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The dynamics of effector T cells and Foxp3+ regulatory T cells in the promotion and regulation of autoimmune encephalitis

Thomas Korn[1], **Ana C. Anderson**[1], **Estelle Bettelli**[1], and **Mohamed Oukka**[2]

1*Brigham and Women's Hospital, Center for Neurologic Diseases Harvard Medical School, Boston, MA 02115, USA*

2*Brigham and Women's Hospital, Harvard Medical School, 65 Landsdowne Street, Cambridge, MA 02139, USA*

Abstract

The Th1/Th2 paradigm of T helper cell subsets had to be revised when IL-17 producing T cells (Th17) were identified as a distinct T helper cell lineage. Th17 cells are very efficient inducers of tissue inflammation and crucial initiators of organ specific autoimmunity. Whereas Th17 cells promote autoimmune tissue inflammation, Foxp3$^+$ regulatory T cells (T-reg) are necessary and sufficient to prevent autoimmunity throughout the life span of an individual. Here, we review recent findings of how responses of effector T cells and T-reg cells with a defined antigen specificity develop in autoimmune encephalomyelitis. Moreover, Th17 cells and Foxp3$^+$ T-reg seem to be dichotomously related in that TGF-β induces Foxp3 in naïve T cells, but TGF-β and IL-6 together drive the generation of Th17 cells. Thus, we give an overview of how Th17 cells, induced Foxp3$^+$ T-reg, as well as how naturally occurring T-reg cells might cooperate to promote and regulate autoimmune inflammation of the central nervous system (CNS). The monitoring of the population dynamics of these T cell-subsets in reporter mice *in vivo* will enable us to revisit the pathogenic concept of autoimmune inflammation in the CNS and design rational and phase-specific therapeutic interventions.

Keywords

Th17 cell; Foxp3; regulatory T cell; experimental autoimmune encephalomyelitis

1. Introduction

Since the identification of the forkhead box transcription factor Foxp3 as an essential transcription factor in CD4$^+$ regulatory T cells (T-reg), CD4$^+$Foxp3$^+$ T-reg have been well characterized as a distinct lineage of T cells (Fontenot and Rudensky, 2005; Hori et al., 2003; Schubert et al., 2001). Their thymic origin as well as their importance for the maintenance of peripheral tolerance under non-inflammatory conditions throughout the life span of an individual, have been confirmed (Fontenot et al., 2005a; Kim et al., 2007; Lahl et al., 2007). When Foxp3$^+$ T-reg are depleted in an adult individual, fatal multi-organ autoimmunity finally results (Kim et al., 2007) and the phenotype of this disease is virtually indistinguishable from

Correspondence: Mohamed Oukka, PhD, Tel.: 617-768-8629; Fax: 617-525-5566, e-mail: moukka@rics.bwh.harvard.edu, Thomas Korn, MD, Tel. 617-525-5353; Fax: 617-525-5566, e-mail: tkorn@rics.bwh.harvard.edu.

genetic deficiency of Foxp3 that is characterized by a massive lymphoproliferative syndrome (IPEX syndrome in humans (Bennett et al., 2001; Wildin et al., 2001) and scurfy phenotype in mice (Godfrey et al., 1991)). It is less clear whether $Foxp3^+$ T-reg can be *de novo* generated or selectively expanded *in vivo* in the peripheral immune compartment. Thus, it has been an important question how T-reg responses develop in relation to antigen-specific responses of effector T cells in autoimmune diseases and in which compartment, the peripheral lymphoid tissue or the target organ, regulation takes place.

Only recently, another $CD4^+$ T cell subset, a T helper cell subset, has been recognized as an independent T cell lineage distinct from Th1 and Th2 cells. Based on the predominant production of IL-17 by this new T helper cell subset, it has been termed Th17 and its importance in the induction of organ-specific autoimmune diseases has been shown (Bettelli et al., 2007; Weaver et al., 2007). Twenty years ago, Mosmann and Coffman proposed that T helper cells could be categorized into two distinct subsets, T helper type 1 (Th1) and Th2. The hallmark criteria for one or the other subset were specific cytokine profiles (Mosmann and Coffman, 1989). Th1 cells produce large quantities of interferon (IFN)-γ whereas Th2 cells produce interleukin 4 (IL-4), IL-5, IL-13 and IL-25 (Fort et al., 2001). Accordingly, Th1 cells induce immune responses of the delayed type hypersensitivity type (DTH) and are very efficient in clearing intracellular pathogens whereas Th2 cells are essential in promoting eosinophilic and humoral immune responses and have a role in host defense against parasitic infections. With the discovery of the T cell derived cytokine IL-17 that cannot be categorized according to this scheme, the Th1/Th2 paradigm had to be revisited. The identification of TGF-β plus IL-6 as the differentiating factors (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006a) and of IL-23 as essential growth and maintenance factor for IL-17 producing T helper cells (Cua et al., 2003) suggested that this T cell subset might be a distinct lineage. This was confirmed when ROR-γt was defined as a master transcription factor of Th17 cells (Ivanov et al., 2006). Th17 cells play a role in host defense against certain extracellular pathogens, particularly gram-negative bacteria and fungi, but also in autoimmunity. Here, Th17 cells and not Th1 cells seem to be the major T helper cell subset initiating tissue inflammation.

Regarding the substantial role of $CD4^+Foxp3^+$ T-reg in preventing spontaneous autoimmunity and of Th17 cells in driving autoimmune pathology, it was very surprising that $CD4^+Foxp3^+$ T-reg might have a close relationship with Th17 cells, in that TGF-β induces the expression of Foxp3 in naïve T cells, but promotes the generation of Th17 cells in the presence of IL-6 (Bettelli et al., 2006). This dichotomous pathway has been clearly demonstrated *in vitro*. In this review, we will give a brief overview of the differentiation of naturally occurring $Foxp3^+$ T-reg, induced $CD4^+$ $Foxp3^+$ T-reg, and Th17 cells and the role of Th17 cells in CNS autoimmunity as well as their functional relationship with $Foxp3^+$ T-reg.

2. Regulatory T cells

2.1. Generation of $Foxp3^+$ T-reg in the thymus

Naturally occurring T-reg are generated in the thymus. This process requires T cell receptor (TcR) triggering in the presence of costimulation (Fontenot et al., 2005c; Tai et al., 2005). However, TGF-β and IL-2 are dispensable (Fontenot et al., 2005b; Marie et al., 2005). Although still diverse, the TcR repertoire of naturally occurring T-reg may be skewed towards self tissue antigens. (Pacholczyk et al., 2006). Notably, the expression of AIRE that regulates the expression of tissue antigens in the thymus and thus, is responsible for the negative selection of autoreactive T-cell clones (recessive or deletional tolerance), seems to be required for the 'positive selection' of regulatory T-cells (Chen et al., 2005). The expression of a functionally intact Foxp3 protein is not needed for thymocytes to be selected into the T-reg compartment (Gavin et al., 2007; Hsieh et al., 2006; Lin et al., 2007). Thus, thymocytes bearing autoreactive TcRs that are supposed to become T-reg are not negatively selected in the absence of a

functional Foxp3 protein. But the lack of Foxp3 prevents the sufficient stabilization of the transcriptional program of T-reg (Williams and Rudensky, 2007). Accordingly, in the presence of a non-functional Foxp3 gene product, the peripheral immune compartment is populated with a T cell subset that has the TcR repertoire and the genetic signature of T-reg cells, but cannot stabilize the functional phenotype of T-reg cells (Lin et al., 2007). Subsequently, these T cells develop a highly autoreactive potential and induce very aggressive autoimmune disease (Williams and Rudensky, 2007). Collectively, these data suggest that whereas the expression of Foxp3 is dispensable for the thymic selection process of T-reg, it is necessary and sufficient in order to stabilize the regulatory phenotype of $Foxp3^+$ T cells in the peripheral immune compartment. Accordingly, forced expression of Foxp3 in $CD4^+CD25^-$ T-cells converts these cells into *bona fide* T-reg (Hori et al., 2003), and conversely, loss of Foxp3 in T-reg results in effector like T-cells with increased production of Th1 cytokines and IL-17 and an increased potential for tissue infiltration (Williams and Rudensky, 2007). As a result of thymic selection, the peripheral $CD4^+$ T cell population contains a fraction of 6 to 10% naturally occurring $Foxp3^+$ T-reg cells. In order to be maintained in the secondary lymphoid tissue, this pool of naturally occurring T-reg is dependent on continuous TcR triggering and costimulation in the presence of IL-2 (Gavin et al., 2002; Knoechel et al., 2005; Lohr et al., 2003). Thus, whereas dispensable for the thymic generation of T-reg, IL-2 is required for the maintenance of a functional T-reg pool in the secondary lymphoid tissue (Malek, 2003). Whether or not $Foxp3^+$ T-reg cells can be *de novo*-generated from $Foxp3^-$ T cells *in vivo* and which are the factors inducing Foxp3 in the peripheral immune compartment *in vivo* needs to be further investigated.

2.2. Induction of Foxp3+ T-reg in the periphery

The conversion of naive $CD4^+CD25^-$ T-cells into $CD4^+CD25^+$ T-reg can be driven by TGF-β and TcR stimulation in the presence of co-stimulation *in vitro* (Chen et al., 2003). When transferred into *wildtype* hosts, 10% of congenically marked $CD4^+CD25^-$ T-cells converted into Foxp3 expressing $CD4^+CD25^+$ T-reg after six weeks. This conversion occurred irrespective of whether or not the host mice were lymphopenic or not. This suggested that homeostatic proliferation of the donor cells was unimportant for the conversion process (Liang et al., 2005). However, the presence of costimulatory molecules of the B7 family in the host mice was an absolute requirement. It has also been shown that continuous low dose administration of antigen without inflammatory stimuli induced the conversion of $CD4^+CD25^-$ T-cells into $CD4^+CD25^+$ T-reg (Apostolou and von Boehmer, 2004). In addition, antigen presentation by immature or tolerogenic dendritic cells (DCs) or presentation of antigen that is targeted to DCs via the DEC-205 antigen might provide the cytokine milieu that is required to induce T-reg (Kretschmer et al., 2005; Steinman et al., 2003). Besides TcR engagment, this conversion always required TGF-β signaling in the starting T-cell population.

For all these experiments, it has been a concern that the starting T cell-populations were not rigorously sorted for the absence of Foxp3 so that expansion of contaminating $Foxp3^+$ T cells in the starting population could not be distinguished from true conversion of $Foxp3^-$ into $Foxp3^+$ T cells. Nevertheless, in summary these data suggest that antigen exposure in the absence of inflammation and a high load of TGF-β in the environment might drive the preferential expansion of the $Foxp3^+$ T-reg-pool in the peripheral immune compartment. It is possible that conversion of $Foxp3^-$ into $Foxp3^+$ T cells or rather preferential induction of $Foxp3^+$ expressing T cells actually occurs *in vivo* in certain microenvironments or niches presumably under conditions of high TGF-β concentrations. One example of this kind of microenvironment is the lamina propria of the colon where, under steady state conditions, the fraction of $Foxp3^+$ T cells in the local CD4 population is between 15 and 20 percent and thus, three times as high as in the spleen. Upon polyclonal stimulation with anti-CD3 antibody *in vivo*, the $Foxp3^+$ T-reg population is preferentially enlarged by *de novo* generation or expansion

and constitutes three quarters of all $CD4^+$ T cells in the colonic lamina propria (Kamanaka et al., 2006).

3. Origin and dynamics of Foxp3⁺ T-reg cells in autoimmunity

In the current understanding of the pathogenic process in experimental autoimmune encephalomyelitis (EAE), antigen specific priming of encephalitogenic T cells happens in secondary lymphoid tissue outside the central nervous system (CNS). Myelin-specific T cells then traffic to the CNS where they are re-activated (Flugel et al., 2001; Wekerle et al., 1994). The efficiency of the activation *in situ* as well as the acquisition of further effector functions determines the maintenance of specific effector populations within the CNS. It has been unclear whether T-reg cells behave in a similar manner. Only recently, tools with which we can address the questions whether T-reg cells respond in an antigen specific manner and what is the source of T-reg cells during organ-specific autoimmunity, i. e. whether they are *de novo* induced or expanded from naturally occurring T-reg cells, have become available. In our laboratory, we developed $MOG_{35–55}/IA^b$ (MHC class II) tetramers in order to track T-reg with this specificity in *Foxp3gfp*.KI mice. These mice are engineered to express GFP in all T cells that are positive for Foxp3. However, their TcR repertoire is unbiased and identical to that of *wildtype* C57BL/6 mice. We found that upon immunization with $MOG_{35–55}$ in complete Freund's adjuvant (CFA), Foxp3 $^+$tetramer$^+$ T-reg could be visualized in the secondary lymphoid tissue (Korn et al., 2007b). This indicated that even under inflammatory conditions, the antigen-specific T-reg pool would expand. Using adoptively transferred rigorously sorted $Foxp3^-$ T-cells from *Foxp3gfp*.KI mice as indicator cells, we were unable to detect conversion into $Foxp3^+$ T-cells *in vivo* under inflammatory conditions, i. e. after immunization with autoantigen emulsified in CFA. No Foxp3 expression was induced in the transferred T cell population, either in the secondary lymphoid organs or in the inflamed CNS target tissue suggesting that strongly inflammatory conditions prevent the conversion of naive T-cells into T-reg. However, T-reg cells still expand in an antigen specific manner in the lymphoid tissue.

It has been unclear whether T-reg traffic into the target organ. Several reports suggested that T-reg might prevent T-eff from trafficking into the target organ, but remain outside the CNS themselves (Kohm et al., 2002; Tischner et al., 2006). Here, *ex vivo*-tetramer staining in *Foxp3gfp*.KI mice illustrated that antigen-specific T-reg reach the CNS and even further expand in the inflamed target tissue (Korn et al., 2007b). Triggering of their TcR, probably in the context of a specific cytokine milieu, is necessary in order for T-reg to express homing receptors for the site of inflammation. CD103, CCR4, CCR5, and CCR6 are crucial for T-reg homing to inflamed target tissues in different animal models of transplantation, chronic infection, and autoimmune disease (Kleinewietfeld et al., 2005; Lee et al., 2005; Yurchenko et al., 2006). Conversely, genetic deficiency in CD103 or CCR5 prevents T-reg from reaching the site of inflammation resulting in an essentially altered disease course in a model of chronic skin inflammation (Suffia et al., 2005; Yurchenko et al., 2006).

In summary, in inflammatory autoimmunity, T-reg cells respond to the eliciting autoantigen. However, in a highly inflammatory environment, antigen-specific T-reg cells are not *de novo*-induced, but expand from the pool of naturally occurring T-reg cells. Similar to encephalitogenic effector T cells, T-reg traffic to the CNS and according to their antigen-specificity, become re-activated *in situ*.

4. IL-17 and Th17 cells

4.1. The cytokines involved in the differentiation of Th17 cells

TGF-β is necessary for the induction of $Foxp3^+$ expressing T-reg *in vitro* and *in vivo*. However, TGF-β is a pleiotropic cytokine with multiple functions in T cell development and homeostasis.

Nevertheless, in the prevailing understanding TGF-β has been placed in the category of immunosuppressive cytokines since it clearly inhibits T cell activation and differentiation of Th1 and Th2 cells (Gorelik and Flavell, 2002) and accordingly, TGF-β1 deficient mice develop an inflammatory response syndrome at the age of two weeks and usually die at the age of four weeks (Kulkarni et al., 1993). In light of this, it was surprising that TGF-β is necessary for the differentiation of a specific subset of IL-17-producing T helper cells that is now named Th17.

Three independent studies, including our own, independently observed that a combination of the pro-inflammatory cytokine IL-6 and TGF-β could induce the differentiation of Th17 cells from naïve T cells *in vitro* (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006a). The importance of TGF-β in this process has been shown *in vivo* as well. We have demonstrated that mice, which express TGF-β under the IL-2-promoter produced TGF-β upon *ex vivo* stimulation, but developed Th17 cells *in vivo* under inflammatory conditions and elevated concentrations of IL-6 (Bettelli et al., 2006). The increased number of Th17 cells in these animals resulted in the exacerbation of EAE. Consequently, CD4-DNTGFβRII mice, which express a dominant negative mutant of the TGF-β receptor are deficient in Th17 cells and are resistant to the development of EAE (Veldhoen et al., 2006b). Although the exact source of TGF-β for this process *in vivo* is not yet defined, it has recently been demonstrated that mice in which the TGF-β1 gene can be selectively inactivated in T cells develop a lethal inflammatory disorder. The disease is characterized by enhanced Th1 and Th2 responses indicating that T-reg cell-derived TGF-β might be required to control these responses. Interestingly, ablation of TGF-β production from T cells in these mice resulted in a defect in the generation of Th17 cells and the development of EAE (Li et al., 2007). Although the present study does not elucidate which T cell subset or which combination of T cell subsets (T-reg, Th3 cells (Chen et al., 1994), non T-reg) represent the major source of TGF-β, it clearly indicates that T cell-derived TGF-β plays a critical role in the differentiation of Th17 cells *in vivo*. Identifying the T cell-subset that is the essential source of TGF-β will certainly be critical to our understanding of how Th17 cells are generated and regulated *in vivo*.

IL-6 is a cytokine that is strongly induced in cells of the innate immune system upon stimulation of specific pattern recognition receptors such as toll like receptors and C-type lectin receptors. Thus, upon encounter of microbial constituents, IL-6 becomes available in large amounts. In the presence of TGF-β, this re-directs the “default” differentiation of naïve T cells towards Th17 cells. The analysis of IL-6 deficient animals also points to an important role of IL-6 in the differentiation of Th17 cells in autoimmunity since these animals are resistant to the development of EAE (Bettelli et al., 2006; Eugster et al., 1998; Mendel et al., 1998; Okuda et al., 1998; Samoilova et al., 1998) and do not develop a competent Th17 response (Bettelli et al., 2006; Korn et al., 2007a). Therefore, under inflammatory conditions and constant surveillance by regulatory mechanisms, IL-6 and TGF-β are key cytokines for the differentiation of Th17 cells *in vivo*.

4.2. Cytokine network regulating Th17 cell responses

4.2.1. IL-23 and IL-1 enhance Th17 responses—The identification of IL-23, a member of the IL-12 family of cytokines, was instrumental in the discovery of Th17 cells since IL-23 is essential in maintaining and expanding Th17 cell populations. In addition to the prototypic IL-12, the IL-12 family of cytokines now includes IL-23 and IL-27. IL-12 is a heterodimeric cytokine composed of p40 and p35 subunits and signals through a receptor complex made of IL-12Rβ1 and IL-12Rβ2 subunits (Kastelein et al., 2007). IL-23 consists of a specific p19 subunit and shares the p40 subunit with IL-12. IL-23 signals through a heterodimeric receptor complex consisting of IL-12Rβ1 and IL-23R (Kleinschek et al., 2007). Both IL-23 and IL-12 are produced by activated APC such as DC and macrophages. Based on the similarities of the cytokines and their receptors, IL-23 and IL-12 were predicted to have similar functions.

However IL-12R- and IL-23R-signaling are conveyed through STAT4 and STAT3, respectively. In contrast to IL-12, which enhances Th1 responses, IL-23 through STAT3 is essential in maintaining Th17 cells (Yang et al., 2007). Experiments including the analysis of IL-23p19 deficient mice showed a defect in the Th17 cell subset in the absence of IL-23 (Cua et al., 2003; Langrish et al., 2005). Subsequently, Cua and colleagues suggested that IL-23 was an important cytokine for the differentiation of Th17 cells. However, it is now clear that IL-23 is not able to drive the differentiation of naive T cells into the Th17 pathway and naïve T cells do not express the IL-23 receptor (Mangan et al., 2006). But still, IL-23 clearly promotes the proliferation of IL-17-producing cells in the pool of activated memory cells (Aggarwal et al., 2003; Langrish et al., 2005) and thus is important to stabilize and maintain the Th17 phenotype (Veldhoen et al., 2006b).

Besides IL-23, other cytokines may have a role in stabilizing the phenotype of Th17 cells. For example, in IL-1RI deficient mice, the Th17 response is severely compromised and the animals are resistant to EAE. Thus, IL-1 may contribute to the recruitment and maintenance of Th17 cells (Sutton et al., 2006). Although IL-23 can expand Th17 cells independently of IL-1, it has been shown that IL-23 and TNF may also cooperate to induce IL-1 and drive the expansion of Th17 cells (Sutton et al., 2006). Moreover, in animal models of rheumatoid arthritis, IL-17 induces IL-1 in the inflamed synovial tissue (Koenders et al., 2005). Hence, IL-17 may be part of a positive feedback loop driven by IL-1.

4.2.2. IL-2, IL-25 and IL-27 dampen Th17 responses—Th1 and Th2 cells cross-inhibit each other's differentiation. Similarly, both hallmark cytokines of Th1 and Th2 cells, i. e. IFN-γ and IL-4, inhibit the IL-23-driven expansion of Th17 cells (Harrington et al., 2005; Park et al., 2005). Besides IL-23 as a major promoter of Th17 responses and IL-4 and IFN-γ as inhibitors of Th17 development, there is now evidence for a complex cytokine network that controls and eventually shuts down Th17 responses. Recent reports suggest that the IL-12 family member IL-27 is a negative regulator of Th17 cell development. IL-27 is a heterodimeric cytokine composed of Epstein-Barr virus-induced gene 3 (EBI3) and p28 chains. Similar to IL-12 and IL-23, IL-27 is produced by dendritic cells and macrophages. IL-27 signals through a receptor complex composed of the IL-27 receptor chain (IL-27R; also called WSX-1 or TCCR) and the gp130 chain shared with the IL-6 receptor (Hunter, 2005; Pflanz et al., 2002). The absence of IL-27-mediated signaling enhances the generation of Th17 cells, increases the number of IL-17-expressing T cells in tissue infiltrates, and exacerbates neuroinflammation. Hence, IL-27 receptor deficient mice exhibit increased immunopathology in EAE and chronic Toxoplasmosis (Batten et al., 2006; Stumhofer et al., 2006). Furthermore, IL-27-mediated inhibition of Th17 cells is independent of IFN-γR- and IL-6R-signaling, and independent of T-bet, but requires intact STAT1 signaling (Batten et al., 2006; Stumhofer et al., 2006).

In addition to IL-27 which is mainly produced by myeloid cells, IL-25 (IL-17E) which is produced by myeloid cells, but also by Th2 cells, contributes to down-regulate Th17 responses. Indeed, *Il25*$^{-/-}$ mice are highly susceptible to EAE due to an enhanced Th17 response. Conversely, treatment with recombinant IL-25 or IL-25 delivered by a viral vector system is sufficient to suppress EAE in wild type mice (Kleinschek et al., 2007). Although IL-25 is produced by activated Th2 cells, resident cells of the innate immune system like microglial cells are believed to be the major source of IL-25 in CNS autoimmunity. IL-25 inhibits Th17 responses indirectly by inducing IL-13 which decreases the production of IL-23, IL-1 and IL-6 in antigen presenting cells (Kleinschek et al., 2007).

Interestingly, IL-2 which is an important growth factor for Th1 and Th2 cells and is indispensable for the maintenance of Foxp3^{+} T-reg cells in the peripheral immune compartment, inhibits the differentiation of Th17 cells (Veldhoen et al., 2006a). IL-2 through STAT5 inhibits the differentiation of naïve T cells into Th17 cells and accordingly, IL2

deficient and STAT5 deficient $CD4^+$ T cells have a strong propensity to differentiate into Th17 cells. Among others, this may be one of the reasons why *Il2*$^{-/-}$ and *Stat5*$^{-/-}$ mice suffer from early-onset multi-organ autoimmunity. Further understanding of the mechanisms involved in the inhibition of IL-17 by IL-2 and more importantly, identification of factors and signaling pathways involved in the potential reciprocal regulation of induced T-reg and Th17 cells *in vivo* will provide valuable information on ways how to therapeutically manipulate the generation of these cells.

5. The role of Th17 cells in EAE

Since IL-17 was recognized to be increased in human autoimmune diseases like Multiple Sclerosis (Lock et al., 2002; Matusevicius et al., 1999), rheumatoid arthritis (Aarvak et al., 1999), and psoriasis (Teunissen et al., 1998) as well as in animal models of autoimmunity, much attention has been focused on defining the role of Th17 cells in the pathogenic process of tissue inflammation (Steinman, 2007). In the last 3 years the importance of Th17 cells in the pathogenesis of organ-specific autoimmune inflammation has been demonstrated in different animal models. In particular, EAE was instrumental in defining the role of Th17 cells in autoimmunity. Mice deficient in IL-23p19 are resistant to EAE and IL-17-deficient animals develop a delayed and attenuated disease (Komiyama et al., 2006). Furthermore, the administration of an IL-17-blocking antibody to mice immunized with a myelin antigen prevents chemokine expression in the brain and the subsequent development of EAE (Hofstetter et al., 2005; Langrish et al., 2005). These findings were paradigm changing, since previously, Th1 cells were considered to be almost exclusively responsible for driving autoimmune tissue damage (O'Garra et al., 1997). This concept was challenged when it became clear that IFN-γ and IFN-γ-receptor deficient mice as well as mice that lack molecules critically involved in the differentiation and stabilization of the Th1 phenotype like IL-12p35, IL-12 receptor-β2, and IL-18 were not protected from EAE, but developed more severe disease (Gran et al., 2002; Gutcher et al., 2006; Krakowski and Owens, 1996; Tran et al., 2000; Zhang et al., 2003). Furthermore, it was suggested that Th17 cells were more potent than Th1 cells in transferring EAE to naïve wild type host animals (Langrish et al., 2005). Finally, it was shown that IL-23 and not IL-12 was essential for mounting an autopathogenic T cell response (Cua et al., 2003). When IL-23 is not available in order to maintain and expand a population of already primed Th17 cells, EAE is markedly attenuated (Cua et al., 2003). Certain adjuvants like zymosan, a constituent of fungal cell walls, are potent inducers of Th17 cells, but do not result in a sufficiently robust IL-23 production by dendritic cells resulting in a merely temporary Th17 response in the secondary lymphoid tissue without substantial tissue inflammation (Veldhoen et al., 2006b). Furthermore, resident microglial cells and infiltrating macrophages are producers of IL-23 (Cua et al., 2003). Collectively, these findings suggest that Th17 cells are essential inducers of autoimmune tissue inflammation and that IL-23p19 is not only required to shape a stable Th17 population in the secondary lymphoid tissue, but also to maintain an encephalitogenic Th17 population in the CNS.

Although Th17 cells are potent inducers of autoimmunity, it is clear that Th1 cells are also involved in the development of autoimmune responses. However, to date, it is not established what are the kinetics and the specific roles of these two T helper cell subsets during the development of an autoimmune response. Rankings in the pathogenicity of Th17 vs. Th1 cells have to be considered with caution. First, Th17 might be recruited to the target tissue with faster kinetics. Second, Th17 through the induction of chemokines might more readily attract other effector cells to the target organ. Third, Th1 might have to cooperate with Th17 cells in order to induce tissue inflammation. In the natural course of $MOG_{35–55}$-induced EAE, the number of Th17 producing $CD4^+$ T cells in the CNS peaks earlier than that of Th1 cells (Korn et al., 2007b). Thus, Th17 cells might constitute the first wave of effector T cells migrating to the CNS controlling the recruitment of further waves of effector T cells, especially Th1 cells.

In support of this hypothesis, IL-17 is an inducer of MCP-1 (Park et al., 2005) that plays a prominent role in the recruitment of mononuclear cells to the CNS (Fife et al., 2000). By inducing IP-10, Th17 cells might drive the migration of Th1 cells that express the IP-10 receptor CXCR3 (Liu et al., 2006) into the inflamed tissue. This gives rise to the concept of a specific temporal sequence of various T helper cell subsets infiltrating the CNS. Support for this idea comes from studies on the T cell response against *Mycobacterium tuberculosis*. Using specific vaccination strategies against infection with *M. tuberculosis*, it has been recognized that the elicitation of an early Th17 response is essential in promoting a delayed and sustained Th1 response that finally controls the pathogen (Khader et al., 2007). Based on the currently available data, it is intriguing to speculate that a similar pattern of T helper cell dynamics drives immunopathology in organ-specific autoimmunity.

Whereas *in vitro*-differentiation of naive T cells leads to stable lineage commitment, a considerable population of cells secreting both IL-17 and IFN-γ can consistently be observed *in vivo* in the CNS. This suggests that there may be transitional stages of lineage commitment *in vivo*. Notably, there are some reports proposing that lineage commitment of T helper cells may be occurring in the CNS. CNS-derived antigen presenting cells appear to have the capacity to differentially activate and possibly further differentiate Th17 cells, Th1 cells and T-reg cells depending on the phase of EAE (Deshpande et al., 2007). Based on experiments in a relapsing EAE-model where naïve T cells specific for a relapse-inducing PLP-epitope were primed in the CNS to become Th17 cells, it was suggested that myeloid dendritic cells that accumulate in the CNS during EAE skew T cell differentiation towards Th17 cells *in vivo* (Bailey et al., 2007). Furthermore, whereas $CD11c^+$ antigen presenting cells isolated at the onset of disease promoted the proliferation and IL-17-production from naïve T cells, there was no induction of proliferation anymore when $CD11c^+$ antigen presenting cells were recovered at the onset of recovery (Deshpande et al., 2007). These findings are beginning to shed light on the impact of the APC-compartment in the CNS to shape the local Th17 vs. Th1 response. However until reporter mice for IL-17 become available that would allow tracking of IL-17 producing T cells in the CNS *in vivo*, functional analyses of Th17 cells and also T cells that produce IL-17 and IFN-γ simultaneously remain difficult to interpret (Suryani and Sutton, 2007). It will be interesting to define the relation of IL-17/IFN-γ producing T-cells with Th17 cells and Th1 cells from the target organ as well as their relevance for driving the disease process in EAE.

6. The relationship between T-reg and Th17 cells

In vitro, it is clear now that whereas TGF-β induces Foxp3 in naive T cells and thus bona fide T-reg, the combination of TGF-β plus the acute phase protein IL-6 leads to induction of Th17 cells. Thus, the combination of TGF-β plus IL-6 is at present considered as the standard differentiating condition for the generation of Th17 cells from naive T cells. Moreover, TGF-β plus IL-6 induces ROR-γt that has been shown to be the critical lineage determining transcription factor of Th17 cells (Ivanov et al., 2006). The induction of Th17 cells is dichotomously related to the induction of $Foxp3^+$ T-reg and IL-6 is one of the switching factors identified so far that re-directs T cell differentiation from the “default” T-reg pathway into the Th17 pathway. As mentioned above, IL-2 inhibits the generation of Th17 cells, but is required for the maintenance of T-reg cells. IL-2 is also a growth factor for Th1 and Th2 cells further strengthening the argument that a reciprocal relationship might exist between T-reg cells and Th17 cells, but not between T-reg cells and Th1 or Th2 cells.

In order to explore the relevance of these findings *in vivo*, IL-6 deficient mice have been re-analyzed. It has long been known that IL-6 deficient mice resist EAE. A multitude of possible explanations for this phenomenon have been offered ranging from impaired priming of autoantigen specific T cells upon immunization to defective trafficking and re-activation of T cells in the target organ. Recently, we showed by crossing IL-6 deficiency into *Foxp3gfp*.KI

reporter mice that the lack of IL-6 leads to an increased proportion of $Foxp3^+$ T-reg in the peripheral repertoire of these mice (Korn et al., 2007a). Upon immunization with $MOG_{35–55}$, the fraction of $Foxp3^+$ T-reg in the $CD4^+$ T cell compartment is even further increased. This is due to the fact that in *Il6*$^{-/-}$ mice, MOG-specific T-reg are *de novo* generated from naive MOG-specific T cells or preferentially expanded over T-eff. At the same time, the generation of Th17 is defective. Thus, the resistance of *Il6*$^{-/-}$ mice to EAE is due to an immune response in which antigen-specific T-reg are expanded at the expense of the generation of Th17 cells. This suggests that a reciprocal developmental pathway between $Foxp3^+$ T-reg and Th17 cells may be relevant *in vivo* and that IL-6 might be a major switch factor between the generation/expansion of T-reg and Th17 cells. At present, it is not yet resolved whether the high fraction of $Foxp3^+$ T-reg in *Il6*$^{-/-}$ mice is due to expansion or true *de novo* generation of T-reg in the peripheral immune compartment. Thus, the proof of whether the same naive T cell can differentiate into T-reg vs. Th17 cell depending on whether the antigenic stimulus is given under non-inflammatory or inflammatory conditions is still lacking. Adoptive transfer experiments with trackable congenic reporter T cells from *Foxp3gfp*.KI mice will help answer these questions. These experiments have so far only been carried out using CD25 as a T-reg marker and here, antigenic exposure in the absence of inflammatory stimuli suggested that conversion of naive $Foxp3^–$ T cells into $Foxp3^+$ T-reg cells was possible (Apostolou and von Boehmer, 2004).

Collectively, these findings support the idea that under inflammatory conditions as induced by adjuvant or microbial agents, an antigen-specific T-reg response still develops alongside the expansion of antigen-specific T-eff. In the absence of conversion, T-reg expand from the pool of naturally ocurring T-reg. Thus, in an inflammatory environment – and IL-6 might be a major defining factor of this condition – *de novo* generation of T-reg is shut off and Th17 rapidly expand reaching the threshold of disease induction. Moreover, IL-6 not only determines the initial decision in favor of the generation and expansion of pathogenic Th17 cells, but IL-6 also overrides the suppressive action of an already established pool of naturally occurring T-reg in the peripheral immune compartment (Pasare and Medzhitov, 2003). Thus, conversely to *Il6*$^{-/-}$ mice, high levels of IL-6 should be a susceptibility factor for the development of autoimmunity. In accordance with this idea, mice that have a genetically determined high frequency of autoreactive T-cells in the peripheral repertoire eventually develop autoimmune arthritis since after activation, these autoreactive T cells start producing IL-17 and IL-6. This reinforces the further generation of Th17 cells and overcomes T-reg-mediated inhibition resulting in spontaneous autoimmune arthritis (Hirota et al., 2007).

7. Th17 cells and T-reg cells at the site of tissue inflammation

In situ T-reg that bear antigen-specific TcRs are activated and eventually acquire an IL-10 producing phenotype (Herman et al., 2004). In EAE, antigen-specific (MOG $tetramer^+$) T-reg are not essentially delayed in their appearance in the CNS as compared to T-eff (Korn et al., 2007b). However, the number of MOG-specific T-eff in the CNS increases faster than that of MOG-specific T-reg. This suggests that there might be a time window of inefficient regulation in the initial stages of T-cell activation *in situ*. Inefficent suppressive function of T-reg and resistance to suppression of T-eff in the target tissue are potential and not mutually exclusive explanations for this situation. In diabetes, intra-islet T-reg seem to express lower levels of Foxp3 than their lymph node derived counterparts (Wan and Flavell, 2007) and it has been reported that genetically engineered T-reg with low expression of Foxp3 partially lost their capacity to suppress polyclonal T-eff responses *in vitro* and *in vivo* while retaining their anergic state *in vitro* (Wan and Flavell, 2007). Similarly, we have found that the expression level of Foxp3 on a per cell basis was lower in CNS-derived T-reg at the peak of EAE as compared to recovery (unpublished data). However, during any disease phase, CNS-derived T-reg were still efficient in suppressing naive MOG TcR tg responder cells and splenic MOG-specific T-eff

in vitro (Korn et al., 2007b). This led us to speculate whether T-eff in the target organ might be resistant to T-reg-mediated regulation in a cell autonomous way. T-eff themselves might generate a cytokine milieu that impairs T-reg-mediated regulation. Notably, CNS-derived $CD4^+Foxp3^-$ T-eff are resistant to T-reg-mediated suppression when isolated at the onset or at the peak of disease, but are at least partially susceptible when isolated during recovery. The phenomenon of an ongoing destructive autoimmune response despite the presence of $Foxp3^+$ T-reg has also been reported for rheumatoid arthritis (Cao et al., 2006; Ruprecht et al., 2005; van Amelsfort et al., 2004). Intriguingly, the fraction of IL-17^+ T-eff is very high in the CNS in the initial disease phase and through the peak of disease and drops at the transition to recovery while the fraction of IFN-γ-producing $CD4^+$ T cells remains at a high level. The initial disease phases with the highest fraction of Th17 cells are coincident with the most severely impaired T-reg-mediated suppression of CNS-derived T-eff. Mechanistically, IL-6 and TNF, which are produced by CNS-derived T-eff, but not T-eff from the peripheral lymphoid compartment (Langrish et al., 2005) might be responsible for the resistance of T-eff to T-reg-mediated suppression (Korn et al., 2007b). IL-6 and TNF have also been reported to be present in high amounts in the synovial fluid of rheumatoid arthritis-joints. IL-6 may not only be derived from T cells, but high amounts of IL-17 also trigger the production of IL-6 from other myeloid cells in a feedback loop. IL-6 as well as TNF are targets of IL-17 signaling. IL-6 induces hyperproliferation of T-eff. Both T-eff and T-reg differentially express components of the IL-6 receptor thereby providing a potential molecular basis for a qualitatively different response to IL-6 (Oberg et al., 2006).

In summary, we propose that IL-6 and possibly other factors are instrumental in balancing T-reg cells and pathogenic Th17 cells. In the peripheral immune compartment, the mechanism of IL-6 may be twofold: First, IL-6 may skew the differentiation of naive T cells into the Th17 pathway. Second, it may override the suppressive effect of an already established pool of $Foxp3^+$ T-reg to allow for the antigen-specific expansion of effector cells. In the target tissue, the potential of T-reg to control an effector T cell response may vary depending on the composition of the T-eff population. Whereas until the peak of disease the T-eff population is dominated by Th17 cells that either produce IL-6 themselves or by production of IL-17, drive the secretion of IL-6 by myeloid cells, the composition of the $CD4^+$ T-eff population in the CNS changes after the peak of disease in that the fraction of IFN-γ producing T-cells increases. Notably, the resistance of CNS-derived T-eff to T-reg-mediated suppression is highest during the onset until the peak of disease and decreases during recovery. Thus, T-eff recovered from the CNS during the recovery phase are suppressible by CNS-derived T-reg. These data suggest that Th1-dominated T-eff populations might be more easily controlled by T-reg than Th17-dominated T-eff populations. The induction of IL-6 and TNF and possibly other cytokines induced by Th17 cells might be the mechanism underlying the resistance of Th17 cells to T-reg mediated suppression. Thus, the sequential change in the composition of the T-eff population might not only determine the extent of immunopathology, but also the controllability of the T-eff population by T-reg at the site of inflammation.

8. Summary and future perspective

The concept of how T cell-mediated organ-specific autoimmune diseases are triggered has essentially been revisited with the discovery of Th17 cells. At least in relevant rodent models for human autoimmune diseases, Th17 cells are essential in initiating autoimmune tissue inflammation and it is indispensable for starting the disease process that Th17 cells are induced in the secondary lymphoid tissue and maintained in the target organ. IL-6 plus TGF-β have been shown to be the differentiating factors for Th17 cell-generation and IL-23 is a crucial maintenance factor for Th17 cells. The cytokine network that is required to induce, amplify, maintain and regulate Th17 responses is not yet fully understood, but major promoting factors like TGF-β, IL-6, IL-21, IL-23, and IL-1 on one hand, and negative regulators like IL-4, IFN-

γ, IL-25, and IL-27 have been identified. It is very likely that the discovery of new cytokines or the identification of new functions for old cytokines will further enhance our concept of the regulation of Th17 cells. As far as the regulation of autoaggressive T cell-responses is concerned, many questions have arisen with the observation that there might be a close link between pathogenic Th17 cells and $Foxp3^+$ T-reg cells. First, IL-6 and possibly other cytokines are a switch factor to skew the TGF-β-driven generation of $Foxp3^+$ T-reg cells in the peripheral immune compartment into the Th17 pathway. Second, T-reg cells, by producing TGF-β, might contribute to the *de novo* generation of Th17 cells in a highly inflammatory environment where cytokines like IL-6 are available in high amounts. In addition, it still needs meticulous investigation how stable the phenotype of $Foxp3^+$ T-reg cells actually is at sites of tissue inflammation. At the site of inflammation, T-reg cells eventually control T cell-driven inflammation. However, the efficiency of T-reg-mediated suppression of effector T cell populations may depend on the composition of the effector T cell population as far as the fraction of Th17 and Th1 cells in the effector T cell population is concerned. Associated with this, the amount of inflammatory cytokines in the local milieu like IL-6 and TNF may be key to decide whether or not T-reg-mediated suppression actually is operative. Since Th17 cells drive the production of IL-6 and TNF from myeloid cells in the inflammatory infiltrate, it is conceivable that Th17 cells are less amenable to T-reg-mediated suppression than Th1 cells.

Collectively, the amendment of the Th1/Th2 paradigm by new subsets of T cells has resolved some inconsistencies of the Th1/Th2 paradigm, but has also made the understanding of the pathogenic process of organ-specific autoimmune diseases more complicated. Better understanding of the population dynamics and kinetics of Th17 and Th1 cells, and possibly their interplay and susceptibility towards regulatory mechanisms at the site of inflammation will be crucial to define phase specific approaches for therapeutic interventions in order to prevent immunopathology in chronic autoimmune diseases.

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