or SF APCs from the same patient. To analyze T cell responsiveness to suppression, FACS sorted $CD4^+CD25^+CD127^{low}$ Treg were added and inhibition of T cell proliferation was measured. As a control for cell density, in some wells additional Teff instead of Treg were added (+Teff), which did not result in evident suppression. However, in the presence of Treg at either a 1 to 8 (1:8) or a higher 1 to 4 ratio (1:4) proliferation of both $CD4^+$ (Figure 1 A) and $CD8^+$ T cells (Figure 1 B) was clearly inhibited in co-cultures with PB APCs (black bars). In contrast, in the presence of SF APCs (gray bars) suppression of T cell proliferation was much lower, indicating that in the presence of SF APCs T cells become less responsive to suppression. Thus, our *ex vivo* data suggest that APCs present at the site of autoimmune inflammation are able to induce T cell resistance to suppression.

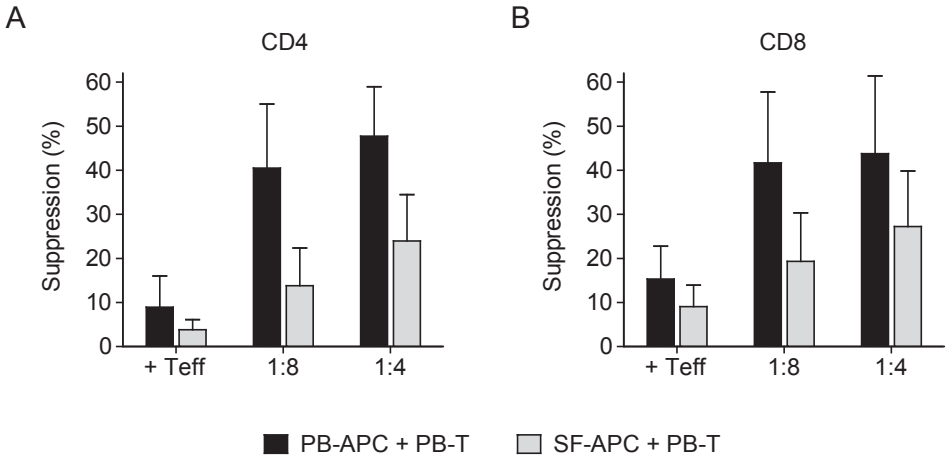


Figure 1 | SF APCs induce T cell resistance to suppression. **A-B**, PB $CD3^+$ T cells, PB and SF $CD3^-$ APC and SF $CD4^+CD25^+CD127^{low}$ Treg were sorted by flow cytometry. T cells and APCs were co-cultured in the absence or presence of Treg and at day 4 proliferation of CFSE labeled T cells was analysed. Suppression of $CD4^+$ T cell (***A***) and $CD8^+$ T cell proliferation (***B***) in the presence of PB (*black bars*) or SF derived APCs (*gray bars*) and Treg at a 1 to 8 (1:8) and 1 to 4 (1:4) ratio, or additional Teff at a 1 to 4 ratio (+Teff). The results show percentage of suppression in the presence of Treg or additional Teff relative to T cells cultured alone, mean ± SEM of n = 4.

DIFFERENT COMPOSITION OF APCs IN INFLAMED JOINTS COMPARED TO PB

Since our data indicated that APCs in SF are able to induce T cell resistance to suppression, we next aimed to identify the APC subset responsible. Therefore, we characterized APCs in SF compared to PB by FACS analysis. In contrast to PB, percentages of B cells in SF were very low (Supplementary Figure 1), suggesting that B cells do not play a major role in resistance to suppression observed in the presence of SF APCs. To further identify the cell type responsible, we therefore phenotypically analyzed the remaining Lin^-($CD3^-CD19^-CD56^-$) $HLA\text{-}DR^+$ population according to previously described classifications[11] (Figure 2 A). In general, SF appeared to contain more Lin^- $HLA\text{-}DR^+$ APCs compared to PB (Figure 2B). When further analyzing these SF APCs we detected both $CD14^+$ monocytes as well as a large population of $CD14^-$ DCs (Figures 2 A and C). These SF $CD14^-$ DCs consisted of both $CD1c^+$ cDCs as well as $CD123^+$ pDCs (Figures 2 A and D). However, the main population of DCs present in SF were $CD141^+$ cDCs (Figures 2 A and D). In contrast, in PB the percentage of $CD14^-$ DCs was lower and the majority of Lin^- $HLA\text{-}DR^+$ cells consisted of $CD14^+$ monocytes (Figure 2 C). Furthermore, the subtypes of DCs present in PB were different from SF. In PB $CD1c^+$ cDCs and $CD123^+$ pDCs were the main DC populations present, whereas $CD141^+$ cDCs that are highly present in SF were only marginally detected in PB (Figure 2 D). Together, these *ex vivo* data demonstrate clear differences in the number and subtypes of APCs present in SF compared to PB and reveal a unique composition of DCs at the site of autoimmune inflammation with high percentages of $CD141^+$ cDCs. Given this distinction in DC subsets and the observation that DCs have superior T cell activating capacity compared to other APC subsets[14], it is tempting to speculate that DCs are responsible for the induction of

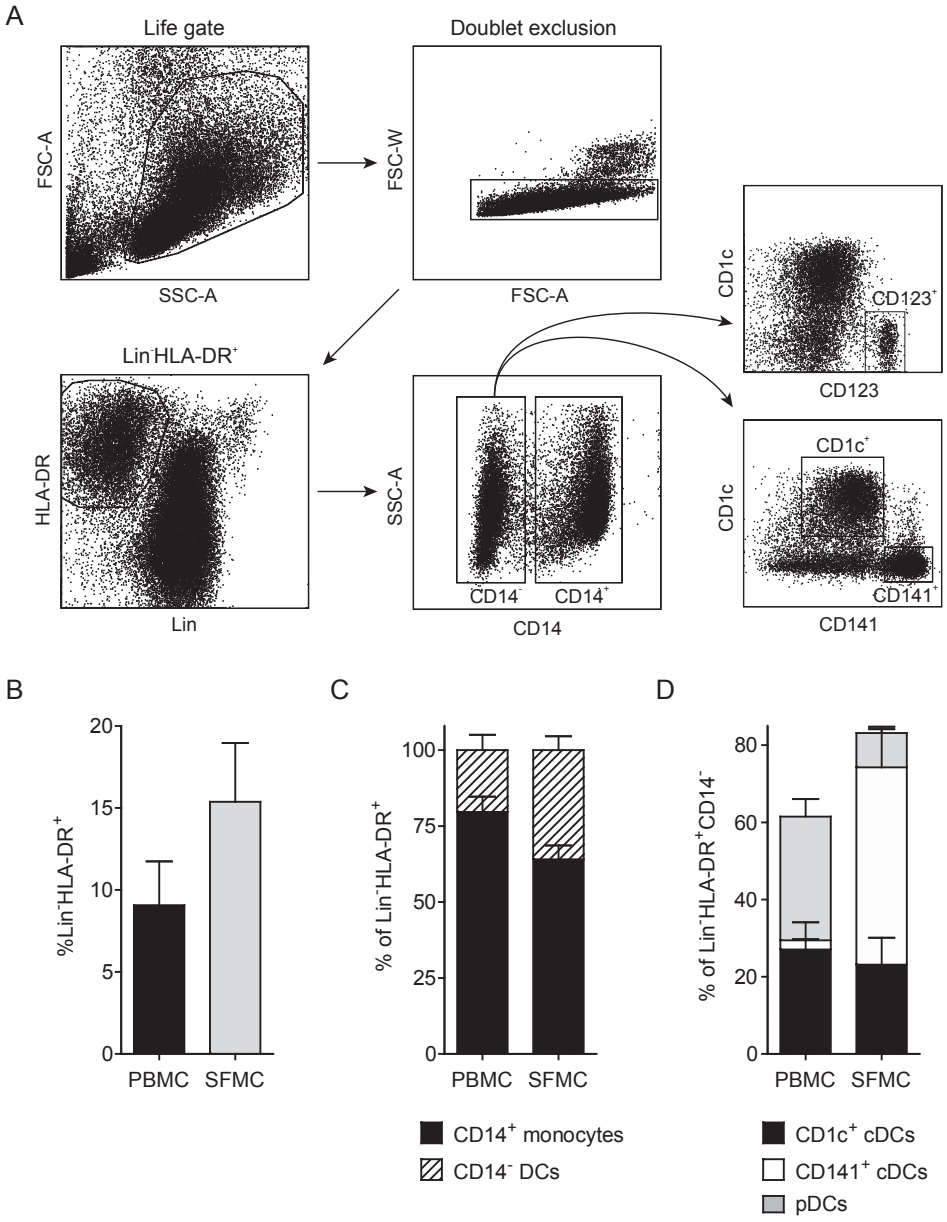


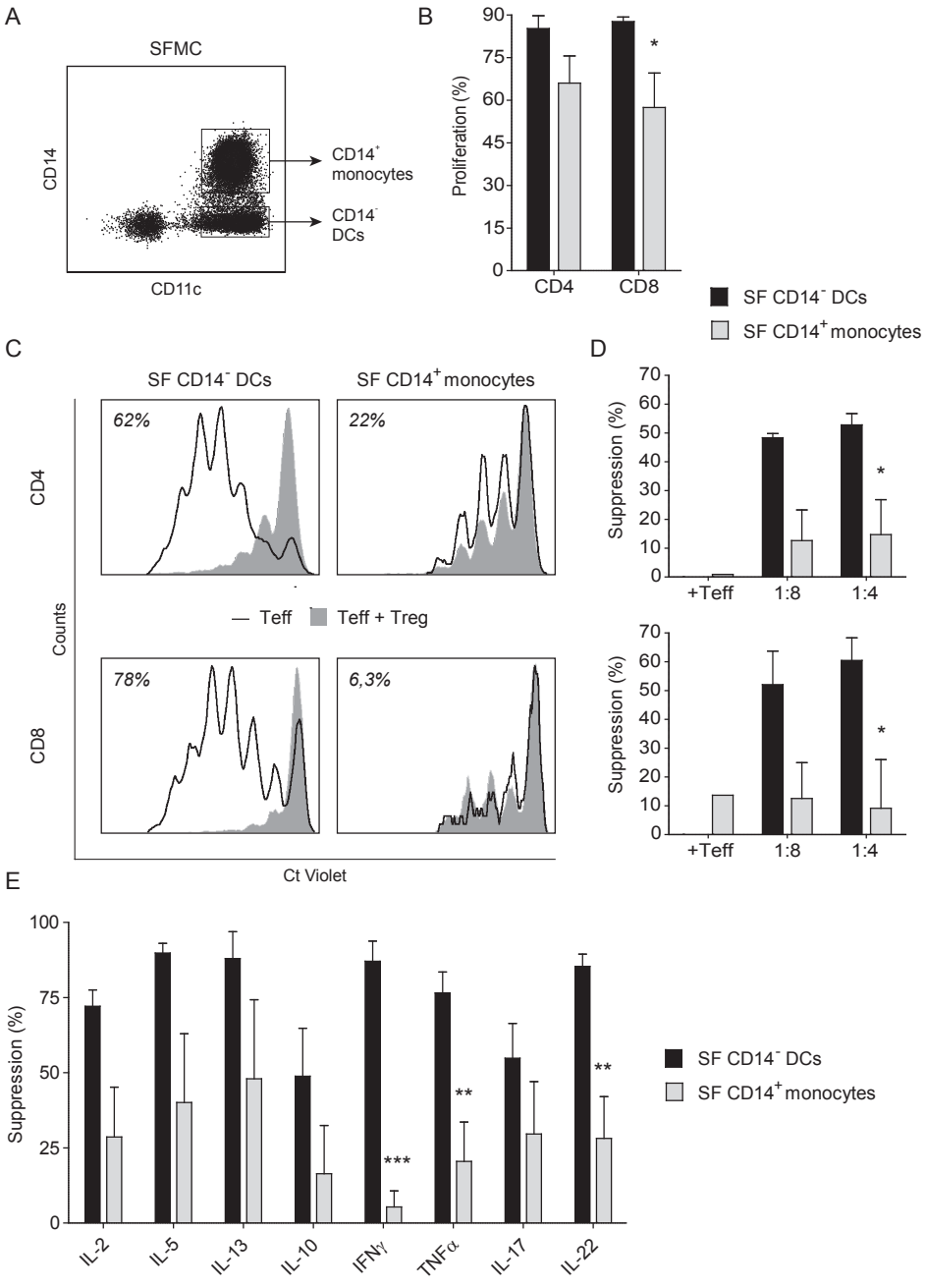
Figure 2 | Different composition of APCs in SF compared to PB. **A-D**, Paired PBMC and SFMC were analysed *ex vivo* by flow cytometry. **A**, Gating strategy used to analyze $CD14^-$ monocytes and $CD14^+$ DC subsets in SF compared to PB. **B**, Percentage total $Lin^-HLA\text{-}DR^+$ APCs in PBMC (*black bar*) and SFMC (*gray bar*), mean ± SEM of n = 5. **C**, Percentage $CD14^+$ monocytes (*black bars*) and $CD14^-$ DCs (*hatched bars*) of $Lin^-HLA\text{-}DR^+$ PBMC and SFMC, mean ± SEM of n = 5. **D**, Percentage $CD1c^+$ cDCs (*black bars*), $CD141^+$ cDCs (*white bars*) and pDCs (*gray bars*) of $Lin^-HLA\text{-}DR^+CD14^-$ PBMC and SFMC, mean ± SEM of n = 2.

resistance to suppression observed in the presence of SF APCs. However, monocytes from SF of both RA[5] and JIA patients[3] have a highly activated phenotype with enhanced expression of costimulatory molecules. As a result, also these cells could induce profound T cell activation, which might lead to reduced responsiveness to Treg mediated suppression[5;15]. Therefore we identified both DCs and monocytes from the site of inflammation in JIA that may have the capacity to induce resistance to suppression and we next performed suppression assays with sorted DCs and monocytes from SF to further investigate this.

MONOCYTES FROM THE SITE OF INFLAMMATION INDUCE T CELL RESISTANCE TO SUPPRESSION

The distribution and activation status of DCs and monocytes differs in SF compared to PB and both types of APCs could play a role in inducing resistance to suppression. To investigate whether SF DCs and/or monocytes are able to induce resistance to suppression, we therefore sorted these two main APC populations present in SF based on CD14 expression (Figure 3 A) and co-cultured them with PB T cells from the same patient. We first analyzed proliferation of PB T cells in the presence of these different subsets of SF APCs. Both $CD4^+$ and $CD8^+$ T cells proliferated more in the presence of SF DCs (black bars) compared to monocytes (gray bars) (Figure 3 B), in line with a potent antigen presenting capacity described for DCs[14]. To investigate responsiveness of Teff to suppression, FACS sorted $CD4^+CD25^+CD127^{low}$ Treg were added to the same APC and T cell co-cultures. Although $CD4^+$ and $CD8^+$ T cells highly proliferated in the presence of SF DCs (Figures 3 B and C; left panel, open histogram), this proliferation was still efficiently suppressed by the presence of Treg (Figure 3 C; left panel, filled histogram). In contrast, in the presence of SF monocytes, Treg mediated suppression of both $CD4^+$ and $CD8^+$ T cell proliferation was almost completely abrogated (Figure 3 C; right panel). This severely reduced suppression of cell proliferation in the presence of SF monocytes was detected at both a 1 to 8 and a 1 to 4 ratio of Treg added to effector cells (Figure 3 D). Furthermore, suppression of a broad range of cytokines measured in the culture supernatant was highly decreased in the presence of SF monocytes (Figure 3 E). In conclusion, although SF DCs induce high levels of T cell activation, Treg mediated suppression of cell proliferation and cytokine production remains intact. In contrast, monocytes

Figure 3 | SF monocytes induce T cell resistance to suppression. **A-E**, PB $CD3^+$ T cells, SF $CD11c^+CD14^+$ monocytes, SF $CD11c^+CD14^-$ DCs and SF $CD4^+CD25^+CD127^{low}$ Treg were sorted by flow cytometry. T cells and APCs were co-cultured in the absence or presence of Treg. At day 4 proliferation of cell trace labeled T cells was analysed and supernatant was collected to analyze cytokine production. **A**, Gating strategy applied to sort $CD11c^+CD14^+$ monocytes and $CD11c^+CD14^-$ DCs from SF. **B**, Proliferation of $CD4^+$ and $CD8^+$ T cells in the presence of SF $CD14^-$ DCs (*black bars*) or SF $CD14^+$ monocytes (*gray bars*), mean ± SEM of n = 4, *P < .05. **C**, Proliferation profile of $CD4^+$ (*upper panel*) and $CD8^+$ T cells (*lower panel*) upon co-culture with SF $CD14^-$ DCs (*left panel*) or SF $CD14^+$ monocytes (*right panel*) in the absence (*open histogram*) or presence of Treg at a 1 to 4 ratio (*filled histogram*), one representative of n = 4. **D**, Suppression of $CD4^+$ T cell *(upper panel*) and $CD8^+$ T cell proliferation (*lower panel*) in the presence of SF $CD14^-$ DCs (*black bars*) or SF $CD14^+$ monocytes (*gray bars*) and Treg at a 1 to 8 (1:8) and 1 to 4 ratio (1:4), or additional Teff at a 1 to 4 ratio (+Teff). The results show percentage of suppression in the presence of Treg or additional Teff relative to T cells cultured alone, mean ± SEM of n = 4, *P < .05. **E**, Suppression of cytokine production in the presence of SF $CD14^-$ DCs (*black bars*) or SF $CD14^+$ monocytes (*gray bars*) and Treg added at a 1 to 4 ratio. The results show percentage of suppression in the presence of Treg relative to T cells cultured alone, mean ± SEM of n = 4, **P < .01, ***P < .001. ►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22%
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CD8
Counts
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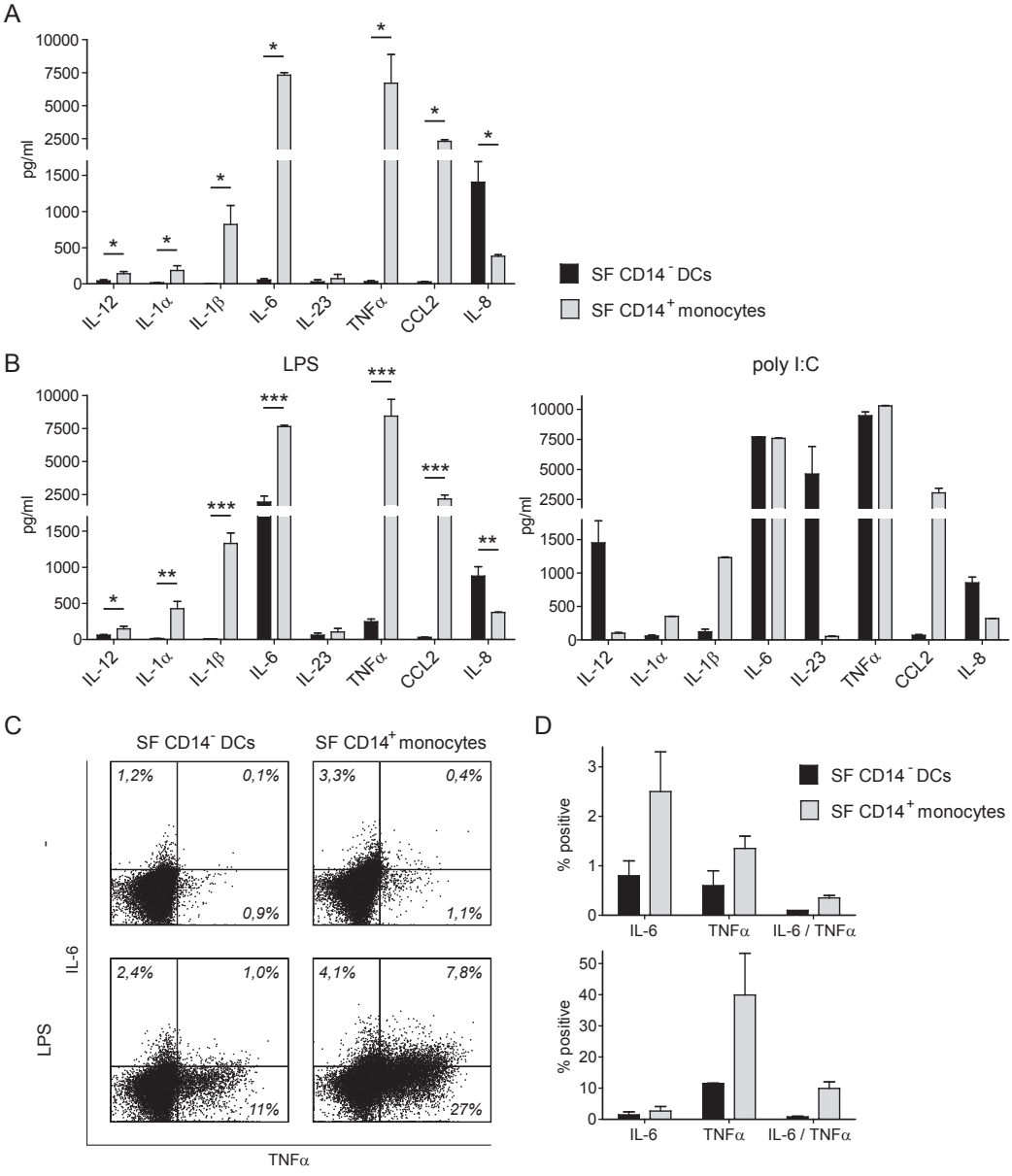
Suppression (%)
+Teff
1:8
1:4
E
Suppression (%)
IL-2
IL-5
IL-13
IL-10
IFNγ
TNFα
IL-17
IL-22
SF CD14- DCs
SF CD14+ monocytes

from the site of inflammation reduce responsiveness of Teff to suppression leading to severely impaired suppression of both cell proliferation and cytokine production. Thus, monocytes from the site of autoimmune inflammation, and not DCs, induce T cell resistance to suppression.

SF MONOCYTES HAVE A PROINFLAMMATORY PHENOTYPE WITH HIGH TNFα AND IL-6 EXPRESSION

Since our results showed that monocytes from the site of autoimmune inflammation are able to induce resistance to suppression, we further phenotypically and functionally characterized these cells *ex vivo*. First we analyzed cytokine production in overnight cultures after sorting SF monocytes and DCs as described in Figure 3 A. Whereas IL-8 production was higher in DC cultures, monocytes produced high levels of a broad range of proinflammatory cytokines, including IL-1α, IL-1β, IL-6, TNFα and CCL-2 (Figures 4 A en B). Interestingly, production of these cytokines by SF monocytes was high even in the absence of any stimulus, especially for TNFα and IL-6 (Figure 4 A). In contrast, DCs produced TNFα and IL-6 only after *in vitro* stimulation, upon exposure to LPS and especially Poly(I:C) (Figure 4 B). We confirmed this increased IL-6 and TNFα production by SF monocytes compared to DCs by intracellular cytokine staining both *ex vivo* and upon LPS stimulation (Figures 4 C and D). Together, these data indicate that SF monocytes have strong proinflammatory capacities by producing high levels of proinflammatory cytokines, such as TNFα and IL-6.

To investigate whether these functional data correlate with a more proinflammatory phenotype, we next characterized the different subsets of human monocytes in SF compared to PB. Initially human monocytes were categorized into $CD14^{++}CD16^{-}$ classical monocytes and a $CD16^{+}$ subset that was considered proinflammatory based on its proinflammatory cytokine production[16;17] and the observation that these cells are expanded under inflammatory conditions[17;18], including RA[17;19]. However, more recently two separate populations within the $CD16^{+}$ subset have been identified; a nonclassical $CD14^{+}CD16^{++}$ population and a $CD14^{++}CD16^{+}$ intermediate subset[9;11]. This latter subset was found to be responsible for the increase in $CD16^{+}$ monocytes observed in PB of RA patients[20;21]. However, data on the presence of these different monocyte subsets at the actual the site of inflammation; the inflamed joint, are still missing. Therefore, we characterized the different subsets of human monocytes; $CD14^{++}CD16^{-}$ classical monocytes, $CD14^{+}CD16^{++}$ nonclassical monocytes and $CD14^{++}CD16^{+}$ intermediate monocytes in SF compared to PB (Figure 4 E). We observed a significant decrease in the amount of $CD14^{++}CD16^{-}$ classical monocytes in SF compared to PB, which was accompanied by a specific increase in the $CD14^{++}CD16^{+}$ intermediate population (Figure 4 F). Thus, SF monocytes have a more proinflammatory phenotype compared to their PB counterparts. Together our data show that monocytes from the site of autoimmune inflammation display strong proinflammatory characteristics reflected phenotypically by high CD16 expression as well as functionally by spontaneous production of proinflammatory cytokines. In contrast DCs isolated from inflamed joints do not produce large quantities of proinflammatory cytokines directly *ex vivo*.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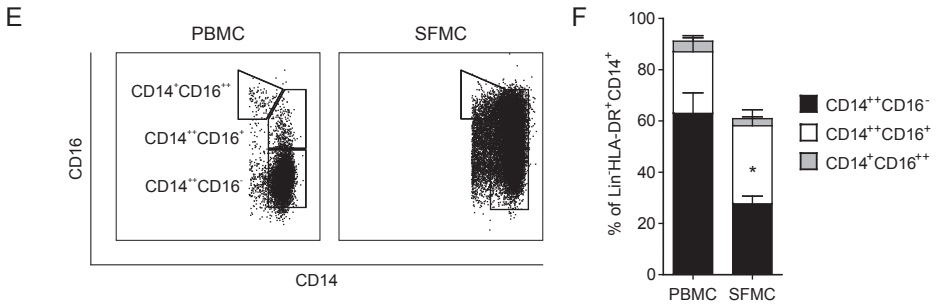


Figure 4 | SF monocytes display proinflammatory phenotypical and functional characteristics. **A-B**, SF $CD11c^+CD14^+$ monocytes and $CD11c^+CD14^-$ DCs were sorted by flow cytometry. **A**, Cytokine production in overnight cultures of SF $CD11c^+CD14^-$ DCs (*black bars*) or SF $CD14^+$ monocytes (*grey bars*), mean ± SEM of n = 4, *P < .05. **B**, Cytokine production in overnight cultures of SF $CD11c^+CD14^-$ DCs (*black bars*) or SF $CD14^+$ monocytes (*grey bars*) upon stimulation with LPS (*left panel*), mean ± SEM of n = 6, *P < .05, **P < .01, ***P < .001, or Poly(I:C) (*right panel*), mean ± SEM of n = 2. **C-D**, Paired PBMC and SFMC were cultured 4 hours in the absence (*upper panel*) or presence of LPS (*lower panel*) and TNFα and IL-6 expression was measured by intracellular cytokine staining. **C**, TNFα and IL-6 expression in SF $CD14^-$ DCs (*left panel*) or $CD14^+$ monocytes (*right panel*), one representative of n = 2. **D**, Percentage of IL-6^+, TNFα^+ and IL-6^+TNFα^+ SF $CD14^-$ DCs (*black bars*) or SF $CD14^+$ monocytes (*grey bars*), mean ± SEM of n = 2. **E-F**, PBMC and SFMC were analysed *ex vivo* by flow cytometry. **E**, Distribution of $CD14^{++}CD16^-$, $CD14^{++}CD16^+$ and $CD14^+CD16^{++}$ monocytes in paired PBMC and SFMC, one representative of n= 4. **F**, Percentage $CD14^{++}CD16^-$ (*black bars*), $CD14^{++}CD16^+$ (*white bars*) and $CD14^+CD16^{++}$ (*grey bars*) of $Lin^-HLA^-DR^+CD14^+$ PBMC and SFMC, mean ± SEM of n = 5, *P < .05.

DISCUSSION

Because of their profound anti-inflammatory properties Treg are considered for therapeutic application in patients with autoimmune disease[22]. More recently, also Treg extrinsic factors have been identified as a cause of regulatory defects in human autoimmune disease and especially resistance of Teff to suppression contributes to disturbed immune regulation in autoimmune inflammation[2]. For a Treg based strategy to be successful, this resistance to suppression should therefore be targeted as well. As a result, more information on how Teff resistance to suppression is induced and maintained is required. *Ex vivo* data from patients with autoimmune disease, especially from the site of inflammation, are thereby indispensable. In this study, using mononuclear cells from inflamed joints of JIA patients, we were able to show that local APCs present at the site of autoimmune inflammation induce T cell resistance to suppression. Moreover, we established that proinflammatory monocytes, and not DCs, are responsible for this induction of resistance to suppression.

We are the first to demonstrate with *ex vivo* patient material that APCs present in inflamed joints interfere with T cell regulation. These data are in line with *in vitro* experiments indicating that highly activated APCs can interfere with Treg-mediated suppression[5;8]. In addition, in systemic lupus erythematosus (SLE) it has been shown that APCs from patients with active disease can impair Treg-mediated inhibition of Teff[23]. However, in these experiments total T cell depleted PBMC were used as APCs and therefore no conclusion could be drawn on the subtype

of APC responsible for interfering with suppression. In contrast, we characterized APC subsets present at the site of autoimmune inflammation and were able to show that SF monocytes and not DCs are able to induce unresponsiveness to suppression. Besides phenotypic data showing that SF monocytes have an enhanced activation status[3;5] and a role for these cells in promoting Th17 cells[24] these are the first functional data showing that monocytes from inflamed joints interfere with T cell regulation in arthritis.

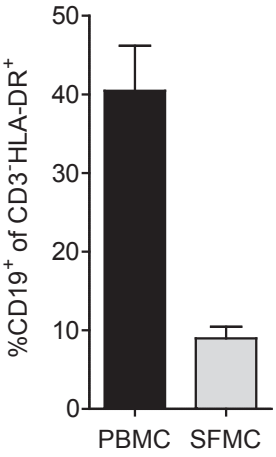
We further characterized SF monocytes and observed that a large proportion of these cells belong to the $CD14^{++}CD16^{+}$ intermediate subset. This finding correlates with a study in RA reporting an increased percentage of monocytes expressing CD16 in SF compared to PB[19]. However, in this study no distinction between different subsets of $CD16^{+}$ monocytes was made, since heterogeneity of the $CD16^{+}$ population has only recently been recognized[9;11]. At present two populations within the $CD16^{+}$ monocyte population are being identified: $CD14^{+}CD16^{++}$ nonclassical monocytes and a $CD14^{++}CD16^{+}$ intermediate subset[9;11]. This latter population of monocytes has been found to be expanded under many inflammatory conditions[9], including in PB of RA patients[20;21]. However, until now it has been unclear whether these cells actively contribute to the ongoing inflammation or just expand secondary to inflammatory stimuli. The high proportion of $CD14^{++}CD16^{+}$ intermediate monocytes in SF suggests that this $CD16^{+}$ subset specifically contribute to induction of resistance to suppression. We have some preliminary data supporting this hypothesis, since $CD14^{+}CD16^{++}$ nonclassical monocytes isolated from inflamed joints failed to induce resistance to suppression (data not shown). These data suggest that $CD14^{++}CD16^{+}$ intermediate monocytes have an active role in chronic inflammation by interfering with T cell regulation. Interestingly, Rossol et al. showed that $CD14^{++}CD16^{+}$ monocytes specifically expand Th17 cells[21] and these cells have been known to be less responsive to suppression compared to other T helper subsets[25-28]. However our *ex vivo* data revealing impaired suppression of a broad range of cytokines in the presence of SF monocytes argue against specific expansion of Th17 cells as a mechanism behind the observed resistance to suppression.

Proinflammatory cytokines might play a role in the capacity of SF monocytes to induce resistance to suppression. We detected extensive production of proinflammatory cytokines in overnight cultures of SF monocytes, whereas cytokine production by SF DCs, which failed to induce resistance to suppression, was low. Moreover, in contrast to what has been reported for monocytes from healthy controls[29;30], production of proinflammatory cytokines by SF monocytes was high even in the absence of any stimulus, especially for TNFα and IL-6. We have previously shown that incubation of PBMC from healthy donors with TNFα and IL-6 leads to reduced responsiveness of these cells to suppression[3]. In addition, in a mouse model for multiple sclerosis (MS) Teff from the inflamed central nervous system were found to be refractory to suppression and this resistance to suppression was associated with high TNFα and IL-6 production[31]. Finally, murine *in vitro* experiments have shown that activated APCs can render Teff unresponsive to suppression, partially dependent on IL-6[8]. Therefore, TNFα and IL-6 are likely candidates to play a role in the mechanism by which SF monocytes induce resistance to suppression.

In conclusion, our data show that monocytes from the site of autoimmune inflammation are able to induce Teff resistance to suppression, leading to impaired Treg mediated control of T cell

proliferation and cytokine production. These data shed new light on the importance of monocytes in autoimmune pathology. In addition, these results have clinical implications as they point towards monocytes and their ability to induce resistance to suppression as a new target to treat autoimmune inflammation. Addressing these inflammatory monocytes might create a window of opportunity for a Treg based therapy to be successful in patients with autoimmune disease.

SUPPLEMENTARY INFORMATION



Supplementary Figure 1 | Low levels of B cells in SF compared to PB. Paired PBMC and SFMC were analysed *ex vivo* by flow cytometry. Percentage of $CD19^+$ B cells in $CD3^-HLA\text{-}DR^+$ PBMC (*black bar*) and SFMC (*gray bar*), mean ± SEM of n = 3.

REFERENCES

1. Buckner JH. Mechanisms of impaired regulation by CD4(+)CD25(+)FOXP3(+) regulatory T cells in human autoimmune diseases. Nat.Rev. Immunol. 2010;10:849-859.
2. Wehrens EJ, Prakken BJ, Van Wijk F. T cells out of control-impaired immune regulation in the inflamed joint. Nat.Rev.Rheumatol. 2012;18:34-42.
3. Wehrens EJ, Mijnheer G, Duurland CL et al. Functional human regulatory T cells fail to control autoimmune inflammation due to PKB/c-akt hyperactivation in effector cells. Blood 2011;118:3538-3548.
4. Haufe S, Schepp C, Kuemmerle-Deschner J et al. Impaired suppression of synovial fluid CD4(+) CD25(-) T cells from patients with juvenile idiopathic arthritis by CD4(+) CD25(+) regulatory T cells. Arthritis Rheum. 2011;63:3153-3162.
5. Van Amelsfort JM, Van Roon JA, Noordegraaf M et al. Proinflammatory mediator-induced reversal of CD4+,CD25+ regulatory T cell-mediated suppression in rheumatoid arthritis. Arthritis Rheum. 2007;56:732-742.
6. Cavanagh LL, Boyce A, Smith L et al. Rheumatoid arthritis synovium contains plasmacytoid dendritic cells. Arthritis Res. Ther. 2005;7:R230-R240.
7. Smolewska E, Stanczyk J, Brozik H et al. Distribution and clinical significance of blood dendritic cells (BDC) in children with juvenile idiopathic arthritis. Ann.Rheum.Dis. 2008;67:762-768.
8. Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science 2003;299:1033-1036.
9. Wong KL, Tai JJ, Wong WC et al. Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. Blood 2011;118:e16-e31.
10. Zawada AM, Rogacev KS, Rotter B et al. SuperSAGE evidence for CD14++CD16+ monocytes as a third monocyte subset. Blood 2011;118:e50-e61.
11. Ziegler-Heitbrock L, Ancuta P, Crowe S et al. Nomenclature of monocytes and dendritic cells in blood. Blood 2010;116:e74-e80.
12. Petty RE, Southwood TR, Baum J et al. Revision of the proposed classification criteria for juvenile idiopathic arthritis: Durban, 1997. J.Rheumatol. 1998;25:1991-1994.
13. De Jager W, Hoppenreijs EP, Wulffraat NM et al. Blood and synovial fluid cytokine signatures in patients with juvenile idiopathic arthritis: a cross-sectional study. Ann.Rheum.Dis. 2007;66:589-598.
14. Lipscomb MF, Masten BJ. Dendritic cells: immune regulators in health and disease. Physiol Rev. 2002;82:97-130.
15. Baecher-Allan C, Viglietta V, Hafler DA. Inhibition of human CD4(+)CD25(+high) regulatory T cell function. J.Immunol. 2002;169:6210-6217.
16. Belge KU, Dayyani F, Horelt A et al. The proinflammatory CD14+CD16+DR++ monocytes are a major source of TNF. J.Immunol. 2002;168:3536-3542.
17. Ziegler-Heitbrock L. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. J.Leukoc.Biol. 2007;81:584-592.
18. Fingerle G, Pforte A, Passlick B et al. The novel subset of CD14+/CD16+ blood monocytes is expanded in sepsis patients. Blood 1993;82:3170-3176.
19. Kawanaka N, Yamamura M, Aita T et al. CD14+,CD16+ blood monocytes and joint inflammation in rheumatoid arthritis. Arthritis Rheum. 2002;46:2578-2586.
20. Cooper DL, Martin SG, Robinson JI et al. FcgammaRIIIa Expression on Monocytes in Rheumatoid Arthritis: Role in Immune-Complex Stimulated TNF Production and Non-Response to Methotrexate Therapy. PLoS.One. 2012;7:e28918.
21. Rossol M, Kraus S, Pierer M, Baerwald C, Wagner U. The CD14(bright) CD16+ monocyte subset is expanded in rheumatoid arthritis and promotes Th17 expansion. Arthritis Rheum. 2012;64:671-677.
22. Bluestone JA. Regulatory T-cell therapy: is it ready for the clinic? Nat.Rev.Immunol. 2005;5:343-349.
23. Yan B, Ye S, Chen G et al. Dysfunctional CD4+,CD25+ regulatory T cells in untreated active systemic lupus erythematosus secondary to interferon-alpha-producing antigen-presenting cells. Arthritis Rheum. 2008;58:801-812.
24. Evans HG, Gullick NJ, Kelly S et al. In vivo activated monocytes from the site of inflammation in humans specifically promote Th17 responses. Proc.Natl.Acad.Sci.U.S.A 2009;106:6232-6237.
25. Annunziato, F. Phenotypic and functional features of human Th17 cells. J.Exp.Med. 2007;204:1849-1861.

26. Chauhan SK, El AJ, Ecoiffier T et al. Autoimmunity in dry eye is due to resistance of Th17 to Treg suppression. J.Immunol. 2009;182:1247-1252.

27. O'Connor RA, Taams LS, Anderton SM. Translational mini-review series on Th17 cells: CD4 T helper cells: functional plasticity and differential sensitivity to regulatory T cell-mediated regulation. Clin.Exp.Immunol. 2010;159:137-147.

28. Stummvoll GH, DiPaolo RJ, Huter EN et al. Th1, Th2, and Th17 effector T cell-induced autoimmune gastritis differs in pathological pattern and in susceptibility to suppression by regulatory T cells. J.Immunol. 2008;181:1908-1916.

29. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. Nat.Immunol. 2007;8:942-949.

30. Walter GJ, Evans HG, Menon B et al.. Interaction with activated monocytes enhances cytokine expression and suppressive activity of human $CD4^+CD45RO^+CD25^+CD127^{low}$ regulatory T cells. Arthritis Rheum. 2012, *in press*

31. Korn T, Reddy J, Gao W et al. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. Nat. Med. 2007;13:423-431.

7

T CELLS OUT OF CONTROL: IMPAIRED IMMUNE REGULATION IN THE INFLAMED JOINT

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ABSTRACT

Since the discovery of FOXP3$^+$ regulatory T cells (Treg) over 15 years ago, intensive research has focused on their presence, phenotype and function in autoimmune disease. Whether deficiencies in Treg underlie autoimmune pathology and whether, or how, therapeutic approaches based on these cells might be successful is still the subject of debate. The potential role of Treg extrinsic factors, such as proinflammatory cytokines and resistance of effector T cells (Teff) to suppression, as the cause of regulatory defects in chronic autoimmune inflammation is an intensive area of research. It is now clear that, at the site of inflammation, antigen presenting cells (APCs) and proinflammatory cytokines drive Teff skewing and plasticity, and that these T cells can become unresponsive to regulation. In addition, expansion and function of Treg is affected by the inflammatory environment; indeed, new data suggest that, in certain conditions, Treg promote inflammation. This Review summarizes the latest findings on changes in Teff homeostasis in autoimmune disease and focuses on how mechanisms that normally regulate these cells are affected in the inflamed joints of patients with arthritis. These findings have important clinical implications and will affect the development of new therapeutic strategies for autoimmune arthritis.

INTRODUCTION

Our understanding of autoimmune diseases, including rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA), has been broadened by the identification of new key players in autoimmune inflammation. The discovery of $CD25^+FOXP3^+$ regulatory T cells (Treg)[1], capable of suppressing T cell activation, proliferation and effector function, opened an exciting new area of immunological research. $FOXP3^+$ Treg originate from the thymus and are therefore commonly referred to as natural Treg; they can, however, also be induced in the periphery from naive T cells in the presence of transforming growth factor β (TGFβ) and are then termed induced Treg (Figure 1, part 1)[2]. Because of their suppressive function, Treg are crucial in maintaining self-tolerance and preventing autoimmune responses[2]. As a result, their discovery stimulated extensive research into whether deficiencies in the number or function of Treg underlie human autoimmune disease, such as RA[3;4]. Accumulating data are now showing, however, that it is not just a question of insufficient or nonfunctional Treg in the perpetuation of autoimmune arthritis. The proinflammatory environment in affected joints can also interfere with T cell regulation[5]. In particular, findings in 2011 indicated that resistance of effector T cells (Teff) to suppression has an important role in synovial inflammation[6;7]. In addition, insights into T cell plasticity, especially in inflamed joints[8;9], have extended our understanding of autoimmune pathology. Here we summarize our current knowledge on how T cells lose control in autoimmune inflammation. We discuss how proinflammatory mediators can induce T cell plasticity and interfere with T cell regulation, especially in the inflamed joints of patients with arthritis. We also focus on how highly activated T cells influence antigen presenting cells (APCs) by modifying their infiltration and differentiation. Finally, we consider how these ineffective regulatory mechanisms might contribute to the chronicity of inflammation, which should be kept in mind when developing new therapies for autoimmune arthritis.

T CELL SUBSETS IN AUTOIMMUNE ARTHRITIS

In RA, the synovium becomes infiltrated by multiple types of immune cells, including granulocytes, monocytes and/or macrophages, B cells and high levels of $CD4^+$ and $CD8^+$ T cells, which are mostly activated memory cells, leading to the production of high levels of proinflammatory cytokines[10;11]. Genetic association studies have generated evidence for an important role of T cells, particularly $CD4^+$ T cells, in RA pathogenesis. For example, HLA-DR1 and HLA-DR4, which are involved in antigen presentation and T cell selection, have been associated with the development of RA[10]. In addition, in two spontaneous mouse models of arthritis, adoptive transfer of T cells from diseased mice induced disease in recipient mice[11;12]. Classically, type 1 T helper (Th1) cells, which produce IFNγ, were thought to drive RA pathology. Since the discovery of type 17 T helper (Th17) cells (characterized by the production of IL-17), however, this concept of disease has been revised as these cells seem to be even more important in promoting autoimmune disease[11;13]. In RA, IL-17 promotes synovial inflammation by enhancing the influx of inflammatory cells, such as neutrophils, and is a major contributing factor to bone and cartilage damage[10;14].

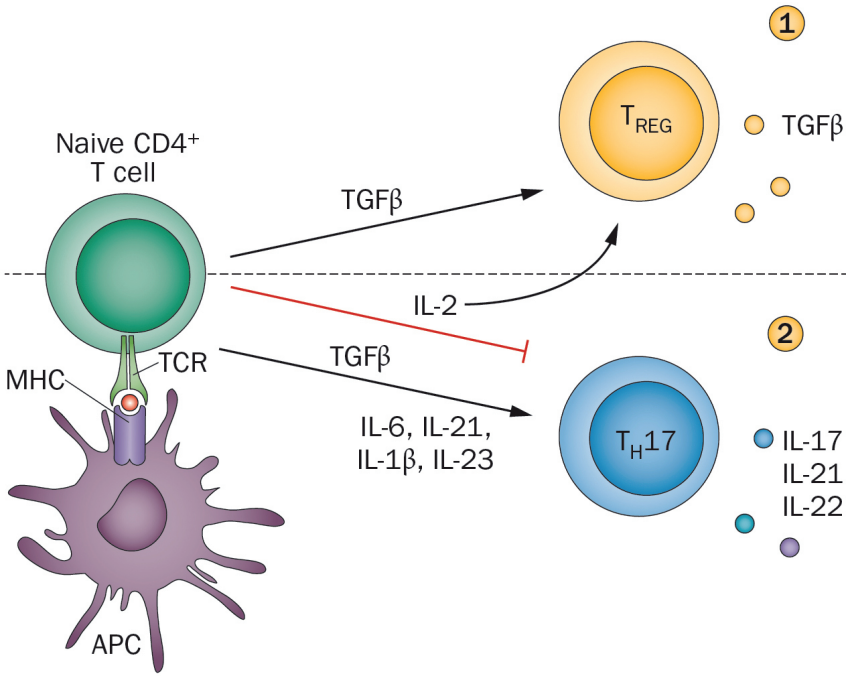

Figure 1 | Reciprocal development of peripherally induced Treg and Th17 cells. Under specific circumstances, including high levels of TGFβ, Treg can be induced in the periphery from naive cells upon interaction with APCs (***1***). Development of Th17 cells is also dependent on TGFβ and proinflammatory cytokines, such as IL-6 and IL-21, whereas it is inhibited by IL-2 (***2***). Therefore, Treg can promote the development of Th17 cells in a proinflammatory environment by producing TGFβ and consuming IL-2.

SYNOVIAL FLUID

The relative contribution and commitment of $CD4^+$ T cell subsets in RA pathology was investigated using epigenetic immune lineage analysis[15]. Epigenetic information is not encoded by changes in DNA sequence but by differential methylation of the DNA and chromatin modification, which results in heritable but plastic modifications. These epigenetic processes have been shown to be key determinants in T helper (Th) cell differentiation and stability[16]. By analyzing DNA methylation levels of several key genes in Th cell differentiation, Janson and colleagues established that demethylation of the *Ifnγ* locus is significantly enhanced in $CD4^+$ T cells isolated from the synovial fluid of patients with RA[15], which is indicative of enhanced skewing towards the Th1 lineage. In line with this finding, $CD4^+$ T cells in the synovial fluid were found to predominantly produce IFNγ in response to type II collagen, one of the main constituents of articular cartilage[17]. Interestingly, both studies also reported Th17 cell responses at the site of inflammation, although this phenotype was less pronounced than Th1 skewing. Moreover, in patients with JIA, higher levels of IFNγ than IL-17 were observed and Th17 cells were found to co-produce IFNγ in inflamed joints[8;9;18]. However, as described above, Th17 cells are key players

in joint pathology and their lower levels in the inflamed joint compared with Th1 cells might result from limited expansion of these cells at the inflammatory site[19].

In addition to *Ifnγ* demethylation, Janson *et al.*[15] also observed increased demethylation of the *Foxp3* locus in synovial fluid CD4$^+$ T cells. Thus, in addition to Th1 skewing, an enhanced commitment towards the Treg lineage is also observed. This observation is in line with studies showing that FOXP3$^+$ Treg are enriched in the synovial fluid of patients with RA and JIA: these studies also confirm the high levels of demethylation of the *Foxp3* Treg specific demethylated region (TSDR), suggesting stable FOXP3 expression[5;6]. Enhanced expression of the antigen Ki67 (a marker of cell proliferation) in these cells[5] (and Wehrens *et al.* unpublished observation) further suggest that Treg are expanding locally in the inflamed joints. Although little is known about the role of CD8$^+$ T cells in joint pathology, high levels of these cells are present in synovial fluid, mainly with an effector memory phenotype, and produce marked amounts of proinflammatory cytokines, such as tumour necrosis factor α (TNFα) and IFNγ[6,20]. Furthermore, in patients with JIA high levels of synovial fluid CD8$^+$ T cells are correlated with a more progressive course of disease than is observed in patients with lower levels of these cells[21].

SPECIFIC T CELL SKEWING IN INFLAMED JOINTS

Notably, in the peripheral blood of patients with RA, the level of demethylation of *Ifnγ* and *Il-17a* is the same as in healthy controls[15]. Thus, skewed CD4$^+$ T cell commitment occurs specifically at the site of inflammation, likely as a result of an interaction between CD4$^+$ T cells and local APCs, which are found in increased numbers in inflamed joints[22–28]. Moreover, synovial fluid monocytes[22;24;26] and dendritic cells (DCs)[23;25–28] have a more activated phenotype with enhanced expression of the maturation markers CD40, CD80, CD86 and HLA-DR than those isolated from peripheral blood. Upon interaction with CD4$^+$ T cells these monocytes derived from synovial fluid specifically induce Th17 responses, in contrast to monocytes isolated from peripheral blood[22]. In experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis, DCs promote maintenance of Th17 cells at the site of inflammation via production of IL-23[29]. In addition, the acute phase protein serum amyloid A, which is highly present during inflammation, induces Treg proliferation without abrogating their suppressive function in a monocyte-dependent manner[30]. Thus, in an inflammatory environment, monocytes may also induce expansion of Treg, potentially explaining the high levels of proliferating Treg observed in the inflamed joints of patients with arthritis[5,6]. In conclusion, Treg, Th1 and Th17 cells are key T cell subsets in joint inflammation. Although it is unlikely that priming of naive T cells takes place at the site of inflammation, T cell expansion, differentiation and effector functions are probably further promoted following interactions with local activated APCs (Figure 2).

TREG IN AUTOIMMUNE INFLAMMATION

Given their central role in maintaining self-tolerance and suppressing inflammation, research has focused on the clinical application of Treg in patients with autoimmune disease, including RA[31]. However, whether Treg are actually deficient in these patients remains controversial[31;32]. Indeed, as described above, high levels of Treg are present in the inflamed joints of patients with arthritis and numerous publications have shown that synovial fluid Treg are functional

in vitro[6;7;33–36]. Nevertheless, the proinflammatory environment might interfere with their effectiveness at the site of inflammation. For example, highly activated APCs in inflamed joints might reduce Treg function, as strong and prolonged T cell stimulation through the concomitant stimulation of the T cell receptor and CD28 signaling pathways can impair Treg-mediated suppression[24;37;38]. In addition, in an experimental model of systemic lupus erythematosus (SLE)[39] and in patients with SLE[40], APCs were found to impair Treg-mediated inhibition through production of the proinflammatory cytokines IL-6 and IFNα, respectively.

TNFα

Research over the past 8 years has particularly focused on the effects of TNFα on Treg function, probably because its receptor (TNF receptor II (TNFRII)) is preferentially expressed on the surface of Treg in both mice and humans[41–43]. Extensive data on the effect of TNFα on Treg function have now been retrieved from both human and experimental studies, with partially conflicting results. Indeed, a direct TNFRII-mediated impairment of human Treg function has been described *in vitro*[43–45] and, from the reverse perspective, inhibition of TNFα improves Treg-mediated suppression both *in vitro*[5] and in patients treated with infliximab[43;45;46]. Interestingly, a novel Treg population with enhanced suppressive capacity seems to be induced in patients treated with either infliximab[45;47] or adalumimab[48], although cytotoxic T-lymphocyte protein 4 (CTLA-4) expression by Treg remains unaffected[49]. By contrast, data from mouse models revealed that TNFα can actually boost Treg expansion[50;51] and, as such, protect mice from subsequent induction of autoimmune disease[51]. TNFα might, therefore, have different effects on Treg - reducing their suppressive function - but, at the same time, promoting their expansion. The latter could be another mechanism behind the high levels of proliferating Treg observed in the inflamed joints of patients with arthritis[5;6]. However, the relationship between TNFα levels and Treg number still remains a conundrum: in humans TNFα has a negative effect on Treg expansion *in vitro*[52] and, in the inflamed joints of patients with arthritis, a negative correlation between TNFα levels and the percentage of $FOXP3^+$ T cells was observed[5]. Furthermore, in mice, TNFα was found to increase Treg suppressive capacity[50;51], which contradicts with the data obtained from humans[5;43;46]. Thus, mouse and human Treg might respond differently to TNFα and so far all human data point towards a negative effect of TNFα on Treg function. In our opinion, the effect of TNFα is species specific: TNFα induced expansion of Treg only occurs in mice, whereas, in humans, TNFα negatively affects both Treg expansion and function. Other cytokines, such as IL-2, IL-7 and IL-15 have also been described to interfere with human Treg function[24;35]. Thus, in the inflamed joint of patients with arthritis, proinflammatory cytokines, in particular TNFα, and highly activated APCs are thought to interfere with Treg function (Figure 3, part 1).

OTHER INHIBITORY PATHWAYS

The proinflammatory synovial environment may also hinder other pathways of T cell regulation. For instance, the programmed cell death protein 1 (PD-1) is up-regulated on synovial fluid T cells, but these cells display impaired responsiveness to PD-1-mediated restriction of proliferation and cytokine production. This phenotype can be mimicked *in vitro* by culturing cells from

healthy donors in the presence of synovial fluid[53], suggesting that soluble proinflammatory mediators have a role in impaired responsiveness to PD-1 signaling. In addition, Hidalgo *et al*.[54] showed that CD130, which is involved in IL-6 signaling, is not down-regulated in synovial tissue, presumably owing to the local presence of IL-10. The specific cytokine environment therefore suppresses negative feedback mechanisms in the inflamed synovium, allowing for continuous IL-6 signaling and unresponsiveness to PD-1-mediated inhibition[54]. All together, these data implicate that T-cell regulation, and specifically Treg function, are diverted by the proinflammatory environment in the inflamed joint.

TEFF RESISTANCE TO SUPPRESSION

In addition to the impairment of Treg function described above, resistance of Teff to suppression also markedly contributes to uncontrolled inflammation. In 2003, *in vitro* mouse experiments showed that activated APCs could render Teff unresponsive to suppression by a mechanism dependent, in part, on IL-6[55]. The phenomenon of Teff becoming refractory to suppression was additionally suggested *in vivo* in EAE[56;57]. Treg isolated from the site of inflammation in EAE were functional, as these cells suppressed naive Teff *in vitro*. However, suppression was reduced following co-culture with primed Teff from the inflamed central nervous system, demonstrating that Teff from the site of inflammation are less responsive to suppression[56;57]. Notably, these primed Teff produced high levels of TNFα and IL-6; adding these cytokines to naive Teff reversed their suppression by Treg[56].

Resistance of Teff to suppression was also reported for cells from the site of inflammation in patients with JIA. We[6] and others[7] have shown that Treg-mediated suppression is impaired when cells are isolated from the inflamed joints of these patients. Interestingly, this finding is not a result of a functional defect in synovial fluid Treg, as these cells suppressed Teff isolated from peripheral blood to a similar level as was observed in healthy controls[6;7]. Instead Teff from the site of inflammation showed reduced responsiveness to suppression, which, although not associated with a memory phenotype of the cells, did correlate with their activation status[6;7]. More specifically, we established that protein kinase B (PKB)/c-akt hyperactivation contributed to resistance of synovial fluid Teff to suppression. In our opinion, PKB/c-akt hyperactivation results from the proinflammatory synovial environment, because TNFα and IL-6, both highly present in inflamed joints, induce PKB/c-akt activation and subsequent resistance to suppression in Teff from healthy donors[6]. These findings are in agreement with the before mentioned studies in EAE showing a role for TNFα and IL-6 in Teff resistance to suppression[55;56]. In patients with JIA, unresponsiveness to suppression was not only observed in $CD4^+$ T cells, but also highly apparent in synovial fluid $CD8^+$ T cells[6], in line with their correlation to a more severe outcome of disease[21]. Furthermore, although this resistance to suppression is intrinsic to T cells and maintained *in vitro* even in the absence of synovial fluid APCs[6], it seems that synovial fluid APCs are responsible for the initial induction of resistance to suppression in JIA (Wehrens *et al*. unpublished observations).

In patients with RA, resistance of Teff to suppression - although to a lesser degree than resistance to suppression observed in the synovial fluid of patients with JIA - was also detected in peripheral blood of patients when compared with healthy controls[58], perhaps owing to a more

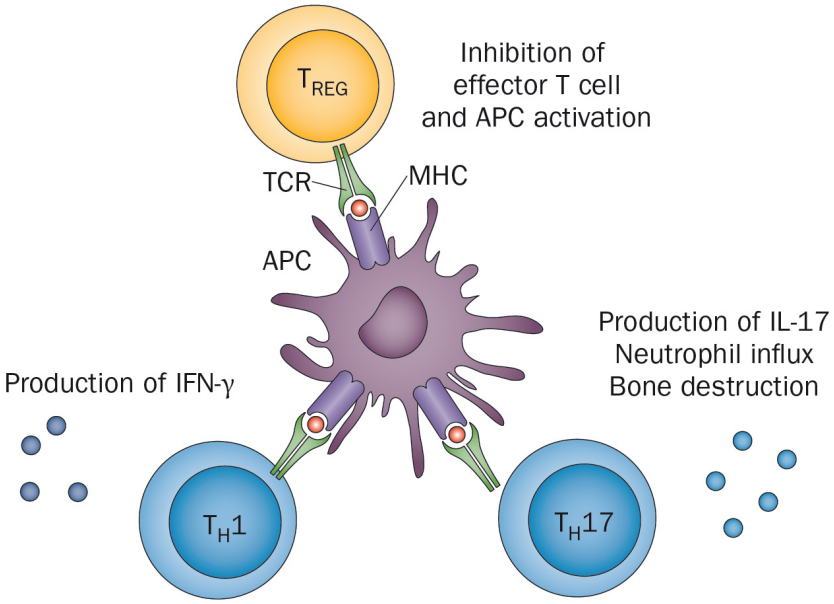

Figure 2 | The main $CD4^+$ T cell subsets in synovial inflammation. Th1 cells, which produce IFNγ, and Treg, which can inhibit Teff and APC activation, are key T cell subsets in the inflamed joints of patients with arthritis. Th17 cells, which produce IL-17, contribute to synovial inflammation by enhancing neutrophil infiltration and bone destruction. The differentiation, expansion and effector function of these T cells are modulated by interaction with local APCs.

systemic pathology in RA than JIA. In other autoimmune diseases, including type I diabetes mellitus (T1DM), SLE and inflammatory bowel disease, resistance of Teff to suppression has been described as well, both in patients[59–63] and in animal models[39;64–67] (Table 1). Teff resistance to suppression therefore seems to be a general mechanism involved in autoimmune pathology and chronic inflammation. In line with this conclusion, Th17 cells, identified as key players in autoimmune inflammation, are less susceptible to suppression than other Th subsets[57;68–70]. In conclusion, Teff in the inflamed joints of patients with arthritis are refractory to suppression, which probably contributes to uncontrolled synovial inflammation (Figure 3, part 2).

INFLAMMATION INDUCED T CELL PLASTICITY

TREG

The proinflammatory environment is known to interfere with Treg-mediated suppression, but it might also induce plasticity of these cells. Purified human Treg can down-regulate FOXP3 expression in the presence of proinflammatory cytokines, such as IL-1β and IL-6, *in vitro*[71;72]. This loss of FOXP3 expression is associated with reduced suppressive function[72] and, more importantly, production of IL-17[71;72]. Although it is impossible to exclude outgrowth of contaminating $FOXP3^-$ cells in a human system, data from transgenic mice confirmed that cells expressing FOXP3 tagged with green fluorescent protein could differentiate into effector cells that produce IL-17

Table 1 | Resistance of effector cells to suppression in human and experimental autoimmune disease

Disease	Subject	Suppression assay	Type of effector cell analyzed	Resistant compared to	Suggested mechanism	Reference
JIA	Human	*In vitro* allogeneic	SF $CD4^+CD25^-$Teff	Teff from HC	Enhanced activation	7
		In vitro autologous	SF $CD4^+$ and $CD8^+$ Teff	PB Teff from the same patient	PKB/c-akt hyperactivation (in response to TNFα and IL-6)	6
RA	Human	*In vitro* allogeneic	PB $CD4^+CD25^-$Teff	Teff from HC	Increased TRAIL expression (inducing Treg apoptosis)	58
T1D	NOD mice	*In vitro* syngeneic	SP $CD4^+CD25^-$Teff	Teff from pre-diabetic mice	ND	67
	DO11.10 RIP-mOVA mice	*In vivo* syngeneic	LN $CD4^+CD25^-$Teff	Teff from pre-diabetic mice	High IL-21 production	64
	NOD mice	*In vitro* allogeneic	SP $CD4^+CD25^-$Teff	Teff from B6 mice	ND	65
	Human	*In vitro* allogeneic	PB $CD4^+CD25^-$Teff	Teff from HC	ND	61
	Human	*In vitro* allogeneic	PB $CD4^+CD25^-$Teff	Teff from HC	ND	60
SLE	MRL/lpr and NZB/WF1 mice	*In vitro* allogeneic	SP and LN $CD4^+CD25^-$Teff	Teff from CBA/Ca mice	ND	66
	MRL/lpr mice	*In vitro* allogeneic	LN $CD4^+CD25^-$Teff	Teff from CBA/J mice	ND	39
	Human	*In vitro* allogeneic	PB $CD4^+CD25^-$Teff	Teff from HC	ND	63
	Human	*In vitro* allogeneic	PB $CD4^+CD25^-$Teff	Teff from HC	ND	62
IBD	Human	*In vitro* allogeneic	LP $CD4^+$ Teff	Compared to LP and PB Teff from HC	High expression of Smad7 (interfering with TGFβ signaling)	59
EAE	*Foxp3gfp*.KI mice	*In vitro* autologous and allogeneic	CNS $CD4^+$FOXP3/ GFP^-Teff	SP Teff from the same mice and Teff from 2D2 mice	High TNFα and IL-6 production	56
	C57BL/6 mice	*In vitro* allogeneic	CNS $CD4^+CD25^-$Teff	Teff from 2D2 mice	ND	57

Abbreviations: CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; HC, healthy controls; IBD, inflammatory bowel disease; JIA, juvenile idiopathic arthritis; LN, lymph node; LP, lamina propria; ND, not determined; PKB, protein kinase B; RA, rheumatoid arthritis; SF, synovial fluid; SLE, systemic lupus erythematosus; SP, splenic; T1D, type 1 diabetes; Teff, effector T cells; TGFβ, transforming growth factor β; TNFα, tumour necrosis factor α; TRAIL, TNF-related apoptosis inducing ligand; Treg, regulatory T cell.

and IFNγ in an autoimmune inflammatory environment[73;74]. Together these data suggest that Treg from patients with autoimmune disease exhibit enhanced plasticity because of ongoing inflammation. In patients with multiple sclerosis[75] and T1DM[76], increased numbers of peripheral blood FOXP3$^+$ T cells co-express IFNγ and, as a result, display reduced suppressive capacity. These unstable Treg are thought to arise as a consequence of their proinflammatory environment, as exposure to IL-12 *in vitro* also induced IFNγ production in FOXP3$^+$ cells from healthy donors[75;76].

How these findings translate to Treg cells present in the inflamed joints of patients with arthritis is still unclear and might be obscured by the transient up-regulation of FOXP3 expression in human activated Teff[77;78]. Although IFNγ levels are very low, low levels of IL-17 expression can be found in FOXP3$^+$ T cells isolated from synovial fluid of patients with arthritis, but it is not clear how these levels compare to Treg from peripheral blood[5]. In addition, we did not observe any IFNγ and IL-17 production in synovial fluid FOXP3$^+$ T cells in our cohort of patients with JIA (Wehrens *et al.* unpublished observation). Furthermore, FOXP3$^+$ cells from synovial fluid of patients with JIA have high TSDR demethylation levels, similar to FOXP3$^+$ cells from peripheral blood[5;6], suggesting that these cells have a relatively stable phenotype despite their proinflammatory environment. Data in mice have now shown that only a minor subpopulation of FOXP3$^+$ T cells with a fully methylated TSDR region become unstable[79;80], whereas the majority of FOXP3$^+$ T cells are resistant to conversion into Teff and have a fully demethylated TSDR region[80]. Notably, these unstable FOXP3$^+$ cells are mainly present within peripherally induced and not natural Treg and preferentially expand under inflammatory conditions[80]. Thus, unstable peripherally induced Treg might predominantly contribute to Treg plasticity observed in the presence of proinflammatory cytokines[71;72;74]. It is not possible to distinguish between natural and induced Treg in humans, however, but in support of the data from mice studies, only a subpopulation of human FOXP3$^+$ cells was found to be unstable and displayed impaired suppressive capacity[72;81]. Natural FOXP3$^+$ Treg might, therefore, be more stable than initially recognized. Although no data so far indicate that synovial fluid Treg are unstable, it is still possible that in the inflamed synovium some Treg display enhanced plasticity due to the highly proinflammatory environment (Figure 3, part 3).

TH17 CELLS

Although it is still unclear whether Treg plasticity takes place in the inflamed joint, plasticity of Th17 cells in the synovium of patients with JIA has been reported. Compared with peripheral blood, a high proportion of Th17 cells in synovial fluid co-express IFNγ[8;9;18] and display both Th17 (RORC)[8;9] and Th1 (T-bet) transcription factors[9]. Instability of these synovial fluid Th17 cells is confirmed *ex vivo* as, following culture, a substantial proportion start to produce IFNγ, whereas Th17 cells from peripheral blood remain IL-17 single positive[8]. However, Th17 cells derived from peripheral blood can convert into Th1 cells when cultured in the presence of synovial fluid in a mechanism dependent on high levels of IL-12 in the synovial fluid[8;9]. Thus, Th17 cells in the synovium of patients with JIA probably become unstable owing to their proinflammatory environment containing high levels of IL-12. This inflammatory mediated conversion of Th17 has also been observed *in vivo* in mouse models of autoimmune disease[82–84]. CD161 expression by a proportion of Th1 cells in the synovium[9] and clonal overlap of these cells with Th17 cells and

Th17 cells co-expressing IFNγ[8;9] suggest that unstable Th17 cells give rise to a population of Th1 cells in the inflamed joints, potentially explaining the more pronounced Th1 responses in the inflamed synovium described earlier[8;16;17].

T CELLS MODULATE APCs: CLOSING THE LOOP

Highly activated APCs at the site of autoimmune inflammation influence T cell homeostasis, which is reflected by the preferential induction of Th17 cells by monocytes from the inflamed joints of patients with RA[22]. Studies in mice and humans over the past 3 years have demonstrated that T cells also influence the infiltration and differentiation of APCs. Human monocytes express high levels of IL-17 receptor and IL-17 serves a chemoattractant for these cells. More specifically, synovial fluid from patients with RA, which contains high levels of IL-17, induces monocyte migration, which is abrogated by antibodies against IL-17 or its receptors[85]. Thus, in inflamed joints, monocytes induce Th17 cell differentiation whereas IL-17 produced by these cells promotes the infiltration of new monocytes into the synovium (Figure 3, part 4). In addition to enhancing monocyte infiltration, the differentiation of these cells into DCs is also promoted by cytokines produced by Teff (Figure 3, part 5). Human $CD4^+$ T cells induce differentiation of monocyte-derived DCs *in vitro*, which is triggered by production of granulocyte-macrophage colony stimulating factor (GM-CSF) and TNFα[86]. Furthermore, during inflammation *in vivo* the generation of monocyte-derived DCs seems to be dependent on $CD4^+$ T cells producing GM-CSF[87]. When acute inflammatory arthritis is induced in mice that lack GM-CSF producing $CD4^+$ T cells, the numbers of monocyte-derived DCs in draining lymph nodes are significantly reduced. As a result, histological arthritis scores in these mice are moderated, indicating that T cell dependent differentiation of monocyte-derived DCs contribute to the severity of joint inflammation[87].

In addition to promoting DC differentiation in general, one study now indicates that human $CD4^+$ T cells can actually modulate the type of DC that is generated, depending on the subset of Th cell and the cytokines it produces[86]. DCs differentiated in the presence of Th1, Th2 or Th17 cells differ in phenotype and cytokine production. As a result, following co-culture with naive T cells they preferentially induce the same type of Th cell that they were generated with. Some evidence now indicates that this T cell instructed DC differentiation also occurs during inflammation *in vivo*, as DCs isolated from psoriatic skin lesions in which Th1 and Th17 cells predominate resemble those that are generated in the presence of Th1 and Th17 cells *in vitro*[86]. Together, these data suggest that at the site of autoimmune inflammation, a loop exists in which T cells act back on APCs, not only by enhancing their recruitment (Figure 3, part 4), but also by promoting and probably modulating their differentiation into DCs (Figure 3, part 5). As a result, the proinflammatory type of immune response is further reinforced. Reduced killing of activated monocytes might also contribute to this ongoing loop of inflammation. Under homeostatic conditions effector cells can induce monocyte apoptosis, but this response is impaired in patients with RA, especially in cells from synovial fluid[88], which might result from resistance of synovial fluid monocytes to FAS-induced apoptosis[89]. Finally, although Treg normally inhibit APC function, APCs become less responsive to suppression following activation[90]. Therefore, it will be very interesting to investigate whether Treg are capable of suppressing the highly activated APCs

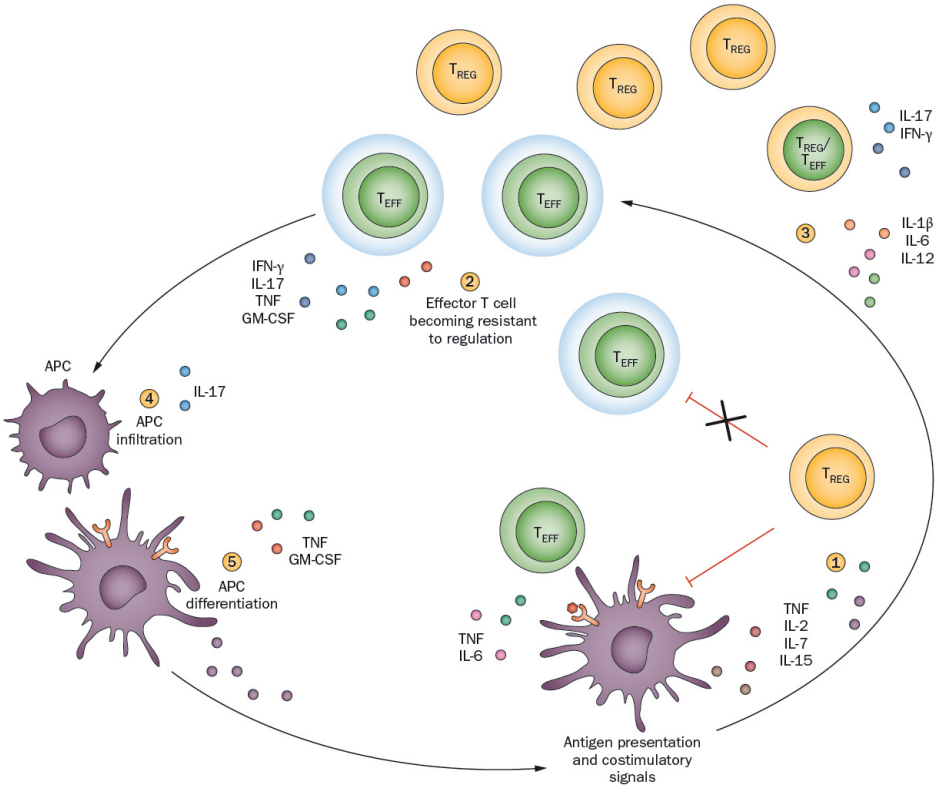

Figure 3 | Perpetuating loop of uncontrolled synovial inflammation. In the inflamed joints of patients with arthritis, activated APCs and proinflammatory cytokines impair the function of Treg (***1***) and induce resistance of Teff to suppression (***2***). In the presence of proinflammatory APCs, a small population of unstable Treg might convert into pathogenic Teff (***3***). Cytokines produced by highly activated uncontrolled Teff further drive the inflammatory response by recruiting monocytes to the site of inflammation (***4***) and promoting their differentiation into dendritic cells (***5***).

found in the inflamed joints of patients with arthritis. In conclusion, insufficiently controlled Teff in the inflamed synovium (Figure 3, part 2) can further enhance the ongoing inflammation by promoting monocyte infiltration (Figure 3, part 4) and differentiation into DCs (Figure 3, part 5).

TREG AND CHRONIC INFLAMMATION

So far we have described several pathways involved in T cell regulation that are up-regulated at the site of autoimmune inflammation. However, their regulatory outcome is clearly restricted by the proinflammatory environment. In particular, FOXP3$^+$ Treg are present at high levels in the inflamed joints of patients with arthritis[5;6], but ineffective in controlling Teff, at least partly owing to the resistance of Teff to suppression[6;7]. Besides being ineffective, could these Treg actually contribute to ongoing inflammation? For example, as induction of pathogenic Th17 cells is dependent on TGFβ[84;91–93] but inhibited by IL-2[84;94], Treg could promote Th17 differentiation by

producing TGFβ and consuming IL-2[84;95] (Figure 1). Indeed, in mice, highly purified FOXP3$^+$ Treg induce Th17 differentiation, in a manner dependent on production of TGFβ[73;96] or consumption of IL-2[98], or both[97]. Moreover, this Th17 induction specifically occurs under inflammatory conditions both *in vitro*[73;96] and *in vivo*[97;99]. In addition, in humans, Treg not only fail to suppress IL-17 levels in culture, but actually increase the percentage of IL-17 producing cells[100]. Of note, Treg enhance IL-17 production by effector memory T cells in these assays, suggesting that in inflamed joints of patients with arthritis, in which memory T cells predominate[6;7], Treg could promote Th17 cellular responses. In addition, if Treg become unstable and subsequently convert into pathogenic Teff producing proinflammatory cytokines, there could be a highly detrimental effect on autoimmune inflammation. Indeed, mouse Treg become pathogenic following down-regulation of FOXP3 and induce diabetes in RAG2$^{-/-}$ mice[101]. Finally, although Treg inhibit proinflammatory cytokine production by monocytes[88;102], they do not induce apoptosis of these cells, as shown for Teff[88]. Therefore, reduced killing of activated monocytes might also be a negative consequence of high levels of Treg present at the site of inflammation. Thus, besides being ineffective in controlling the inflammatory response, through a variety of mechanisms, Treg might actually contribute to the ongoing inflammation.

CONCLUSION

In the past decade, biologic agents that specifically target essential mediators of autoimmune inflammation have been introduced in the clinic to treat autoimmune disease. Of these, monoclonal antibodies against TNFα (infliximab and adalimumab), or the soluble TNFα receptor (etanercept), have been very promising in the treatment of autoimmune arthritis. Anti-TNFα therapy is still not curative and only partially effective in the majority of patients[103]. The search for new treatment options therefore continues. Over the past years the therapeutic application of Treg has been thoroughly investigated because of their profound anti-inflammatory properties[31]. However, the local proinflammatory environment can interfere with Treg function in patients with autoimmune inflammation and resistance of Teff to suppression could restrict the effectiveness of Treg targeted approaches[6;7;58;59;61;63]. Looking to the future, more information on how this resistance is induced and maintained will be essential in the development of more effective therapeutic strategies. Data on whether and how current therapies target resistance of Teff to suppression will also help to further understand its importance in autoimmune pathology. Indeed, alternative targets might be more efficient in the treatment of autoimmune arthritis or could enhance effectiveness of Treg based therapies. Moreover, Treg could promote the development of pathogenic Teff in a proinflammatory environment[71–73;75]. Overall, Treg based therapies should be approached with great caution in patients with ongoing inflammation and a combination with a profound anti-inflammatory strategy might be indispensable in preventing adverse events.

KEY POINTS

- The study of immune regulation at the site of inflammation is required to improve our understanding of autoimmune pathology
- At the site of autoimmune inflammation, proinflammatory mediators interfere with T cell regulation and may induce T cell plasticity
- Treg are less functional, or might even become pathogenic, in an autoimmune inflammatory environment, which should be kept in mind when developing Treg based therapies
- Resistance of Teff to suppression markedly contributes to the disturbed immune balance in the inflamed joints of patients with arthritis
- In autoimmune inflammation, a perpetuating loop exists in which APCs instruct T cell differentiation and function, and Teff promote and shape the infiltration and differentiation of APCs

REFERENCES

1. Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155, 1151–1164 (1995).
2. Sakaguchi, S. *et al.* Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol. Rev.* 212, 8–27 (2006).
3. Notley, C. A. & Ehrenstein, M. R. The yin and yang of regulatory T cells and inflammation in RA. *Nat. Rev. Rheumatol.* 6, 572–577 (2010).
4. Valencia, X. & Lipsky, P. E. CD4+CD25+FoxP3+ regulatory T cells in autoimmune diseases. *Nat. Clin. Pract. Rheumatol.* 3, 619–626 (2007).
5. Herrath, J. *et al.* The inflammatory milieu in the rheumatic joint reduces regulatory T-cell function. *Eur. J. Immunol.* 41, 2279–2290 (2011).
6. Wehrens, E. J. *et al.* Functional human regulatory T cells fail to control autoimmune inflammation due to PKB/c-akt hyperactivation in effector cells. *Blood* 118, 3538–3548 (2011).
7. Haufe, S. *et al.* Impaired suppression of synovial fluid CD4+ CD25− T cells from patients with juvenile idiopathic arthritis by CD4+ CD25+ regulatory T cells. *Arthritis Rheum.* 63, 3153–3162 (2011).
8. Cosmi, L. *et al.* Evidence of the transient nature of the Th17 phenotype of CD4+CD161+ T cells in the synovial fluid of patients with juvenile idiopathic arthritis. *Arthritis Rheum.* 63, 2504–2515 (2011).
9. Nistala, K. *et al.* Th17 plasticity in human autoimmune arthritis is driven by the inflammatory environment. *Proc. Natl Acad. Sci. USA* 107, 14751–14756 (2010).
10. McInnes, I. B. & Schett, G. Cytokines in the pathogenesis of rheumatoid arthritis. *Nat. Rev. Immunol.* 7, 429–442 (2007).
11. Toh, M. L. & Miossec, P. The role of T cells in rheumatoid arthritis: new subsets and new targets. *Curr. Opin. Rheumatol.* 19, 284–288 (2007).
12. Lundy, S. K., Sarkar, S., Tesmer, L. A. & Fox, D. A. Cells of the synovium in rheumatoid arthritis. T lymphocytes. *Arthritis Res. Ther.* 9, 202 (2007).
13. Steinman, L. A brief history of TH17, the first major revision in the TH1/TH2 hypothesis of T cell-mediated tissue damage. *Nat. Med.* 13, 139–145 (2007).
14. Van den Berg, W. B. & Miossec, P. IL-17 as a future therapeutic target for rheumatoid arthritis. *Nat. Rev. Rheumatol.* 5, 549–553 (2009).
15. Janson, P. C. *et al.* Profiling of CD4+ T cells with epigenetic immune lineage analysis. *J. Immunol.* 186, 92–102 (2011).
16. Wilson, C. B., Rowell, E. & Sekimata, M. Epigenetic control of T-helper-cell differentiation. *Nat. Rev. Immunol.* 9, 91–105 (2009).
17. Snir, O. *et al.* Multifunctional T cell reactivity to native and glycosylated type-II collagen in rheumatoid arthritis. *Arthritis Rheum.* 64, 2482–2488 (2012).
18. Nistala, K. *et al.* Interleukin-17-producing T cells are enriched in the joints of children with arthritis, but have a reciprocal relationship to regulatory T cell numbers. *Arthritis Rheum.* 58, 875–887 (2008).
19. Santarlasci, V. *et al.* Rarity of human T helper 17 cells is due to retinoic acid orphan receptor-dependent mechanisms that limit their expansion. *Immunity.* 36, 201–214 (2012).
20. Cho, B. A. *et al.* Characterization of effector memory CD8+ T cells in the synovial fluid of rheumatoid arthritis. *J. Clin. Immunol.* 32, 709–720 (2012).
21. Hunter, P. J. *et al.* Biologic predictors of extension of oligoarticular juvenile idiopathic arthritis as determined from synovial fluid cellular composition and gene expression. *Arthritis Rheum.* 62, 896–907 (2010).
22. Evans, H. G. *et al. In vivo* activated monocytes from the site of inflammation in humans specifically promote Th17 responses. *Proc. Natl Acad. Sci. USA* 106, 6232–6237 (2009).
23. Smolewska, E. *et al.* Distribution and clinical significance of blood dendritic cells (BDC) in children with juvenile idiopathic arthritis. *Ann. Rheum. Dis.* 67, 762–768 (2008).
24. Van Amelsfort, J. M. *et al.* Proinflammatory mediator-induced reversal of CD4+, CD25+ regulatory T cell-mediated suppression in rheumatoid arthritis. *Arthritis Rheum.* 56, 732–742 (2007).
25. Cavanagh, L. L. *et al.* Rheumatoid arthritis synovium contains plasmacytoid dendritic cells. *Arthritis Res. Ther.* 7, R230–R240 (2005).
26. Gattorno, M. *et al.* Distinct expression pattern of IFN-α and TNF-α in juvenile idiopathic arthritis synovial tissue. *Rheumatology (Oxford)* 46, 657–665 (2007).

27. Lande, R. *et al*. Characterization and recruitment of plasmacytoid dendritic cells in synovial fluid and tissue of patients with chronic inflammatory arthritis. *J. Immunol*. 173, 2815–2824 (2004).

28. Thomas, R. & Quinn, C. Functional differentiation of dendritic cells in rheumatoid arthritis: role of CD86 in the synovium. *J. Immunol*. 156, 3074–3086 (1996).

29. Poppensieker, K. *et al*. CC chemokine receptor 4 is required for experimental autoimmune encephalomyelitis by regulating GM-CSF and IL-23 production in dendritic cells. *Proc. Natl Acad. Sci. USA* 109, 3897–3902 (2012).

30. Nguyen, K. D. *et al*. Serum amyloid A overrides Treg anergy via monocyte-dependent and Treg-intrinsic, SOCS3-associated pathways. *Blood* 117, 3793–3798 (2011).

31. Esensten, J. H., Wofsy, D. & Bluestone, J. A. Regulatory T cells as therapeutic targets in rheumatoid arthritis. *Nat. Rev. Rheumatol*. 5, 560–565 (2009).

32. Buckner, J. H. Mechanisms of impaired regulation by CD4+CD25+FOXP3+ regulatory T cells in human autoimmune diseases. *Nat. Rev. Immunol*. 10, 849–859 (2010).

33. De Kleer, I. *et al*. CD4+CD25bright regulatory T cells actively regulate inflammation in the joints of patients with the remitting form of juvenile idiopathic arthritis. *J. Immunol*. 172, 6435–6443 (2004).

34. Mottonen, M. *et al*. CD4+ CD25+ T cells with the phenotypic and functional characteristics of regulatory T cells are enriched in the synovial fluid of patients with rheumatoid arthritis. *Clin. Exp. Immunol*. 140, 360–367 (2005).

35. Ruprecht, C. R. *et al*. Coexpression of CD25 and CD27 identifies FoxP3+ regulatory T cells in inflamed synovia. *J. Exp. Med*. 201, 1793–1803 (2005).

36. Van Amelsfort, J. M., Jacobs, K. M., Bijlsma, J. W., Lafeber, F. P. & Taams, L. S. CD4+CD25+ regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. *Arthritis Rheum*. 50, 2775–2785 (2004).

37. Baecher-Allan, C., Viglietta, V. & Hafler, D. A. Inhibition of human CD4+CD25+high regulatory T cell function. *J. Immunol*. 169, 6210–6217 (2002).

38. Ashley, C. W. & Baecher-Allan, C. Cutting Edge: Responder T cells regulate human DR+ effectorregulatory T cell activity via granzyme B. *J. Immunol*. 183, 4843–4847 (2009).

39. Parietti, V., Monneaux, F., Decossas, M. & Muller, S. Function of CD4+, CD25+ Treg cells in MRL/lpr mice is compromised by intrinsic defects in antigen-presenting cells and effector T cells. *Arthritis Rheum*. 58, 1751–1761 (2008).

40. Yan, B. *et al*. Dysfunctional CD4+, CD25+ regulatory T cells in untreated active systemic lupus erythematosus secondary to interferon-α-producing antigen-presenting cells. *Arthritis Rheum*. 58, 801–812 (2008).

41. Chen, X. *et al*. Cutting edge: expression of TNFR2 defines a maximally suppressive subset of mouse CD4+CD25+FoxP3+ T regulatory cells: applicability to tumor-infiltrating T regulatory cells. *J. Immunol*. 180, 6467–6471 (2008).

42. Chen, X. *et al*. Co-expression of TNFR2 and CD25 identifies more of the functional CD4+FOXP3+ regulatory T cells in human peripheral blood. *Eur. J. Immunol*. 40, 1099–1106 (2010).

43. Valencia, X. *et al*. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. *Blood* 108, 253–261 (2006).

44. Nagar, M. *et al*. TNF activates a NF-κB-regulated cellular program in human CD45RA– regulatory T cells that modulates their suppressive function. *J. Immunol*. 184, 3570–3581 (2010).

45. Bayry, J., Siberil, S., Triebel, F., Tough, D. F. & Kaveri, S. V. Rescuing CD4+CD25+ regulatory T-cell functions in rheumatoid arthritis by cytokine-targeted monoclonal antibody therapy. *Drug Discov. Today* 12, 548–552 (2007).

46. Ehrenstein, M. R. *et al*. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFα therapy. *J. Exp. Med*. 200, 277–285 (2004).

47. Nadkarni, S., Mauri, C. & Ehrenstein, M. R. Anti-TNF-α therapy induces a distinct regulatory T cell population in patients with rheumatoid arthritis via TGF-β. *J. Exp. Med*. 204, 33–39 (2007).

48. McGovern, J. L. *et al*. Th17 cells are restrained by Treg cells via the inhibition of interleukin-6 in patients with rheumatoid arthritis responding to anti-tumor necrosis factor antibody therapy. *Arthritis Rheum*. 64, 3129–3138 (2012).

49. Flores-Borja, F., Jury, E. C., Mauri, C. & Ehrenstein, M. R. Defects in CTLA-4 are associated with abnormal regulatory T cell function in rheumatoid arthritis. *Proc. Natl Acad. Sci. USA* 105, 19396–19401 (2008).

50. Chen, X., Baumel, M., Mannel, D. N., Howard, O. M. & Oppenheim, J. J. Interaction of TNF with

TNF receptor type 2 promotes expansion and function of mouse CD4+CD25+ T regulatory cells. *J. Immunol.* 179, 154–161 (2007).

51. Grinberg-Bleyer, Y. *et al.* Pathogenic T cells have a paradoxical protective effect in murine autoimmune diabetes by boosting Tregs. *J. Clin. Invest* 120, 4558–4568 (2010).
52. Goldstein, I. *et al.* α1β1Integrin+ and regulatory Foxp3+ T cells constitute two functionally distinct human CD4+ T cell subsets oppositely modulated by TNFα blockade. *J. Immunol.* 178, 201–210 (2007).
53. Raptopoulou, A. P. *et al.* The programmed death 1/programmed death ligand 1 inhibitory pathway is up-regulated in rheumatoid synovium and regulates peripheral T cell responses in human and murine arthritis. *Arthritis Rheum.* 62, 1870–1880 (2010).
54. Hidalgo, E. *et al.* The response of T cells to interleukin-6 is differentially regulated by the microenvironment of the rheumatoid synovial fluid and tissue. *Arthritis Rheum.* 63, 3284–3293 (2011).
55. Pasare, C. & Medzhitov, R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science* 299, 1033–1036 (2003).
56. Korn, T. *et al.* Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. *Nat. Med.* 13, 423–431 (2007).
57. O'Connor, R. A., Malpass, K. H. & Anderton, S. M. The inflamed central nervous system drives the activation and rapid proliferation of Foxp3+ regulatory T cells. *J. Immunol.* 179, 958–966 (2007).
58. Xiao, H., Wang, S., Miao, R. & Kan, W. TRAIL is associated with impaired regulation of CD4+CD25- T cells by regulatory T cells in patients with rheumatoid arthritis. *J. Clin. Immunol.* 31, 1112–1119 (2011).
59. Fantini, M. C. *et al.* Smad7 controls resistance of colitogenic T Cells to regulatory T cell-mediated suppression. *Gastroenterology* 136, 1305–1316 (2008).
60. Lawson, J. M. *et al.* Increased resistance to CD4+CD25hi regulatory T cell-mediated suppression in patients with type 1 diabetes. *Clin. Exp. Immunol.* 154, 353–359 (2008).
61. Schneider, A. *et al.* The effector T cells of diabetic subjects are resistant to regulation via CD4+ FOXP3+ regulatory T cells. *J. Immunol.* 181, 7350–7355 (2008).
62. Vargas-Rojas, M. I., Crispin, J. C., Richaud-Patin, Y. & cocer-Varela, J. Quantitative and qualitative normal regulatory T cells are not capable of inducing suppression in SLE patients due to T-cell resistance. *Lupus* 17, 289–294 (2008).
63. Venigalla, R. K. *et al.* Reduced CD4+, CD25- T cell sensitivity to the suppressive function of CD4+, CD25high, CD127low/– regulatory T cells in patients with active systemic lupus erythematosus. *Arthritis Rheum.* 58, 2120–2130 (2008).
64. Clough, L. E. *et al.* Release from regulatory T cell-mediated suppression during the onset of tissue-specific autoimmunity is associated with elevated IL-21. *J. Immunol.* 180, 5393–5401 (2008).
65. D'Alise, A. M. *et al.* The defect in T-cell regulation in NOD mice is an effect on the T-cell effectors. *Proc. Natl Acad. Sci. USA* 105, 19857–19862 (2008).
66. Monk, C. R. *et al.* MRL/Mp CD4+,.CD25- T cells show reduced sensitivity by suppression CD4+, CD25+ regulatory T cells *in vitro*: a novel defect of T cell regulation in systemic lupus erythematosus. *Arthritis Rheum.* 52, 1180–1184 (2005).
67. You, S. *et al.* Autoimmune diabetes onset results from qualitative rather than quantitative age-dependent changes in pathogenic T-cells. *Diabetes* 54, 1415–1422 (2005).
68. Annunziato, F. Phenotypic and functional features of human Th17 cells. *J. Exp. Med.* 204, 1849–1861 (2007).
69. Chauhan, S. K. *et al.* Autoimmunity in dry eye is due to resistance of Th17 to Treg suppression. *J. Immunol.* 182, 1247–1252 (2009).
70. Stummvoll, G. H. *et al.* Th1, Th2, and Th17 effector T cell-induced autoimmune gastritis differs in pathological pattern and in susceptibility to suppression by regulatory T cells. *J. Immunol.* 181, 1908–1916 (2008).
71. Koenen, H. J. *et al.* Human CD25highFoxp3pos regulatory T cells differentiate into IL-17-producing cells. *Blood* 112, 2340–2352 (2008).
72. Beriou, G. *et al.* IL-17-producing human peripheral regulatory T cells retain suppressive function. *Blood* 113, 4240–4249 (2009).
73. Xu, L., Kitani, A., Fuss, I. & Strober, W. Cutting Edge: Regulatory T Cells induce CD4+CD25-Foxp3-T Cells or are self-induced to become Th17 cells in the absence of exogenous TGF-β. *J. Immunol.* 178, 6725–6729 (2007).

74. Yang, X. O. *et al*. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity*. 29, 44–56 (2008).

75. Dominguez-Villar, M., Baecher-Allan, C. M. & Hafler, D. A. Identification of T helper type 1-like, Foxp3+ regulatory T cells in human autoimmune disease. *Nat. Med*. 17, 673–675 (2011).

76. McClymont, S. A. *et al*. Plasticity of human regulatory T cells in healthy subjects and patients with type 1 diabetes. *J. Immunol*. 186, 3918–3926 (2011).

77. Allan, S. E. *et al*. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *Int. Immunol*. 19, 345–354 (2007).

78. Wang, J., Ioan-Facsinay, A., Van der Voort, E. I., Huizinga, T. W. & Toes, R. E. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur. J. Immunol*. 37, 129–138 (2007).

79. Komatsu, N. *et al*. Heterogeneity of natural Foxp3+ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. *Proc. Natl Acad. Sci. USA* 106, 1903–1908 (2009).

80. Miyao, T. *et al*. Plasticity of Foxp3+ T cells reflects promiscuous Foxp3 expression in conventional T cells but not reprogramming of regulatory T cells. *Immunity*. 36, 262–275 (2012).

81. D'Hennezel, E., Yurchenko, E., Sgouroudis, E., Hay, V. & Piccirillo, C. A. Single-cell analysis of the human T regulatory population uncovers functional heterogeneity and instability within FOXP3+ cells. *J. Immunol*. 186, 6788–6797 (2011).

82. Bending, D. *et al*. Highly purified Th17 cells from BDC2.5NOD mice convert into Th1-like cells in NOD/SCID recipient mice. *J. Clin. Invest* 119, 565–572 (2009).

83. Lee, Y. K. *et al*. Late developmental plasticity in the T helper 17 lineage. *Immunity*. 30, 92–107 (2009).

84. Maddur, M. S., Miossec, P., Kaveri, S. V. & Bayry, J. Th17 cells: biology, pathogenesis of autoimmune and inflammatory diseases, and therapeutic strategies. *Am. J. Pathol*. 181, 8–18 (2012).

85. Shahrara, S., Pickens, S. R., Dorfleutner, A. & Pope, R. M. IL-17 induces monocyte migration in rheumatoid arthritis. *J. Immunol*. 182, 3884–3891 (2009).

86. Alonso, M. N. *et al*. TH1, TH2, and TH17 cells instruct monocytes to differentiate into specialized dendritic cell subsets. *Blood* 118, 3311–3320 (2011).

87. Campbell, I. K. *et al*. Differentiation of inflammatory dendritic cells is mediated by NF-κB1-dependent GM-CSF production in CD4 T cells. *J. Immunol*. 186, 5468–5477 (2011).

88. Jagger, A. L. *et al*. FAS/FAS-L dependent killing of activated human monocytes and macrophages by CD4+CD25- responder T cells, but not CD4+CD25+ regulatory T cells. *J. Autoimmun*. 38, 29–38 (2012).

89. Perlman, H. *et al*. Rheumatoid arthritis synovial macrophages express the Fas-associated death domain-like interleukin-1β-converting enzyme-inhibitory protein and are refractory to Fas-mediated apoptosis. *Arthritis Rheum*. 44, 21–30 (2001).

90. Andre, S., Tough, D. F., Lacroix-Desmazes, S., Kaveri, S. V. & Bayry, J. Surveillance of antigen-presenting cells by CD4+ CD25+ regulatory T cells in autoimmunity: immunopathogenesis and therapeutic implications. *Am. J. Pathol*. 174, 1575–1587 (2009).

91. Manel, N., Unutmaz, D. & Littman, D. R. The differentiation of human TH-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORγT. *Nat. Immunol*. 9, 641–649 (2008).

92. Volpe, E. *et al*. A critical function for transforming growth factor-β, interleukin 23 and proinflammatory cytokines in driving and modulating human TH-17 responses. *Nat. Immunol*. 9, 650–657 (2008).

93. Yang, L. *et al*. IL-21 and TGF-β are required for differentiation of human TH17 cells. *Nature* 454, 350–352 (2008).

94. Laurence, A. *et al*. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity* 26, 371–381 (2007).

95. Shevach, E. M. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 30, 636–645 (2009).

96. Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M. & Stockinger, B. TGFβ in the context of an inflammatory cytokine milieu supports *de novo* differentiation of IL-17-producing T cells. *Immunity* 24, 179–189 (2006).

97. Pandiyan, P. *et al*. CD4+CD25+Foxp3+ regulatory T cells promote Th17 cells *in vitro* and enhance host resistance in mouse Candida albicans Th17 cell infection model. *Immunity* 34, 422–434 (2011).

98. Chen, Y. *et al*. Foxp3+ regulatory T cells promote T helper 17 cell development *in vivo* through regulation of interleukin-2. *Immunity* 34, 409–421 (2011).

99. Lohr, J., Knoechel, B., Wang, J. J., Villarino, A. V. & Abbas, A. K. Role of IL-17 and regulatory T lymphocytes in a systemic autoimmune disease. *J. Exp. Med*. 203, 2785–2791 (2006).

100. Evans, H. G., Suddason, T., Jackson, I., Taams, L. S. & Lord, G. M. Optimal induction of T helper 17 cells in humans requires T cell receptor ligation in the context of Toll-like receptor-activated monocytes. *Proc. Natl Acad. Sci. USA* 104, 17034–17039 (2007).

101. Zhou, X. *et al*. Foxp3 instability leads to the generation of pathogenic memory T cells *in vivo*. *Nat. Immunol*. 10, 1000–1007 (2009).

102. Tiemessen, M. M. *et al*. CD4+CD25+Foxp3+ regulatory T cells induce alternative activation of human monocytes/macrophages. *Proc. Natl Acad. Sci. USA* 104, 19446–19451 (2007).

103. Chimenti, M. S., Graceffa, D. & Perricone, R. Anti-TNFα discontinuation in rheumatoid and psoriatic arthritis: is it possible after disease remission? *Autoimmun. Rev.* 10, 636–640 (2011).

8

GENERAL DISCUSSION

TREG IN AUTOIMMUNE DISEASE

OF MICE AND MEN: HUMAN TREG DO NOT INDUCE APOPTOSIS IN TEFF

Despite many years of research into Treg function, the main suppressive mechanisms utilized by Treg remain unclear. This especially holds true for human Treg, since the majority of studies investigating Treg function have been performed in mice. In ***Chapter 3*** we investigated whether a Treg suppressive mechanism described in mice, apoptosis induction in Teff, is also employed by human Treg. We show that human Treg efficiently inhibit Teff proliferation and cytokine production, but do not induce apoptosis in Teff as has been shown for murine Treg[1]. Over the years, more discrepancies between mice and human Treg have been described. Perhaps most obvious differences relate to FOXP3 expression. Compared to mice, expression of FOXP3 less consistently correlates with suppressive function in humans, as it can also be up-regulated in activated T cells without conferring a suppressive phenotype[2-6]. In addition, in humans FOXP3 exists as two isoforms; a full-length form and a splice variant lacking exon 2[7]. These two isoforms have functional differences, since full-length FOXP3, but not the isoform lacking exon 2, interacts with RORα and thereby inhibits Th17 differentiation[8]. Next to these differences in FOXP3 expression the manner in which Treg respond to environmental cues can also differ between mice and human. Both murine and human Treg preferentially express TNF receptor 2 (TNFR)[9-11], but differ in the way they react to TNFα. In mice TNFα has been found to boost Treg expansion and suppressive potential[12;13], whereas in human TNFα negatively affects both Treg function[11;14;15] and expansion[16]. Finally, Treg from mice and humans can differ in the suppressive mechanisms they employ. IL-35 is highly expressed by murine Treg and important for their suppressive function *in vitro* and *in vivo*[17]. In contrast, human Treg do not constitutively express IL-35[18;19], questioning its importance as a suppressive mechanism in humans. Recently, it has been reported that IL-35 is up-regulated in human Treg following long-term activation and is involved in their suppressive capacity under specific *in vitro* conditions[20]. Collectively, these data indicate that IL-35 plays a less vital role in Treg function in human compared to mice. Overall, differences in FOXP3 expression, response to environmental stimuli and suppressive mechanisms have been described for murine and human Treg. As a result, data from mice should always be carefully validated in human cells, especially in light of therapeutic application of Treg. Our result revealing apoptosis independent suppression by human Treg indicates yet another functional difference between mice and human Treg. However, a recent study in mice also questions whether apoptosis induction in Teff is a dominant mechanism employed by murine Treg[21] and this subject is still under debate[22].

TREG SUFFICIENT IN AUTOIMMUNE DISEASE?

Another subject of controversy in the Treg field is whether deficiencies in Treg underlie human autoimmune disease. In ***Chapter 4*** we investigated Treg numbers and function in patients with JIA and show that Treg levels are not decreased in these patients compared to healthy controls. On the contrary, Treg are highly enriched at the site of autoimmune inflammation. Other research groups have also found that Treg are not deficient in RA and JIA[14;23-29]. Moreover, we[24] and others have shown that Treg from PB[27;29] and inflamed joints[25-28] of arthritis patients are fully

functional. However, using cross-over experiments we established that Teff from inflamed joints display reduced responsiveness to suppression, resulting in impaired Treg-mediated control of these cells. This unresponsiveness to suppression has now been reported for Teff from patients with different types of autoimmune diseases, including type 1 diabetes and systemic lupus erythematosus (SLE) (reviewed in Chapter 7, Table 1). Collectively, these data imply that simply enhancing Treg numbers or function for the treatment of autoimmune disease might not be effective. However, Treg employ a variety of suppressive mechanisms[30] and although Treg from inflamed joints display overall suppressive capacity *in vitro*, some specific inhibitory mechanism could still be impaired. For example, Sakaguchi and colleagues[31] have proposed that Treg exert different suppressive functions under steady state and inflammatory conditions. They suggest that Treg repress activation of naive T cells in lymphoid tissues via deprivation of activating signals, for instance by suppressing costimulatory molecule expression on APCs and absorption of IL-2. In contrast, under inflammatory conditions, activated Treg might acquire the capacity to directly kill Teff and activated APC via production of granzymes and/or perforin. Therefore, it will be interesting to investigate whether Treg at the site of autoimmune inflammation produce granzymes and perforin, especially in light of unresponsiveness of Teff to suppression. It seems unlikely that Teff can escape perforin- and granzyme-mediated cytolysis, even if they are refractory to other Treg suppressive mechanisms.

TREG FUNCTIONAL DIFFERENTIATION

Treg functional specialization is thought to extend beyond inflamed versus non-inflamed tissue and might even rely on the specific type of inflammatory response going on. Experiments in mice have shown that Treg can functionally differentiate and express hallmark transcription factors of $CD4^+$ T helper cells. Thus, subsets of Treg express T-bet[32], IRF4[33] or STAT-3[34], which gives them the ability to specifically control Th1-[32], Th2-[33] or Th17- type[34] inflammatory responses, respectively. In addition, Bcl6 expressing Treg specifically control follicular T helper cells and germinal center reactions[35;36]. The mechanisms by which these differentiated Treg control specific $CD4^+$ T helper subsets are still unclear, but might involve up-regulation of the same chemokine receptors allowing for homing to similar sites of inflammation and/or competition for limiting factors. Also in human peripheral blood Treg heterogeneity has been described with subpopulations of functional Treg expressing Th1, Th2, Th17 and Th22 transcription factors and chemokine receptors[37]. In addition, a recent publication by Walter et al. indicates that under inflammatory conditions human Treg produce effector cytokines, such as IL-17 and IFNγ, but still display efficient suppressive capacity[38] (see also Addendum 1). Thus, while maintaining their suppressive function, Treg can differentiate into T helper-like subsets, allowing them to specifically control the type of inflammatory response initiated by the $CD4^+$ T helper cell they resemble. Collectively these data might imply that only specific subsets of Treg will be efficacious in treating Th1 and Th17 driven autoimmune inflammation. Therefore, Treg at the site of human autoimmune inflammation should be further characterized to determine whether they show preferential differentiation towards a certain subset and whether this is associated with a specific suppressive function.

TREG: FRIEND OR FOE IN CHRONIC INFLAMMATION?

As described above a therapeutic approach based on enhancing the total Treg population might not be effective in treating autoimmune disease. However, some findings even raise the question of whether Treg could have detrimental effects in autoimmune inflammation by converting into pathogenic Teff or promoting inflammatory cells.

INFLAMMATION INDUCED TREG INSTABILITY

In vitro experiments have shown that in the presence of proinflammatory cytokines, such as IL-1β and IL-6, highly purified human Treg can lose FOXP3 expression and suppressive function and start to produce IL-17[39;40]. Although it is impossible to exclude outgrow of contaminating FOXP3$^-$ cells in a human system, data from FOXP3-GFP mice confirm that FOXP3 expressing cells can differentiate into FOXP3$^-$ cells producing IL-17 and IFNγ in an autoimmune inflammatory environment[41;42]. There are some indications that also *in vivo,* during autoimmune inflammation, human Treg become unstable, since increased numbers of FOXP3$^+$ Treg from patients with multiple sclerosis[43] and type 1 diabetes[44] co-express IFNγ and, as a result, display reduced suppressive function. It's conceivable that if Treg loose their suppressive capacity and start to produce effector cytokines, such as IL-17 and IFNγ, this could have highly detrimental effects in autoimmune inflammation. Indeed, mouse Treg become pathogenic after down-regulating FOXP3 and induce diabetes in RAG2-/- mice[45]. However, more recent data in mice indicate that only a small population of Treg with a fully methylated TSDR region can become unstable[46;47]. These unstable Treg mainly reside within the peripherally induced population and preferentially expand under inflammatory conditions[47]. Thus, a minor population of not truly committed Treg is thought to predominately contribute to the instability of Treg observed under inflammatory conditions[39;40;42]. In line with these observations in mice, also in humans only a small population of Treg displayed instability and reduced suppressive capacity[39;48]. Moreover, we (Chapter 4) and others[15] have shown that Treg from inflamed joints of JIA patients express high TSDR demethylation levels, similar to Treg from PB, indicating that these cells are relatively stable despite their proinflammatory environment. In line with this, we observed no IL-17 and INFγ production by SF Treg in our experiments. Herrath et al.[15] confirm this low IFNγ production, but do measure some IL-17 production by SF Treg, although it is unclear how these levels compare to PB. In conclusion, although the field is still evolving, accumulating data now indicate that truly committed natural occurring Treg might be more stable than initially recognized, even in a proinflammatory environment. In line with this, Treg from the site of inflammation in JIA appear to be stable and human Treg cultured with proinflammatory APCs produce effector cytokines, but retain suppressive function[38]. However, even if Treg do not convert into pathogenic Teff themselves they could still contribute to the ongoing inflammation.

TREG CONTRIBUTING TO INFLAMMATORY RESPONSES

Treg present in high levels at the site of autoimmune inflammation might fuel the ongoing inflammation by promoting pathogenic effector cells, such as Th17 cells. Differentiation of Th17 cells is dependent on TGFβ[49-52] and inhibited by IL-2[52;53]. Therefore, via production of TGFβ and the capacity to consume IL-2 Treg could promote Th17 differentiation[52;54]. Indeed

highly purified murine Treg induce Th17 differentiation dependent on production of TGFβ[41;55], consumption of IL-2[56] or both[57]. Moreover, this Treg induced Th17 differentiation specifically occurs under inflammatory conditions both *in vitro*[41;55] and *in vivo*[57;58]. Thus, in an autoimmune inflammatory environment Treg could potentially enhance Th17 responses. Moreover, also human Treg appear to be able to enhance Th17 differentiation, since their presence *in vitro* leads to an increase in the percentage of IL-17 producing cells[59]. Finally, whereas Teff induce apoptosis in monocytes, Treg fail to do so[60]. Therefore, reduced killing of activated monocytes can also be a negative consequence of high levels of Treg at the site of autoimmune inflammation. Thus, via various mechanisms Treg might even contribute to the ongoing inflammatory response in patients suffering from autoimmune disease.

TEFF RESISTANCE TO SUPPRESSION

In ***Chapter 4*** we have shown that Teff from inflamed joint of JIA patients are unresponsive to regulation, resulting in severely impaired Treg-mediated control of cell proliferation and cytokine production. This Teff resistance to suppression has now been described in numerous autoimmune diseases, both in patients[61-67] and in animal models[68-73] of these diseases. However, in oligoarticular JIA patients included in our experiments unresponsiveness to suppression could only be detected in Teff from inflamed joints and not systemically, in line with the localized pathology seen in these patients. This indicates that Teff become refractory to regulation in response to inflammatory stimuli and we have further investigated these stimuli as discussed later on. In contrast to other studies reporting Teff resistance to suppression (reviewed in Chapter 7, Table 1), we used total mononuclear cells in our *in vitro* suppression assays to mimic the *in vivo* situation as closely as possible (see also Chapter 1, Table 1). With this approach we noticed that resistance to suppression was also highly apparent in $CD8^+$ T cells from inflamed joints. Since the majority of studies investigating Treg function in autoimmune disease focus on suppression of $CD4^+$ T cells, the role of $CD8^+$ T cells in disturbed immune regulation might be underestimated and deserves future investigation. However, we cannot exclude that resistance of $CD8^+$ T cells to suppression is secondary to the presence of $CD4^+$ T cells in our cultures. Suppression assays with sorted $CD4^+$ and $CD8^+$ T cell populations would resolve this issue.

The observation that Teff become refractory to suppression not only in arthritis[66;67;74], but also in other human autoimmune diseases, including type 1 diabetes[62;63], SLE[64;65] and inflammatory bowel disease[61], indicates that it is a general mechanism underlying autoimmune pathology. Moreover, in genetically prone mice Teff resistance to suppression is observed before clinical overt disease, suggesting that it acts early in disease pathology[69;71]. As a result, unresponsiveness of Teff to suppression could be an attractive target to treat autoimmune inflammation. However, in order to do so, the underlying mechanism of Teff resistance to suppression needs to be clarified.

PKB/C-AKT AND RESISTANCE TO SUPPRESSION

In ***Chapter 4*** we show that Teff from inflamed joints are intrinsically resistant to regulation and this resistance to suppression is at least partially caused by enhanced PKB/c-akt activation in

the cells. This raises the question of how PKB/c-akt hyperactivation imposes unresponsiveness to Treg-mediated suppression. We have established that PKB/c-akt hyperactivated Teff from SF of JIA patients are also refractory to TGFβ-mediated inhibition. In addition, resistant Teff from either Cbl-b$^{-/-}$ mice that have enhanced PKB/c-akt activation[75;76] or transgenic mice expressing a constitutively active form of PKB/c-akt[77] show reduced responsiveness to TGFβ as well. In Cbl-b$^{-/-}$ mice it has further been demonstrated that Teff display defective Smad2 phosphorylation in response to TGFβ[78]. Moreover, PKB/c-akt has been shown to directly interact with Smad3 and inhibit its activity[79;80]. Therefore, PKB/c-akt hyperactivation probably interferes with TGFβ signaling via its downstream targets and as such could impose unresponsiveness to Treg-mediated suppression. In line with this, a direct link between the ability of Teff to respond to TGFβ and their susceptibility to Treg-mediated suppression has been established, since T cells expressing a dominant negative form of TGFβ receptor II (TGFβRII) escape control by Treg[81]. Thus PKB/c-akt hyperactivation might confer resistance to Treg function by rendering Teff unresponsive to TGFβ[82].

Next to interfering with TGFβ signaling, PKB/c-akt hyperactivation might also impair responsiveness to suppression by allowing CD28 independent activation of T cells[82]. The PKB/c-akt pathway is one of the key effector pathways downstream of CD28 signaling and murine TRAF6$^{-/-}$ Teff, displaying PKB/c-akt hyperactivation and resistance to suppression, do not require CD28 ligation for their activation[83]. Since Treg suppression is thought to involve down-regulation of costimulatory molecules on APCs[84-92], thereby depriving T cells from CD28 signaling, Teff that do not require CD28 ligation to become activated will escape this mechanism of suppression. In line with this, one of the first studies describing human Treg already demonstrated that bypassing costimulation *in vitro*, by cross-linking CD28 with a monoclonal antibody, impairs Treg-mediated suppression[93]. In conclusion, although more experimental data are required and the main suppressive mechanisms utilized by Treg are still incompletely understood, PKB/c-akt hyperactivation might confer resistance to suppression by interfering with TGFβ signaling and/or bypassing the requirement for CD28 signaling in T cell activation (Figure 1).

TARGET FOR THERAPEUTIC INTERVENTION?

In ***Chapter 4*** we show that selective inhibition of PKB/c-akt activation is sufficient to restore responsiveness of Teff to suppression. Therefore, PBK/c-akt hyperactivation might be an attractive target to treat autoimmune inflammation by restoring Treg-mediated control of inflammatory Teff. Although we cannot exclude reduced dephosphorylation of PKB/c-akt in Teff from inflamed joints, enhanced PKB/c-akt phosphorylation in T cells is thought to result from phosphoinositide 3 kinase (PI3K) activity upstream of PKB/c-akt[94]. Clinical efficacy of PI3K inhibition has already been shown in experimental models of arthritis[95] and SLE[96]. Since Teff resistance to suppression has been described in arthritis[66;67;74] and SLE patients[64;65] as well as in animal models of SLE[71;72], it is likely that the efficacy of PI3K inhibitors in these models partially results from enhanced responsiveness of Teff to suppression. As shown in our experiments, direct targeting of PKB/c-akt could also be a therapeutic option and clinical trials in cancer patients have demonstrated that low dose administration of PKB/c-akt inhibitors is safe[97]. In addition, targeting PKB/c-akt activation might also have positive effects on Treg, since PKB/c-akt hypoactivation has been

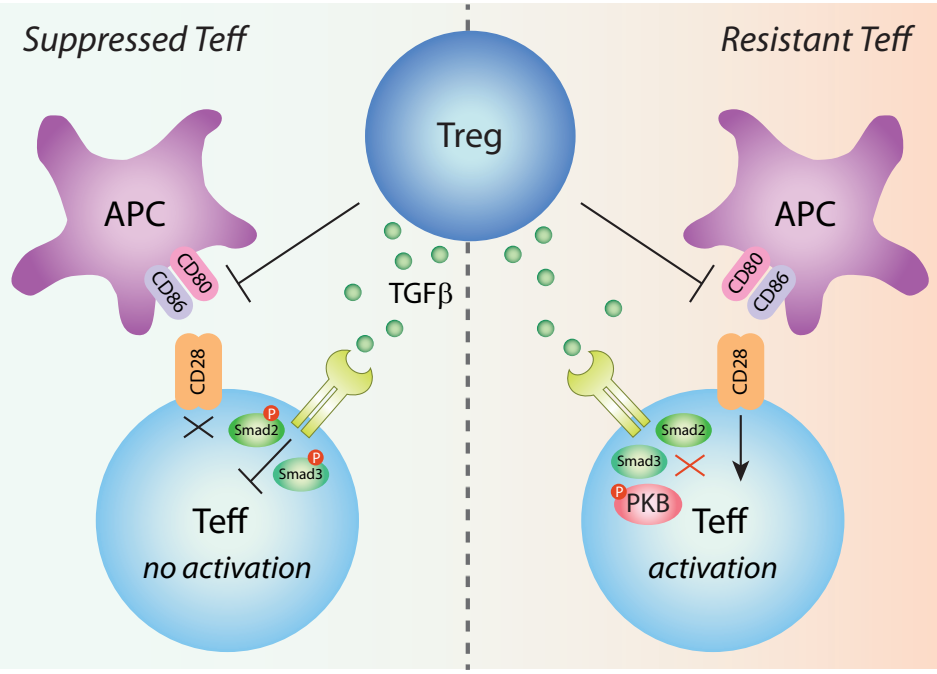

Figure 1 | Proposed mechanism of PKB/c-akt induced resistance to suppression. Treg are thought to repress Teff by down-regulating the costimulatory molecules CD80 and CD86 on APC thereby preventing T cell activation via CD28 and by producing the inhibitory cytokine TGFβ (***left panel***). PKB/c-akt hyperactivation impairs Treg-mediated suppression by interfering with Smad2 and Smad3 phosphorylation thereby abrogating TGFβ signaling and allowing CD28 independent activation of Teff (***right panel***).

shown to be essential for human Treg function[98] and low levels of activated PKB/c-akt support de novo generation of Treg[99;100]. However, the PI3K-PKB/c-akt pathway obviously controls many important cellular functions, including cell growth and survival, in various types of cells[94;101]. Therefore, targeting this pathway can still have considerable side effects on other immune as well as on non-lymphoid cells. Furthermore, if the factors inducing PKB/c-akt hyperactivation in Teff at the site of autoimmune inflammation are not addressed simultaneously, effectiveness of PKB/c-akt targeted approaches might be limited.

INFLAMMATORY MONOCYTES IMPAIR T CELL REGULATION

In ***Chapter 6*** we have investigated the role of local APCs in inducing Teff resistance to suppression in inflamed joints. Classically DCs have been considered professional APCs and are thought to have superior T cell activating capacity compared to other APC subsets[102]. In line with this, we observed enhanced T cell proliferation in the presence of DCs from the site of inflammation, compared to monocytes. However, monocytes from inflamed joints, and not DCs, impair Treg-mediated suppression. These data correspond with the contemporary view that monocytes are not just DCs precursors, but play distinct roles in regulating immune responses[103]. For instance,

it has been shown that monocytes, and not DCs, are required for optimal induction of human Th17 cells[59;104]. In addition, in line with a functional role in shaping immune responses, different subsets of monocytes have been described. Initially a distinction between $CD16^-$ and $CD16^+$ human monocytes has been made[105]. Phenotypically, human $CD14^+CD16^-$ monocytes resemble mouse $Ly6c(Gr1)^+$ monocytes, whereas the $CD14^+CD16^+$ subset in humans corresponds with murine $Ly6c(GR1)^-$ cells in terms of CCR2[106] and CX_3CR1[107] expression[103]. However, functionally the opposite correlation is true, since human $CD14^+CD16^+$ monocyte are the main produces of proinflammatory cytokines, such as TNFα[108;109], and are specifically expanded under inflammatory conditions[109;110]. Human $CD14^+CD16^+$ monocytes are therefore considered proinflammatory and the same holds true for murine $Ly6c^+$ monocytes[107]. Thus, data from mice regarding monocyte phenotype and function cannot directly be translated to human and it is therefore essential to study monocyte subsets and function in human as well.

Our data in Chapter 6 demonstrate that upon co-culture with SF monocytes, Treg-mediated suppression of Teff proliferation and cytokine production is severely impaired. Based on the findings in Chapter 4, it is plausible that the impaired suppression in these assays results from induction of Teff resistance to suppression. However, we can not exclude that SF monocytes act on Treg in these co-cultures, impairing their suppressive function. To confirm a direct effect on Teff, Treg independent suppression should be measured using a TGFβ-based suppression assay (see Chapter 1, Table 1). In addition, measuring PKB/c-akt activation in Teff will also provide more clarity on the subject, since in Chapter 4 we show that unresponsiveness to suppression directly correlates with enhanced PKB/c-akt activation in Teff. However, in support of a primary effect on Teff, *in vitro* experiments in mice showing impaired Treg-mediated suppression in the presence of activated APCs also demonstrate an effect on Teff rather than Treg[111].

In ***Chapter 6*** we further established that the majority of monocytes present in SF of JIA patients are $CD16^+$ in line with findings in RA[112]. More specifically, the more recently identified $CD14^{++}CD16^+$ intermediate monocyte subset[113;114] seems to be expanded in inflamed joints, suggesting that this subset particularly contributes to resistance to suppression. We have some preliminary data in support of this hypothesis, since the other $CD16^+$ subset; $CD14^+CD16^{++}$ non-classical monocytes fail to induce resistance to suppression. These preliminary findings are highly intriguing, since $CD14^{++}CD16^+$ intermediate monocytes have been found to be expanded under many inflammatory conditions[113], including RA[115;116]. However, besides a role for these cells in expanding Th17 cells[116], no other active contribution to autoimmune inflammation has been described. Our data implying that $CD14^{++}CD16^+$ intermediate monocytes impair T cell regulation in JIA, for the first time point towards a role of these cells in disturbed immune regulation in autoimmune inflammation, which should be further investigated.

TNFα AND IL-6: KEY DETERMINANTS OF RESISTANCE TO SUPPRESSION?

Next to APCs, we studied the role of other environmental factors in resistance to suppression as well. In ***Chapter 4*** we show that at the site of inflammation in JIA, in SF taken from inflamed joints, high levels of TNFα and IL-6 are present, in line with previous reports in RA[117] and JIA[118]. In addition, we demonstrate that incubation of PBMC from healthy donors with TNFα and IL-6

leads to enhanced PKB/c-akt activation and reduced responsiveness to suppression. Thus, TNFα and IL-6 are highly present at the site of autoimmune inflammation and are able to induce Teff resistance to suppression. Furthermore, in ***Chapter 6*** we established that SF monocytes that interfere with T cell regulation produce very high levels of TNFα and IL-6 directly *ex vivo*, in contrast to monocytes from PB of healthy controls[38;104]. Therefore, it is reasonable that TNFα and IL-6 play a prominent role in the capacity of SF monocytes to impair T cell suppression and it will be intriguing to investigate how extensive the relative contribution of TNFα and IL-6 is. In conclusion, different datasets described in this thesis point towards an important role for TNFα and IL-6 in Teff resistance to suppression. In line with this, resistance of murine Teff to suppression has also been associated with high TNFα and IL-6 production[70;111].

If TNFα and IL-6 play a prominent role in inducing and/or maintaining Teff resistance to suppression, than blocking these cytokines should lead to enhanced responsiveness to suppression. Indeed in ***Chapter 5*** we show that blocking TNFα, and to a lesser extend IL-6, enhances responsiveness of inflammatory Teff to suppression *in vitro*. Moreover, a reduction in PKB/c-akt activation and resistance to suppression was also observed after anti-TNFα treatment of JIA patients, demonstrating *in vivo* relevance for TNFα induced resistance to suppression. Thus, a biological frequently applied in the treatment of autoimmune arthritis not only affects the innate immune system, but also targets resistance of Teff to suppression. However, anti-TNFα is not able to completely restore immune balance, since patients relapse after discontinuation of therapy[119].

OTHER CANDIDATES?

Next to TNFα and IL-6, other environmental factors at the site of inflammation probably contribute to PKB/c-akt hyperactivation in Teff and subsequent resistance to suppression as well. Besides cytokine signals, both antigen binding to the T cell receptor (TCR) and costimulation can induce strong and sustained activation of PKB/c-akt in T cells[94]. Thus a combination of proinflammatory cytokines, antigen signals and costimulation is likely to be involved in Teff PKB/c-akt hyperactivation at the site of autoimmune inflammation. Obviously, APCs play an essential role in initiating immune responses and are able to deliver all these signals to T cells. Indeed, in Chapter 6 we show that proinflammatory monocytes can interfere with T cell regulation during autoimmune inflammation. Targeting the infiltration and differentiation of these inflammatory monocytes might therefore be an attractive approach to eliminate multiple proinflammatory signals and restore immune balance at the site of inflammation. In line with this, GM-CSF, involved in monocyte differentiation, plays an essential pathogenic role in experimental models of autoimmune disease[120-122]. Moreover, corticosteroids, which rapidly and often durably moderate synovial inflammation in arthritis patients[123], selectively deplete $CD14^+CD16^+$ monocytes[124;125]. This observation illustrates that targeting inflammatory monocytes could indeed have potential in treating autoimmune arthritis. In addition, it has been reported that low expression of CD16 on monocytes before therapy, which did not reflect reduced disease activity, predicts responsiveness to MTX in early RA patients[115]. Thus, inflammatory monocytes might be key variables in determining the outcome of immune regulation and

targeting these monocytes might also enhance effectiveness of other therapeutic approaches. However, clinical effectiveness of autologous stem cell transplantation (ASCT), which is preceded by severe immune ablative conditioning[126], suggests that it takes a multifactorial approach to restore immune balance in patients with autoimmune disease. ASCT has been the only immunosuppressive strategy able to induce long-lasting, drug-free remission in patients with autoimmune disease. Its effectiveness is thought to depend on an almost complete renewal of the immune system together with the induction of a regulatory repertoire[126-131]. Thus, simply targeting some inflammatory mediators might not be sufficient in treating autoimmune inflammation and more research into the critical factors that need to be addressed in order to permanently restore immune balance in patients with autoimmune disease is required.

CONCLUSION

Since their discovery it has been thought that deficiencies in Treg numbers or function underlie human autoimmune pathology and intensive research has focussed on how these cells can be targeted to treat autoimmune inflammation. In this thesis we show that human Treg differ in their suppressive mechanism from murine Treg, again stressing the importance of studying Treg characteristics in human. When analysing Treg function in JIA, we established that Treg are not deficient in these patients, as high levels of functional Treg are present at the site of autoimmune inflammation. Nevertheless, Treg fail to control Teff from inflamed joints due to PKB/c-akt induced resistance of these cells to suppression. This resistance to suppression probably results from high levels of TNFα and IL-6 present at the site of inflammation. In addition, proinflammatory monocytes in inflamed joints contribute to disturbed T cell regulation (summarized in Figure 2). These data indicate that targeting Treg for the treatment of autoimmune disease might be less efficient than initially anticipated. Instead, restoring responsiveness of Teff to suppression either directly, by targeting PKB/c-akt, or indirectly by interfering with the proinflammatory environment might hold greater promise. However, clinical effectiveness of ASCT, which is preceded by very intense immune ablative conditioning, illustrates that probably a multifactorial approach is required to permanently restore immune balance in patients with autoimmune disease. Further research should focus on the critical inflammatory mediators that need to be targeted in order to establish long-lasting disease remission, taking into account the factors interfering with T cell regulation described in this thesis.

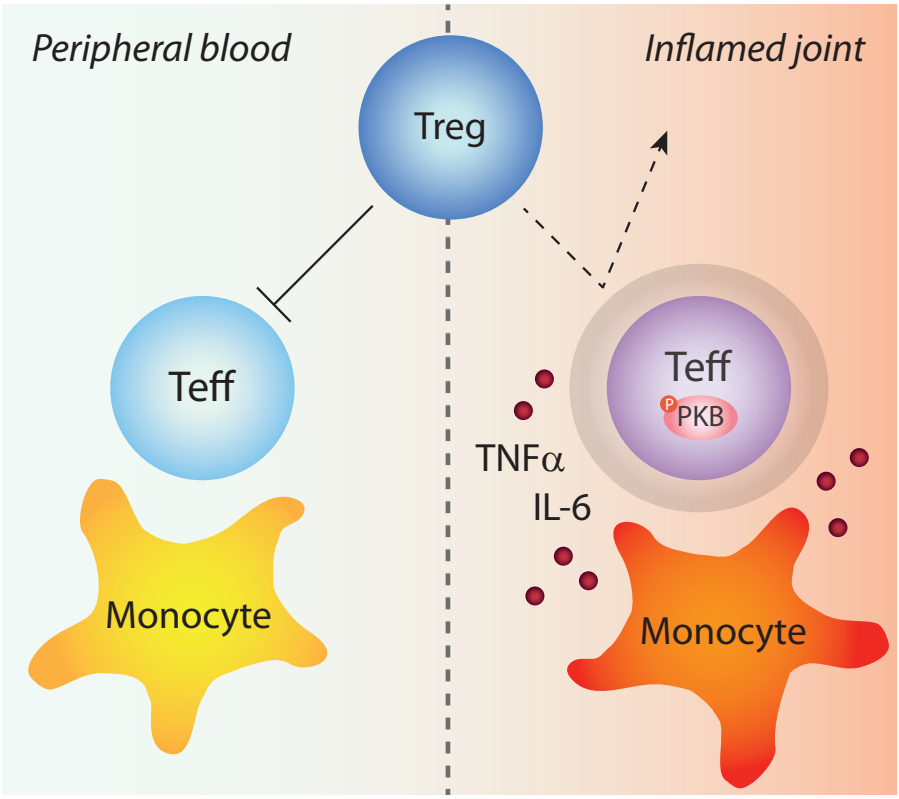

Figure 2 | Impaired T cell regulation in inflamed joints of JIA patients. In peripheral blood of healthy controls Treg efficiently inhibit Teff (***left panel***). In inflamed joints of JIA patients Treg fail to control Teff due to PKB/c-akt induced resistance to suppression resulting from high levels of TNFα and IL-6 and proinflammatory monocytes present (***right panel***).

REFERENCES

1. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. CD4(+)CD25(+)Foxp3(+) regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4(+) T cells. Nat. Immunol. 2007;8:1353-1362.
2. Allan SE, Crome SQ, Crellin NK et al. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. Int.Immunol. 2007;19:345-354.
3. Tran DQ, Ramsey H, Shevach EM. Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype. Blood 2007;110:2983-2990.
4. Wang J, Ioan-Facsinay A, van d, V, Huizinga TW, Toes RE. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. Eur.J.Immunol. 2007;37:129-138.
5. Gavin MA, Torgerson TR, Houston E et al. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. Proc. Natl.Acad.Sci.U.S.A 2006;103:6659-6664.
6. Morgan ME, van Bilsen JH, Bakker AM et al. Expression of FOXP3 mRNA is not confined to CD4+CD25+ T regulatory cells in humans. Hum.Immunol. 2005;66:13-20.
7. Allan SE, Passerini L, Bacchetta R et al. The role of 2 FOXP3 isoforms in the generation of human CD4+ Tregs. J.Clin.Invest 2005;115:3276-3284.
8. Du J, Huang C, Zhou B, Ziegler SF. Isoform-specific inhibition of ROR alpha-mediated transcriptional activation by human FOXP3. J.Immunol. 2008;180:4785-4792.
9. Chen X, Subleski JJ, Kopf H et al. Cutting edge: expression of TNFR2 defines a maximally suppressive subset of mouse CD4+CD25+FoxP3+ T regulatory cells: applicability to tumor-infiltrating T regulatory cells. J.Immunol. 2008;180:6467-6471.
10. Chen X, Subleski JJ, Hamano R et al. Co-expression of TNFR2 and CD25 identifies more of the functional CD4+FOXP3+ regulatory T cells in human peripheral blood. Eur.J.Immunol. 2010;40:1099-1106.
11. Valencia X, Stephens G, Goldbach-Mansky R et al. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. Blood 2006;108:253-261.
12. Chen X, Baumel M, Mannel DN, Howard OM, Oppenheim JJ. Interaction of TNF with TNF Receptor Type 2 Promotes Expansion and Function of Mouse CD4+CD25+ T Regulatory Cells. J.Immunol. 2007;179:154-161.
13. Grinberg-Bleyer Y, Saadoun D, Baeyens A et al. Pathogenic T cells have a paradoxical protective effect in murine autoimmune diabetes by boosting Tregs. J.Clin.Invest 2010;120:4558-4568.
14. Ehrenstein MR, Evans JG, Singh A et al. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. J.Exp.Med. 2004;200:277-285.
15. Herrath J, Muller M, Amoudruz P et al. The inflammatory milieu in the rheumatic joint reduces regulatory T-cell function. Eur.J.Immunol. 2011
16. Goldstein I, Ben-Horin S, Koltakov A et al. alpha1beta1 Integrin+ and regulatory Foxp3+ T cells constitute two functionally distinct human CD4+ T cell subsets oppositely modulated by TNFalpha blockade. J.Immunol. 2007;178:201-210.
17. Collison LW, Workman CJ, Kuo TT et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature 2007;450:566-569.
18. Allan SE, Song-Zhao GX, Abraham T, McMurchy AN, Levings MK. Inducible reprogramming of human T cells into Treg cells by a conditionally active form of FOXP3. Eur.J.Immunol. 2008;38:3282-3289.
19. Bardel E, Larousserie F, Charlot-Rabiega P, Coulomb-L'Hermine A, Devergne O. Human CD4+ CD25+ Foxp3+ regulatory T cells do not constitutively express IL-35. J.Immunol. 2008;181:6898-6905.
20. Chaturvedi V, Collison LW, Guy CS, Workman CJ, Vignali DA. Cutting edge: Human regulatory T cells require IL-35 to mediate suppression and infectious tolerance. J.Immunol. 2011;186:6661-6666.
21. Szymczak-Workman AL, Delgoffe GM, Green DR, Vignali DA. Cutting edge: regulatory T cells do not mediate suppression via programmed cell death pathways. J.Immunol. 2011;187:4416-4420.
22. Pandiyan P, Lenardo M. Comment on "Cutting edge: regulatory T cells do not mediate suppression via programmed cell death pathways". J.Immunol. 2012;188:5203-5204.

23. Flores-Borja F, Jury EC, Mauri C, Ehrenstein MR. Defects in CTLA-4 are associated with abnormal regulatory T cell function in rheumatoid arthritis. Proc.Natl.Acad.Sci.U.S.A 2008;105:19396-19401.

24. De Kleer I, Wedderburn LR, Taams LS et al. CD4+CD25bright regulatory T cells actively regulate inflammation in the joints of patients with the remitting form of juvenile idiopathic arthritis. J.Immunol. 2004;172:6435-6443.

25. Mottonen M, Heikkinen J, Mustonen L et al. CD4+ CD25+ T cells with the phenotypic and functional characteristics of regulatory T cells are enriched in the synovial fluid of patients with rheumatoid arthritis. Clin.Exp.Immunol. 2005;140:360-367.

26. Ruprecht CR, Gattorno M, Ferlito F et al. Coexpression of CD25 and CD27 identifies FoxP3+ regulatory T cells in inflamed synovia. J.Exp.Med. 2005;201:1793-1803.

27. Van Amelsfort JM, Jacobs KM, Bijlsma JW, Lafeber FP, Taams LS. CD4(+)CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. Arthritis Rheum. 2004;50:2775-2785.

28. Cao D, Van Vollenhoven R, Klareskog L, Trollmo C, Malmstrom V. CD25brightCD4+ regulatory T cells are enriched in inflamed joints of patients with chronic rheumatic disease. Arthritis Res.Ther. 2004;6:R335-R346.

29. Lawson CA, Brown AK, Bejarano V et al. Early rheumatoid arthritis is associated with a deficit in the CD4+CD25high regulatory T cell population in peripheral blood. Rheumatology.(Oxford) 2006;45:1210-1217.

30. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. Nat.Rev.Immunol. 2008;8:523-532.

31. Yamaguchi T, Wing JB, Sakaguchi S. Two modes of immune suppression by Foxp3(+) regulatory T cells under inflammatory or non-inflammatory conditions. Semin.Immunol. 2011;23:424-430.

32. Koch MA, Tucker-Heard G, Perdue NR et al. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. Nat.Immunol. 2009;10:595-602.

33. Zheng Y, Chaudhry A, Kas A et al. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. Nature 2009;458:351-356.

34. Chaudhry A, Rudra D, Treuting P et al. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. Science 2009;326:986-991.

35. Chung Y, Tanaka S, Chu F et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. Nat.Med. 2011;17:983-988.

36. Linterman MA, Pierson W, Lee SK et al. Foxp3+ follicular regulatory T cells control the germinal center response. Nat.Med. 2011;17:975-982.

37. Duhen T, Duhen R, Lanzavecchia A, Sallusto F, Campbell DJ. Functionally distinct subsets of human FOXP3+ Treg cells that phenotypically mirror effector Th cells. Blood 2012;119:4430-4440.

38. Walter GJ, Evans HG, Menon B et al. Interaction with activated monocytes enhances cytokine expression and suppressive activity of human $CD4^+CD45RO^+CD25^+CD127^{low}$ regulatory T cells. Arthritis Rheum. 2012, *in press*

39. Beriou G, Costantino CM, Ashley CW et al. IL-17-producing human peripheral regulatory T cells retain suppressive function. Blood 2009;113:4240-4249.

40. Koenen HJ, Smeets RL, Vink PM et al. Human CD25highFoxp3pos regulatory T cells differentiate into IL-17-producing cells. Blood 2008;112:2340-2352.

41. Xu L, Kitani A, Fuss I, Strober W. Cutting Edge: Regulatory T Cells Induce CD4+CD25-Foxp3- T Cells or Are Self-Induced to Become Th17 Cells in the Absence of Exogenous TGF-beta. J.Immunol. 2007;178:6725-6729.

42. Yang XO, Nurieva R, Martinez GJ et al. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. Immunity. 2008;29:44-56.

43. Dominguez-Villar M, Baecher-Allan CM, Hafler DA. Identification of T helper type 1-like, Foxp3+ regulatory T cells in human autoimmune disease. Nat.Med. 2011;17:673-675.

44. McClymont SA, Putnam AL, Lee MR et al. Plasticity of human regulatory T cells in healthy subjects and patients with type 1 diabetes. J.Immunol. 2011;186:3918-3926.

45. Zhou X, Bailey-Bucktrout SL, Jeker LT et al. Foxp3 instability leads to the generation of pathogenic memory T cells in vivo. Nat.Immunol. 2009;10:1000-1007.

46. Komatsu N, Mariotti-Ferrandiz ME, Wang Y et al. Heterogeneity of natural Foxp3+ T cells: a committed regulatory T-cell lineage and an uncommitted minor population

retaining plasticity. Proc.Natl.Acad.Sci.U.S.A 2009;106:1903-1908.

47. Miyao T, Floess S, Setoguchi R et al. Plasticity of Foxp3(+) T cells reflects promiscuous Foxp3 expression in conventional T cells but not reprogramming of regulatory T cells. Immunity. 2012;36:262-275.

48. D'Hennezel E, Yurchenko E, Sgouroudis E, Hay V, Piccirillo CA. Single-cell analysis of the human T regulatory population uncovers functional heterogeneity and instability within FOXP3+ cells. J.Immunol. 2011;186:6788-6797.

49. Manel N, Unutmaz D, Littman DR. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. Nat.Immunol. 2008;9:641-649.

50. Volpe E, Servant N, Zollinger R et al. A critical function for transforming growth factor-beta, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses. Nat.Immunol. 2008;9:650-657.

51. Yang L, Anderson DE, Baecher-Allan C et al. IL-21 and TGF-beta are required for differentiation of human T(H)17 cells. Nature 2008;454:350-352.

52. Maddur MS, Miossec P, Kaveri SV, Bayry J. Th17 cells: biology, pathogenesis of autoimmune and inflammatory diseases, and therapeutic strategies. Am.J.Pathol. 2012;181:8-18.

53. Laurence A, Tato CM, Davidson TS et al. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. Immunity. 2007;26:371-381.

54. Shevach EM. Mechanisms of foxp3+ T regulatory cell-mediated suppression. Immunity. 2009;30:636-645.

55. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity. 2006;24:179-189.

56. Chen Y, Haines CJ, Gutcher I et al. Foxp3(+) regulatory T cells promote T helper 17 cell development in vivo through regulation of interleukin-2. Immunity. 2011;34:409-421.

57. Pandiyan P, Conti HR, Zheng L et al. CD4(+) CD25(+)Foxp3(+) regulatory T cells promote Th17 cells in vitro and enhance host resistance in mouse Candida albicans Th17 cell infection model. Immunity. 2011;34:422-434.

58. Lohr J, Knoechel B, Wang JJ, Villarino AV, Abbas AK. Role of IL-17 and regulatory T lymphocytes in a systemic autoimmune disease. J.Exp.Med. 2006;203:2785-2791.

59. Evans HG, Suddason T, Jackson I, Taams LS, Lord GM. Optimal induction of T helper 17 cells in humans requires T cell receptor ligation in the context of Toll-like receptor-activated monocytes. Proc.Natl.Acad.Sci.U.S.A 2007;104:17034-17039.

60. Jagger AL, Evans HG, Walter GJ et al. FAS/FAS-L dependent killing of activated human monocytes and macrophages by CD4+CD25- responder T cells, but not CD4+CD25+ regulatory T cells. J.Autoimmun. 2012;38:29-38.

61. Fantini MC, Rizzo A, Fina D et al. Smad7 Controls Resistance of Colitogenic T Cells to Regulatory T Cell-Mediated Suppression. Gastroenterology 2009;136:1308-1316.

62. Lawson JM, Tremble J, Dayan C et al. Increased resistance to CD4+CD25hi regulatory T cell-mediated suppression in patients with type 1 diabetes. Clin.Exp.Immunol. 2008;154:353-359.

63. Schneider A, Rieck M, Sanda S et al. The effector T cells of diabetic subjects are resistant to regulation via CD4+ FOXP3+ regulatory T cells. J.Immunol. 2008;181:7350-7355.

64. Vargas-Rojas MI, Crispin JC, Richaud-Patin Y, cocer-Varela J. Quantitative and qualitative normal regulatory T cells are not capable of inducing suppression in SLE patients due to T-cell resistance. Lupus 2008;17:289-294.

65. Venigalla RK, Tretter T, Krienke S et al. Reduced CD4+,CD25- T cell sensitivity to the suppressive function of CD4+,CD25high,CD127low/- regulatory T cells in patients with active systemic lupus erythematosus. Arthritis Rheum. 2008;58:2120-2130.

66. Xiao H, Wang S, Miao R, Kan W. TRAIL is associated with impaired regulation of CD4+CD25- T cells by regulatory T cells in patients with rheumatoid arthritis. J.Clin.Immunol. 2011;31:1112-1119.

67. Haufe S, Schepp C, Kuemmerle-Deschner J et al. Impaired suppression of synovial fluid CD4(+) CD25(-) T cells from patients with juvenile idiopathic arthritis by CD4(+) CD25(+) regulatory T cells. Arthritis Rheum. 2011;63:3153-3162.

68. Clough LE, Wang CJ, Schmidt EM et al. Release from regulatory T cell-mediated suppression during the onset of tissue-specific autoimmunity is associated with elevated IL-21. J.Immunol. 2008;180:5393-5401.

69. D'Alise AM, Auyeung V, Feuerer M et al. The defect in T-cell regulation in NOD mice is an

effect on the T-cell effectors. Proc.Natl.Acad. Sci.U.S.A 2008;105:19857-19862.

70. Korn T, Reddy J, Gao W et al. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. Nat. Med. 2007;13:423-431.

71. Monk CR, Spachidou M, Rovis F et al. MRL/Mp CD4+,CD25- T cells show reduced sensitivity to suppression by CD4+,CD25+ regulatory T cells in vitro: a novel defect of T cell regulation in systemic lupus erythematosus. Arthritis Rheum. 2005;52:1180-1184.

72. Parietti V, Monneaux F, Decossas M, Muller S. Function of CD4+,CD25+ Treg cells in MRL/lpr mice is compromised by intrinsic defects in antigen-presenting cells and effector T cells. Arthritis Rheum. 2008;58:1751-1761.

73. You S, Belghith M, Cobbold S et al. Autoimmune diabetes onset results from qualitative rather than quantitative age-dependent changes in pathogenic T-cells. Diabetes 2005;54:1415-1422.

74. Wehrens EJ, Mijnheer G, Duurland CL et al. Functional human regulatory T cells fail to control autoimmune inflammation due to PKB/c-akt hyperactivation in effector cells. Blood 2011;118:3538-3548.

75. Fang D, Liu YC. Proteolysis-independent regulation of PI3K by Cbl-b-mediated ubiquitination in T cells. Nat.Immunol. 2001;2:870-875.

76. Wohlfert EA, Callahan MK, Clark RB. Resistance to CD4+CD25+ regulatory T cells and TGF-beta in Cbl-b-/- mice. J.Immunol. 2004;173:1059-1065.

77. Pierau M, Engelmann S, Reinhold D et al. Protein kinase B/Akt signals impair Th17 differentiation and support natural regulatory T cell function and induced regulatory T cell formation. J.Immunol. 2009;183:6124-6134.

78. Wohlfert EA, Gorelik L, Mittler R, Flavell RA, Clark RB. Cutting edge: deficiency in the E3 ubiquitin ligase Cbl-b results in a multifunctional defect in T cell TGF-beta sensitivity in vitro and in vivo. J.Immunol. 2006;176:1316-1320.

79. Conery AR, Cao Y, Thompson EA et al. Akt interacts directly with Smad3 to regulate the sensitivity to TGF-beta induced apoptosis. Nat.Cell Biol. 2004;6:366-372.

80. Remy I, Montmarquette A, Michnick SW. PKB/Akt modulates TGF-beta signalling through a direct interaction with Smad3. Nat.Cell Biol. 2004;6:358-365.

81. Fahlen L, Read S, Gorelik L et al. T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. J.Exp. Med. 2005;201:737-746.

82. Wohlfert EA, Clark RB. 'Vive la Resistance!'--the PI3K-Akt pathway can determine target sensitivity to regulatory T cell suppression. Trends Immunol. 2007;28:154-160.

83. King CG, Kobayashi T, Cejas PJ et al. TRAF6 is a T cell-intrinsic negative regulator required for the maintenance of immune homeostasis. Nat.Med. 2006;12:1088-1092.

84. Cederbom L, Hall H, Ivars F. CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. Eur.J.Immunol. 2000;30:1538-1543.

85. Houot R, Perrot I, Garcia E, Durand I, Lebecque S. Human CD4+CD25high regulatory T cells modulate myeloid but not plasmacytoid dendritic cells activation. J.Immunol. 2006;176:5293-5298.

86. Lewkowich IP, Herman NS, Schleifer KW et al. CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. J.Exp.Med. 2005;202:1549-1561.

87. Misra N, Bayry J, Lacroix-Desmazes S, Kazatchkine MD, Kaveri SV. Cutting edge: human CD4+CD25+ T cells restrain the maturation and antigen-presenting function of dendritic cells. J.Immunol. 2004;172:4676-4680.

88. Oderup C, Cederbom L, Makowska A, Cilio CM, Ivars F. Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression. Immunology 2006;118:240-249.

89. Onishi Y, Fehervari Z, Yamaguchi T, Sakaguchi S. Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. Proc.Natl. Acad.Sci.U.S.A 2008;105:10113-10118.

90. Taams LS, Van Amelsfort JM, Tiemessen MM et al. Modulation of monocyte/macrophage function by human CD4+CD25+ regulatory T cells. Hum.Immunol. 2005;66:222-230.

91. Wing K, Onishi Y, Prieto-Martin P et al. CTLA-4 control over Foxp3+ regulatory T cell function. Science 2008;322:271-275.

92. Tiemessen MM, Jagger AL, Evans HG et al. CD4+CD25+Foxp3+ regulatory T cells induce alternative activation of human monocytes/

macrophages. Proc.Natl.Acad.Sci.U.S.A 2007;104:19446-19451.

93. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. CD4+CD25high regulatory cells in human peripheral blood. J.Immunol. 2001;167:1245-1253.

94. Cantrell D. Protein kinase B (Akt) regulation and function in T lymphocytes. Semin. Immunol. 2002;14:19-26.

95. Camps M, Ruckle T, Ji H et al. Blockade of PI3Kgamma suppresses joint inflammation and damage in mouse models of rheumatoid arthritis. Nat.Med. 2005;11:936-943.

96. Barber DF, Bartolome A, Hernandez C et al. PI3Kgamma inhibition blocks glomerulonephritis and extends lifespan in a mouse model of systemic lupus. Nat.Med. 2005;11:933-935.

97. LoPiccolo J, Blumenthal GM, Bernstein WB, Dennis PA. Targeting the PI3K/Akt/mTOR pathway: effective combinations and clinical considerations. Drug Resist.Updat. 2008;11:32-50.

98. Crellin NK, Garcia RV, Levings MK. Altered activation of AKT is required for the suppressive function of human CD4+CD25+ T regulatory cells. Blood 2007;109:2014-2022.

99. Haxhinasto S, Mathis D, Benoist C. The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells. J.Exp.Med. 2008;205:565-574.

100. Sauer S, Bruno L, Hertweck A et al. T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. Proc.Natl.Acad. Sci.U.S.A 2008;105:7797-7802.

101. Koyasu S. The role of PI3K in immune cells. Nat. Immunol. 2003;4:313-319.

102. Lipscomb MF, Masten BJ. Dendritic cells: immune regulators in health and disease. Physiol Rev. 2002;82:97-130.

103. Geissmann F, Auffray C, Palframan R et al. Blood monocytes: distinct subsets, how they relate to dendritic cells, and their possible roles in the regulation of T-cell responses. Immunol.Cell Biol. 2008;86:398-408.

104. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. Nat.Immunol. 2007;8:942-949.

105. Ziegler-Heitbrock HW. Heterogeneity of human blood monocytes: the CD14+ CD16+ subpopulation. Immunol.Today 1996;17:424-428.

106. Weber C, Belge KU, Von Hundelshausen P et al. Differential chemokine receptor expression and function in human monocyte subpopulations. J.Leukoc.Biol. 2000;67:699-704.

107. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity. 2003;19:71-82.

108. Belge KU, Dayyani F, Horelt A et al. The proinflammatory CD14+CD16+DR++ monocytes are a major source of TNF. J.Immunol. 2002;168:3536-3542.

109. Ziegler-Heitbrock L. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. J.Leukoc.Biol. 2007;81:584-592.

110. Fingerle G, Pforte A, Passlick B et al. The novel subset of CD14+/CD16+ blood monocytes is expanded in sepsis patients. Blood 1993;82:3170-3176.

111. Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science 2003;299:1033-1036.

112. Kawanaka N, Yamamura M, Aita T et al. CD14+,CD16+ blood monocytes and joint inflammation in rheumatoid arthritis. Arthritis Rheum. 2002;46:2578-2586.

113. Wong KL, Tai JJ, Wong WC et al. Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. Blood 2011;118:e16-e31.

114. Ziegler-Heitbrock L, Ancuta P, Crowe S et al. Nomenclature of monocytes and dendritic cells in blood. Blood 2010;116:e74-e80.

115. Cooper DL, Martin SG, Robinson JI et al. FcgammaRIIIa Expression on Monocytes in Rheumatoid Arthritis: Role in Immune-Complex Stimulated TNF Production and Non-Response to Methotrexate Therapy. PLoS.One. 2012;7:e28918.

116. Rossol M, Kraus S, Pierer M, Baerwald C, Wagner U. The CD14(bright) CD16+ monocyte subset is expanded in rheumatoid arthritis and promotes Th17 expansion. Arthritis Rheum. 2012;64:671-677.

117. Steiner G, Tohidast-Akrad M, Witzmann G et al. Cytokine production by synovial T cells in rheumatoid arthritis. Rheumatology.(Oxford) 1999;38:202-213.

118. De Jager W, Hoppenreijs EP, Wulffraat NM et al. Blood and synovial fluid cytokine signatures in patients with juvenile idiopathic arthritis: a cross-sectional study. Ann.Rheum.Dis. 2007;66:589-598.

119. Firestein GS. Evolving concepts of rheumatoid arthritis. Nature 2003;423:356-361.

120. Campbell IK, van NA, Segura E et al. Differentiation of inflammatory dendritic cells is mediated by NF-kappaB1-dependent GM-CSF production in CD4 T cells. J.Immunol. 2011;186:5468-5477.

121. Codarri L, Gyulveszi G, Tosevski V et al. RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. Nat.Immunol. 2011;12:560-567.

122. El-Behi M, Ciric B, Dai H et al. The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. Nat.Immunol. 2011;12:568-575.

123. Ravelli A, Martini A. Juvenile idiopathic arthritis. Lancet 2007;369:767-778.

124. Dayyani F, Belge KU, Frankenberger M et al. Mechanism of glucocorticoid-induced depletion of human CD14+CD16+ monocytes. J.Leukoc.Biol. 2003;74:33-39.

125. Fingerle-Rowson G, Angstwurm M, Andreesen R, Ziegler-Heitbrock HW. Selective depletion of CD14+ CD16+ monocytes by glucocorticoid therapy. Clin.Exp.Immunol. 1998;112:501-506.

126. Wulffraat NM, Van Rooijen EM, Tewarie R et al. Current perspectives of autologous stem cell transplantation for severe Juvenile Idiopathic Arthritis. Autoimmunity 2008;41:632-638.

127. De Kleer I, Vastert B, Klein M et al. Autologous stem cell transplantation for autoimmunity induces immunologic self-tolerance by reprogramming autoreactive T cells and restoring the CD4+CD25+ immune regulatory network. Blood 2006;107:1696-1702.

128. Roord ST, De Jager W, Boon L et al. Autologous bone marrow transplantation in autoimmune arthritis restores immune homeostasis through CD4+CD25+Foxp3+ regulatory T cells. Blood 2008;111:5233-5241.

129. Van Wijk F., Roord ST, Vastert B et al. Regulatory T cells in autologous stem cell transplantation for autoimmune disease. Autoimmunity 2008;41:585-91.

130. Delemarre E, Roord S, Wulffraat N, Van Wijk F, Prakken B. Restoration of the immune balance by autologous bone marrow transplantation in juvenile idiopathic arthritis. Curr.Stem Cell Res.Ther. 2011;6:3-9.

131. Muraro PA, Douek DC, Packer A et al. Thymic output generates a new and diverse TCR repertoire after autologous stem cell transplantation in multiple sclerosis patients. J.Exp.Med. 2005;201:805-816.

QUALITY OR QUANTITY? UNRAVELING THE ROLE OF REGULATORY T CELLS IN RHEUMATOID ARTHRITIS

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ABSTRACT

Regulatory T cells (Treg) are crucial for the maintenance of immune tolerance. As in experimental models of autoimmunity the presence or absence of Treg determines disease, qualitative and/or quantitative deficiencies in Treg are suggested as potential cause of human autoimmune diseases such as rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA). Recent studies have shed more light on the function and role of Treg in synovial inflammation in RA and JIA. The proinflammatory environment in the synovium promotes the activation of various forms of Treg, including Treg that can co-produce IL-17, while remaining suppressive *in vitro*. A better understanding of the role of Treg in RA and JIA is pivotal for further development of therapies that can specifically target Treg in order to restore immune tolerance.

About 25 years ago the introduction of methotrexate (MTX) revolutionized the treatment of rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA). In the late nineties this was followed by the second revolution: the introduction of biologicals. Since this, progress has not stopped as new targets for biological therapies emerge and second generations of biologicals are being generated. Still, this development mostly represents progress in terms of diversification and optimization but not a new revolution. The next challenge in the treatment of RA and JIA is to re-establish self-tolerance with the goal to achieve a long-term medication-free remission; in other words a cure[1].

The discovery, first in animal models and later in humans, of regulatory T cells (Treg) offered a complete new perspective into the establishment and maintenance of self-tolerance[2]. Treg regulate immune responses through their ability to suppress other immune cells, such as (effector) T cells, NK cells and antigen presenting cells (APCs). In humans, Treg mostly reside within the population of $CD4^+CD25^+CD127^{low}$ T cells. They are characterized by the expression of the lineage-specific transcription factor FOXP3. In various experimental models Treg indeed make the difference and were shown to be crucial for maintenance of self-tolerance and immune homeostasis. The subsequent discovery that a gene mutation in FOXP3 leads to uncontrolled inflammation autoimmunity in children with IPEX syndrome raised expectations that in human autoimmune diseases chronic inflammation may be caused by a deficiency in the Treg population. However, at a closer look, the answer to the question of the role of Treg in humans turned out to be more complex. Data on peripheral $FOXP3^+$ Treg numbers and function in human autoimmune diseases are contradictory and still subject of debate[3]. To complicate this point further, it has become clear that FOXP3 expressing T cells are not a homogenous population (Table 1). Firstly, FOXP3 is not a definitive marker for human Treg: conventional T cells can temporarily up-regulate FOXP3 upon activation without displaying suppressive function[4], which hampers identification and isolation of human Treg. Moreover, it has been demonstrated that $FOXP3^+$ Treg may have a certain degree of plasticity[5] or instability[6,7] resulting in differentiation into T effector (-like) subtypes with reduced, or complete loss of suppressive function, especially under inflammatory conditions. To add to this conundrum: new data have emerged indicating that Treg are not one of a kind: various subtypes can be determined, including specific populations that share Th cell characteristics but retain their suppressive capacities [8].

In RA and JIA various groups have reported an increased number of Treg in the synovial fluid (SF) of inflamed joints[3,9-11]. This inevitably leads to the question why these Treg in the synovium

Table 1 | The different faces of $FOXP3^+$ T cells in humans

1	'Classical' stable Treg with normal suppressive function
2	Treg that share cytokine production with T helper subsets but retain FOXP3 expression and suppressive function
3	Plastic Treg that start to make proinflammatory cytokines and have reduced suppressive function
4	Unstable Treg that loose FOXP3, differentiate into T effector cells and do not maintain suppressive function
5	T effector cells that temporarily up-regulate FOXP3 but have no suppressive function

are not capable to control inflammation. Is the proinflammatory environment simply too strong for Treg to overcome or are Treg in inflamed synovial fluid not true Treg but effector cells in disguise that have temporarily up-regulated FOXP3?

In this issue of Arthritis and Rheumatism, Walter and co-workers now present important new data that shed light on this puzzle. In elegantly performed *in vitro* studies they show convincingly that activated monocytes induce expression of anti-inflammatory (IL-10) but also proinflammatory (IL-17, IFN-γ, TNF-α) cytokines by human $CD4^+CD45RO^+CD25^+CD127^{low}$ Treg. Importantly, despite their capacity to express proinflammatory cytokines, these Treg maintain a regulatory phenotype *in vitro* and still can effectively suppress T cell proliferation and cytokine production (Figure 1 A). The authors conclude that these cytokine-expressing $FOXP3^+$ T cells are true Treg and thus may still be potent suppressors at sites of inflammation. This is especially intriguing as these Th1- and Th17-like Treg produce exactly those cytokines that are most closely associated with the detrimental proinflammatory immune response in RA, and are being targeted for immune intervention with biologicals.

It has to be noted that Walter et al. made use of an *in vitro* system that mimics the activation of Treg in the SF using APC that are pre-incubated with cytokines that are dominantly present in SF. Directly stimulating such cells with SF from active arthritis joints is practically not feasible, while cells directly isolated from SF are scarcely available in RA. This is an important drawback as phenotype and function of T cells and APCs from the SF compartment likely differ from their PB counterparts because of their residence in a chronic inflammatory environment. Moreover, *in vitro* suppression assays as used by Walter et al. – though being the state of the art technique for testing Treg function - have important limitations. They reflect the suppressive effects on T effector function, while obviously many more cells contribute to joint inflammation in a real-life situation. Lastly, *in vitro* suppression may not adequately reflect *in vivo* suppression.

Despite these obvious limitations, this study provides an important step forward in unraveling the role of Treg in arthritis. The results support recent observations suggesting that Treg present in the inflamed SF are bona fide, stable Treg[9,11]. Furthermore, by showing that Treg cultured in an inflammatory environment not only retain suppressive function but also acquire specific proinflammatory cytokine suppressive capacities, they add to the concept of phenotypical and functional specialization of Treg[8,12]. The emerging picture contains a broad pallet of activated T cells that appears to include not only "true" Th17 and Th1 cells, but also Th1-like and Th-17 like Treg cells that may develop in parallel, co-localize, and act as a counterbalance[8].

Other important questions remain, e.g. on the role of effector cells (Figure 1 B). Various studies have implied that not so much a deficiency in Treg function or number but a resistance of effector T cells may be crucial[3,13]. Interestingly, it seems that APCs play a pivotal role in priming both Treg and T effector cells. Next to APCs it seems likely that other cells, including neutrophils will both affect and be affected by Treg function.

Treg are crucial for restoring immune tolerance in chronic inflammation and the study by Walter et al. underscores that they also are likely to play a regulatory role in RA. Future studies need to reveal the full picture of the different subtypes of Treg, their function and their relation

to other immune cells at the site of inflammation. This is a clear challenge, but worth the efforts as true comprehension of the role of Treg in joint inflammation may hold the clue for a new and truly innovative way to treat RA and JIA.

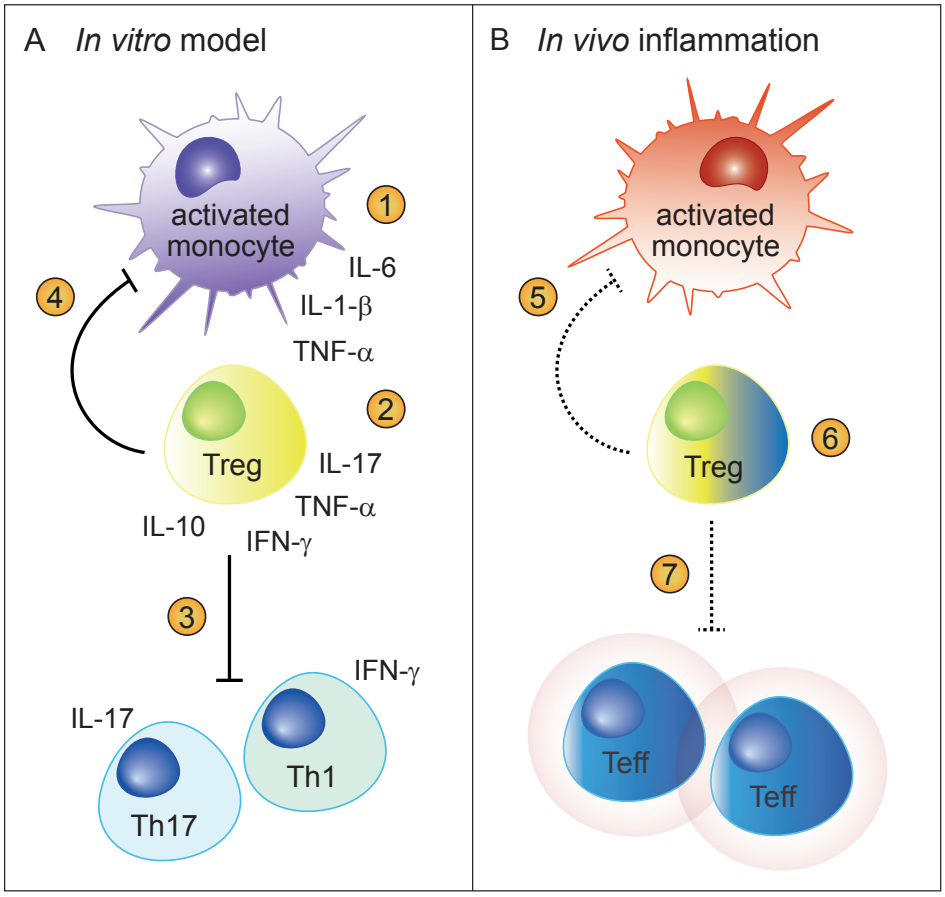

Figure 1 | Treg function under inflammatory conditions. **A**, In this issue of Arthritis and Rheumatism it is shown by Walter et al. that IL-6, TNF-α and IL-1β proinflammatory cytokine secretion by *in vitro* LPS activated monocytes (***1***) induces IL-17, IFN-γ, TNF-α, and IL-10 production by $CD4^{+}CD45RO^{+}CD25^{+}CD127^{low}$ Treg (***2***). Despite their capacity to express proinflammatory cytokines these Treg maintain a regulatory phenotype and effectively suppress *in vitro* T cell proliferation and especially IFN-γ and IL-17 cytokine production by Th1 and Th17 cells (***3***). In addition they suppress proinflammatory cytokine production by the activated monocytes (***4***). **B**, Under *in vivo* inflammatory conditions Treg function may still be hampered by impaired suppression of highly activated APC (***5***), Treg plasticity or instability accompanied by loss of function (***6***), or effector T cells that have become resistant to suppression (***7***).

REFERENCES

1. Benson RA, Patakas A, McQueenie R, Ross K, McInnes IB, Brewer JM, et al. Arthritis in space and time--to boldly go! FEBS Lett 2011;585:3640–3648.
2. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3+ regulatory T cells in the human immune system. Nat Rev Immunol 2010;10:490–500.
3. Buckner JH. Mechanisms of impaired regulation by CD4(+)CD25(+)FOXP3(+) regulatory T cells in human autoimmune diseases. Nat Rev Immunol 2010;10:849–859.
4. Wang J, Ioan-Facsinay A, van der Voort EI, Huizinga TW, Toes RE. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. Eur J Immunol 2006;37:129–138.
5. Dominguez-Villar M, Baecher-Allan CM, Hafler DA. Identification of T helper type 1–like, Foxp3+ regulatory T cells in human autoimmune disease. Nat Med 2011;17:673–675.
6. Koenen HJ, Smeets RL, Vink PM, van Rijssen E, Boots AM, Joosten I. Human CD25highFoxp3pos regulatory T cells differentiate into IL-17-producing cells. Blood 2008;112:2340–2352.
7. Ayyoub M, Deknuydt F, Raimbaud I, Dousset C, Leveque L, Bioley G, et al. Human memory FOXP3+ Tregs secrete IL-17 ex vivo and constitutively express the TH17 lineage-specific transcription factor ROR t. Proc Natl Acad Sci USA 2009;106:8635–8640.
8. Duhen T, Duhen R, Lanzavecchia A, Sallusto F, Campbell DJ. Functionally distinct subsets of human FOXP3+ Treg cells that phenotypically mirror effector Th cells. Blood 2012;119:4430–4440.
9. Wehrens EJ, Mijnheer G, Duurland CL, Klein M, Meerding J, van Loosdregt J, et al. Functional human regulatory T cells fail to control autoimmune inflammation due to PKB/c-akt hyperactivation in effector cells. Blood 2011;118:3538–3548.
10. Van Amelsfort JM, Jacobs KM, Bijlsma JW, Lafeber FP, Taams LS. CD4(+)CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. Arthritis and rheumatism 2004;50:2775–2785.
11. Haufe S, Haug M, Schepp C, Kuemmerle-Deschner J, Hansmann S, Rieber N, et al. Impaired suppression of synovial fluid CD4+CD25- T cells from patients with juvenile idiopathic arthritis by CD4+CD25+ Treg cells. Arthritis and rheumatism 2011;63:3153–3162.
12. Campbell DJ, Koch MA. Phenotypical and functional specialization of FOXP3+ regulatory T cells. Nat Rev Immunol 2011;11:119–130.
13. Wehrens EJ, Prakken BJ, van Wijk F. T cells out of control-impaired immune regulation in the inflamed joint. Nat Rev Rheumatol 2012 ;18:34-42.

SCIENTIFIC ABSTRACT
NEDERLANDSE SAMENVATTING
DANKWOORD
CURRICULUM VITAE
LIST OF PUBLICATIONS

SCIENTIFIC ABSTRACT

FOXP3$^+$ regulatory T cells (Treg) control T cell activation and effector functions and are key players in immune homeostasis and self-tolerance. Given their central role in preventing autoimmune responses, Treg are considered important targets for the treatment of autoimmune inflammation and several strategies are being explored to enhance Treg numbers or function for the treatment of autoimmune disease. In addition, numerous research groups have studied the presence, phenotype and function of Treg in patients with autoimmune disease. Whether deficiencies in Treg underlie human autoimmune pathology, however, is still a subject of debate.

In this thesis we investigated Treg numbers and function in patients with juvenile idiopathic arthritis (JIA), one of the most common autoimmune diseases in children, characterized by chronic inflammation of the joints. In these patients Treg from the site of inflammation can be studied, because during treatment synovial fluid is taken from inflamed joints from which immune cells can be isolated. With this approach we established that Treg are not deficient in JIA patients and are even enriched at the site of autoimmune inflammation. Furthermore, Treg from inflamed joints display efficient suppressive capacity. Still, effector T cells (Teff) from the site of inflammation are insufficiently controlled, because these cells are resistant to suppression. This unresponsiveness to regulation has now been shown for Teff from patients with other autoimmune diseases as well, including type 1 diabetes and inflammatory bowel disease, indicating that it is a general mechanism underlying autoimmune pathology. As a consequence, Teff resistance to suppression might be an attractive target to treat autoimmune inflammation. However, to effectively target unresponsiveness to suppression the underlying mechanism needs to be clarified.

Our experiments using JIA patient material demonstrate that Teff resistance to suppression is, at least partially, caused by PKB/c-akt hyperactivation. Teff from inflamed joints display enhanced PKB/c-akt activation and treatment with a specific PKB/c-akt inhibitor restores responsiveness of the cells to suppression. Proinflammatory cytokines TNFα and IL-6, which are highly present in inflamed joints, are involved in inducing this PKB/c-akt activation and subsequent resistance to suppression. As a result, inhibition of TNFα, and to lesser extend IL-6, reduces PKB/c-akt activation in inflammatory Teff and enhances their responsiveness to suppression. In addition, we show that proinflammatory monocytes present at the site of autoimmune inflammation contribute to impaired T cell regulation as well.

The data described in this thesis indicate that targeting Treg for the treatment of autoimmune disease might be less efficient than initially anticipated. Instead, restoring responsiveness of Teff to suppression, either directly, by targeting PKB/c-akt, or indirectly, by interfering with the proinflammatory environment, might hold greater promise. However, to permanently restore immune balance in patients with autoimmune disease, in other words to cure disease, a multifactorial approach might be required. Future research should focus on the critical inflammatory mediators that need to be targeted in order to establish long-lasting disease remission, taking in to account the factors interfering with T cell regulation described in this thesis.

NEDERLANDSE SAMENVATTING

Juveniele idiopathische artritis (JIA), ook wel jeugdreuma genoemd, komt voor bij ongeveer 1 op de 1.000 kinderen. In deze patiënten valt het immuunsysteem, dat normaal gesproken binnendringende ziekteverwekkers bestrijdt, het gewricht aan. Hierdoor ontstaat een langdurige ontsteking in bijvoorbeeld knieën, enkels en heupen. Omdat het immuunsysteem lichaamseigen cellen aanvalt (auto=zelf), wordt ook wel gesproken van een auto-immuunziekte. Andere voorbeelden van auto-immuunziekten zijn de ziekte van Crohn, waarbij immuuncellen ontstekingen in de darm veroorzaken, en Diabetes type 1, waarbij insulineproducerende cellen worden aangetast. Normaliter worden de aanvallende cellen van het immuunsysteem onderdrukt door regulatoire T-cellen (Treg). Deze cellen vormen zo een belangrijke schakel in het voorkomen van auto-immuunziekte. Omdat in JIA en andere auto-immuunziekten de aanvallende cellen toch de overhand krijgen, is lang gedacht dat er sprake is van te weinig of niet goed functionerende Treg. Er wordt daarom onderzocht of het vermeerderen van Treg of het verbeteren van hun onderdrukkende kracht gebruikt kan worden als therapie voor auto-immuunziekte.

In dit proefschrift hebben we gekeken naar het aantal Treg en hun onderdrukkende werking in patiënten met JIA. Opvallend genoeg vonden we dat er geen tekort is aan Treg in deze patiënten. Integendeel, in de ontstoken gewrichten zijn juist verhoogde aantallen Treg aanwezig en deze cellen hebben een sterk onderdrukkende werking. Ons onderzoek toont echter aan dat de ontstekingscellen in het gewricht zo geactiveerd zijn, dat ze zich niet langer laten onderdrukken. In plaats van de Treg te vermeerderen zouden de ontstekingscellen in het gewricht dus aangepakt moeten worden. In dit proefschrift laten we zien dat lokale omgevingsfactoren, zoals bepaalde typen signaalstoffen en immuuncellen, de activiteit van de ontstekingscellen in het gewricht aanwakkeren. Wanneer we deze omgevingsfactoren verminderen, of direct de activiteit van de cellen remmen, worden de ontstekingscellen wel weer onderdrukt. Deze aanpak kan daarom bijdragen aan een betere behandeling van JIA en andere auto-immuunziekten in de toekomst.

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You won't find faith or hope down a telescope
You won't find heart and soul in the stars
You can break everything down to the chemicals
But you can't explain a love like ours
It's the way we feel, yeah this is real

The Script – Science & Faith

CURRICULUM VITAE

Ellen Wehrens was born on June 5th, 1982 in Born, the Netherlands. In 2000 she completed secondary education at College Sittard and in the same year she started her study Biology at Utrecht University, the Netherlands. As part of this study she wrote a thesis on the role of PPARγ receptors in the immune system under supervision of Dr. Raymond Pieters at the Institute for Risk Assessment Sciences (IRAS), Utrecht University. In addition, she worked on a 9 month research project investigating biological parameters of aggressive behaviour in children suffering from disruptive behaviour disorders under supervision of Dr. Maaike Kempes at the University Medical Center (UMC) Utrecht. She also completed a 9 month laboratory project in which she studied oral tolerance and the role of regulatory T cells in food allergy under supervision of Dr. Femke van Wijk at the IRAS. After receiving her Master's degree *cum laude* in 2005, she started working on a research project investigating endocrine modulation of the immune system at the IRAS. In 2007 she began her PhD training at the Graduate School for Infection and Immunity in the laboratory of Prof. Dr. Berent Prakken and Dr. Femke van Wijk at the Center for Molecular and Cellular Intervention (CMCI) at the UMC Utrecht. During this project she studied immune regulation in a translational setting, mainly focussing on factors interfering with T cell regulation during chronic autoimmune inflammation. The results obtained during this PhD project are presented in this thesis.

LIST OF PUBLICATIONS

B. Prakken, E. Wehrens, F. van Wijk. Quality of Quantity? Unraveling the role of regulatory T cells in rheumatoid arthritis. *Arthritis and Rheumatism* 2012; Accepted Article

E.J. Wehrens, B.J. Prakken, F. van Wijk. T cells out of control - impaired immune regulation in the inflamed joint. *Nature Reviews Rheumatology* 2012; 18:34-42

E.J. Wehrens, G. Mijnheer, C.L. Duurland, M. Klein, J. Meerding, J. van Loosdregt, W. de Jager, B. Sawitzki, P.J. Coffer, B. Vastert, B.J. Prakken*, F. van Wijk*. Functional human regulatory T cells fail to control autoimmune inflammation due to PKB/c-akt hyperactivation in effector cells. *Blood* 2011; 118:3538-48
**equally contributed*

E.J. Wehrens, F. van Wijk, S.T. Roord, S. Albani, B.J. Prakken. Treating arthritis by immunomodulation: is there a role for regulatory T cells? *Rheumatology (Oxford)* 2010; 49:1632-44

Y. Vercoulen*, E.J. Wehrens*, N.H. van Teijlingen, W. de Jager, J.M. Beekman, B.J. Prakken. Human regulatory T cell suppressive function is independent of apoptosis induction in activated effector T cells. *PloS ONE* 2009; 4:e7183
**equally contributed*

F. van Wijk, E.J. Wehrens, S. Nierkens, L. Boon, A. Kasran, R. Pieters, L.M. Knippels. $CD4^+CD25^+$ T cells regulate the intensity of hypersensitivity responses to peanut, but are not decisive in the induction of oral sensitization. *Clinical & Experimental Allergy* 2007; 37:572-81

F. van Wijk, S. Nierkens, W. de Jong, E.J. Wehrens, L. Boon, P. van Kooten, L.M. Knippels, R. Pieters. The CD28/CTLA-4-B7 signaling pathway is involved in both allergic sensitization and tolerance induction to orally administrated peanut proteins. *The Journal of Immunology* 2007; 178:6894-900