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REVIEW

Regulatory T cells in experimental autoimmune disease

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Abstract During the past 10 years, $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg) have been extensively studied for their function in autoimmune disease. This review summarizes the evidence for a role of Treg in suppression of innate and adaptive immune responses in experimental models of autoimmunity including arthritis, colitis, diabetes, autoimmune encephalomyelitis, lupus, gastritis, oophoritis, prostatitis, and thyroiditis. Antigen-specific activation of Treg, but antigen-independent suppressive function, emerges as a common paradigm derived from several disease models. Treg suppress conventional T cells (Tcon) by direct cell contact in vitro. However, downmodulation of dendritic cell function and secretion of inhibitory cytokines such as IL-10 and TGF-β might underlie Treg function in vivo. The final outcome of autoimmunity vs tolerance depends on the balance between stimulatory signals (Toll-like receptor engagement, costimulation, and antigen dose) and inhibitory signals from Treg. Whereas most experimental settings analyze the capacity of Treg to prevent onset of autoimmune disease, more recent efforts indicate successful treatment of ongoing disease. Thus, Treg are on the verge of moving from experimental animal models into clinical applications in humans.

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The discovery of $CD4^+CD25^+$ regulatory T cells

Removal of the thymus in neonatal mice (day 3 thymectomy, d3Tx) leads to the development of organ specific autoimmune disease. This model was used by many investigators in the 80s and 90s and yielded first insights into the fact that the normal T cell repertoire contains autoreactive cells. These self-reactive cells could be inhibited by a subpopulation of $CD4^+$ T cells (reviewed in [1]). The breakthrough in defining the protective cell population came in 1995. Sakaguchi et al. [2] removed CD25 expressing cells from the splenic $CD4^+$ population obtained from healthy BALB/c mice and injected the resulting $CD4^+CD25^-$ T cells into athymic BALB/c mice. These recipients developed autoimmune gastritis. Addition of purified $CD25^+$ cells to the $CD4^+CD25^-$ cells at the time of injection protected from disease [2]. Similarly, transfer of spleen cells containing $CD25^+$ cells into d3Tx mice averted gastritis. In contrast, the prevention of homeostatic proliferation alone, via injection of $CD25^-$ spleen cells or irrelevant T-cell receptor (TCR) transgenic cells, was not protective [3]. Finally, gastritis induction by transfer of clonal T cells directed against the H/K-ATPase, the dominant gastric self-antigen responsible for autoimmune gastritis, could be prevented by $CD4^+CD25^+$ spleen cells. Thus, a new suppressor cell population had been defined, and the term regulatory T cells (Treg) was coined to set them apart from $CD8^+$ suppressor T cells. This term was also chosen to indicate that Treg regulate the normal immune homeostasis by preventing the activation of cells bearing TCR specific for self-antigen.

$CD25^+$ cells constitute 5–10% of $CD4^+$ T cells in lymph nodes (LN), spleen and thymus [2, 4]. The fact that thymic $CD25^+$ cells prevent autoimmunity similarly to peripheral Treg and exhibit the same phenotype as peripheral Treg, leads to the conclusion that Treg constitute a separate lineage of T cells that is educated in the thymus [4]. Because d3Tx animals spontaneously develop gastritis, it was hypothesized that three-day-old mice harbor autoreactive effector T cells (Teff) but not yet $CD25^+$ Treg. Indeed, the spleens of these newborn mice contain very few $CD3^+$ cells and among these, $CD25^+$ cells are hardly detectable [5]. Nevertheless, in LN (which are seeded by T cells before the spleen), 5–7% of $CD4^+$ lymphocytes express CD25 already 2 days after birth [6]. However, the density of CD25 expression is lower on days 2 and 3 after birth when compared to older mice. Due to cell number limitations, these neonatal $CD25^+$ cells were not tested for their suppressive capacity.

Phenotype of $CD4^+CD25^+$ regulatory T cells

Freshly isolated murine Treg can be distinguished from freshly isolated "naïve or resting" $CD4^+CD25^-$ Tcon by a variety of cell surface markers. In addition to CD25, the following molecules are expressed at a higher density on the cell surface of Treg in comparison to Tcon: IL-2Rβ-chain (CD122), CD44, CD54 [4], GITR [7, 8], neuropilin-1 [9], and LAG-3 [10]. CTLA-4 (CD152) is constitutively expressed in the cytoplasma of Treg and is absent in Tcon. After stimulation in vitro, Treg and Tcon express cell surface CTLA-4, though expression is higher on Treg [11, 12]. Treg show a heterogeneous expression of markers associated with recent T cell activation or memory. Most Treg are $CD45RB^{int}$ to $CD45RB^{low}$ indicating that they have seen antigen before [13]. L-selectin (CD62L) is responsible for the entry of T cells into primary lymphoid organs and is highly expressed on resting cells and downregulated on activated T cells. Treg can be divided into two groups: some expressing high, others intermediate or low levels of CD62L. This indicates that some Treg home to LN whereas others migrate to peripheral organs [4, 13]. Such a dual expression pattern is also observed for the α_E integrin (CD103). When α_E integrin pairs with the β7-chain, it binds to E-cadherin and constitutes a receptor responsible for retaining Treg in tissues [14, 15]. In mice, one third of Treg express CD69, a marker for recent activation [13]. The observation that many Treg show a cell surface phenotype reminiscent of recent activation is consistent with the fact that they constantly cycle in vivo, which is probably due to the recognition of self-antigen in the periphery [16].

Most of the markers discussed above are dependent on T-cell activation and can be up- or downregulated on Tcon as well. Thus, the identification of the transcriptional repressor scurfin, encoded by the *Foxp3* gene, in Treg was an important discovery for the field [17–19]. Mice and humans with *Foxp3* mutations abrogating scurfin expression (scurfy mice, $Foxp3^{-/-}$ mice and human IPEX patients) lack Treg and develop severe multiorgan autoimmunity [18, 19]. All $CD25^{hi}$ cells express Foxp3 as shown elegantly in the $Foxp3^{gfp}$ and $Foxp3^{mRFP}$ mice from the Rudensky and Flavell laboratory, respectively. A few $Foxp3^{gfp}$ cells express low levels of CD25, but they are nevertheless equally suppressive, indicating that all $Foxp3^+$ cells are Treg [20, 21]. In addition, the overexpression of Foxp3 in Tcon converts them into immunosuppressive cells very similar to natural Treg [17, 19]. These data lead to the conclusion that Foxp3 is the best marker for Treg to date. Unfortunately, its nuclear localization prevents the use of this molecule for the isolation of Treg. However, recent publications demonstrate that Foxp3 can also be induced in Tcon upon stimulation, with TGF-β being a major inducer. As such $Foxp3^+$ Tcon also show suppressive activity they qualify as induced Treg [21–23]. Furthermore, Treg can be generated from Tcon via presentation of antigen by immature dendritic cells (DC) [24, 25].

Development and homeostasis of Treg

Similar to Foxp3 mutations, other genetic alterations can also lead to a lack of Treg. In all these cases, lymphocyte hyperproliferation and multiorgan autoimmunity ensue. Mice devoid of IL-2Rγ-chain (CD132) do not contain Treg because of defective thymic generation [26]. IL-2Rβ-chain (CD122) expression in the thymus is also important for normal Treg development, while reduced survival of Treg in the periphery is the main reason for autoimmunity in IL-2 and IL-2Rα-chain (CD25) knockout mice [26–29]. Other factors necessary for survival or expansion of Treg are TGF-β and CD28 [30–33]. Treg are selected in the thymus at a specific range of affinity to self-antigen that lies in between positive and negative selection for Tcon (reviewed in [34]).

Purified Treg do not proliferate in vitro upon TCR stimulation unless exogenous IL-2 is added to the culture. Thus, they are called anergic [35]. Such an anergy is not observed in vivo. Initially, various laboratories studied Treg from TCR transgenic mice to show that their Treg proliferate in vivo upon recognition of their respective antigens [36, 37]. Furthermore, after transfer of polyclonal Treg into T-cell-deficient "empty" mice, Treg expand via homeostatic proliferation due to recognition of MHC class II restricted antigen [38, 39]. Finally, by transferring $CD62L^{hi}$ Treg into immunocompetent "full" mice, Salomon et al. found that half of the Treg cycled and developed an activated phenotype. This activation and cycling is most likely due to the recognition of tissue specific self-antigens

in the local LN presented on "resting" DC [16, 40]. Thus, two groups of Treg can be identified in mice: (a) resting Treg that are predominantly $CD62L^{hi}$, $CD44^{int}$, $CD69^{-}$, $CD122^{low}$, $CD134^{int}$, $CD71^{low}$, $CD54^{int}$, $CD5^{int}$, $GITR^{int}$, $CD38^{-}$, and $CD45RB^{int}$ and (b) activated Treg that are $CD62L^{hi/low}$, $CD44^{hi}$, $CD69^{+}$, $CD122^{hi}$, $CD134^{hi}$, $CD71^{hi}$, $CD54^{hi}$, $CD5^{hi}$, $GITR^{hi}$, $CD38^{hi}$, and $CD45RB^{low}$ [16]. While both populations exhibit a similar suppressive capacity in vitro, they most probably display a different homing pattern (LN vs peripheral tissues), and therefore might fulfill different functions in vivo.

Description of the main autoimmune disease models

After their discovery due to their ability to suppress autoimmune gastritis, thyroiditis, or oophoritis, the presence and function of Treg have been studied in mice, rats, and humans. We shall focus on the function of Treg in the most commonly studied diseases in mice. The induction of autoimmunity requires an imbalance of immune stimulation and suppression. Treg depletion in healthy adult mice does not result in autoimmunity [41]. However, if this is accompanied with a further stimulus such as homeostatic proliferation, e.g., in newborn mice or after T cell transfer into T-cell-deficient mice, autoimmune disease ensues [41]. Alternatively, immunization with a tissue-specific antigen together with adjuvant induces experimental allergic/autoimmune encephalomyelitis (EAE), experimental autoimmune thyroiditis (EAT), or experimental autoimmune myasthenia gravis (EAMG) in susceptible mouse strains. Treg have been shown to ameliorate or prevent these diseases [42–44]. T cells expressing a self-antigen-specific TCR induce autoimmunity in the absence of endogenous Treg, e.g., EAE, and diabetes. Finally, we shall summarize the various functional aspects of Treg-mediated suppression to show emerging paradigms. Because requirements and circumstances vary between diseases, different results can be obtained and a generalization of mechanism should be undertaken with caution.

Autoimmune gastritis

Early studies on d3Tx-induced autoimmune gastritis were successful in determining H/K-ATPase α-and β-chains as the main autoantigens similar to the pathogenesis of pernicious anemia in humans. Gastritis can be induced by the transfer of T cells specific for these antigens into T/B-cell-deficient animals or animals that contain TCR transgenic irrelevant T cells. In contrast, normal mice and mice coinjected with splenic Treg are resistant to gastritis induction by H/K-ATPase-specific T cells. Thus, mice containing a polyclonal repertoire of Treg inhibit antigen-specific Teff [3]. Treg depletion is necessary but not sufficient to induce gastritis. It is only when cells from adult mice that had previously received the depleting anti-CD25 mAb PC61 are transferred to T cell-deficient "empty" nude mice, that disease ensues. This indicates that homeostatic proliferation, taking place after transfer into nude mice, provides an activation signal for the transferred cells. Alternatively, this activation signal can also be provided by immunization with H/K-ATPase in incomplete Freund's adjuvant [42]. Autoreactive T cells are most probably activated in the gastric LN by DC that constantly present H/K-ATPase [40]. Subsequently, T cells migrate to the gastric mucosa. After injection of high numbers of polyclonal Treg, they can be detected in the gastric LN and the mucosa. However, they do not prevent expansion of H/K-ATPase-specific T cells in the gastric LN or their migration into the mucosa, thus allowing mild inflammation. In contrast, absence of Treg increases inflammation and destruction of parietal and chief cells with concomitant production of autoantibodies [45]. Polyclonal Treg inhibit gastritis induced by polyclonal or antigen-specific Teff. Moreover, Treg educated in the absence of the H/K-ATPase α-chain suppress gastritis induced by H/K-ATPase α-chain-specific Teff [46]. This can be explained by the presence of Treg directed against other gastric antigens.

Treg suppress the differentiation of autoreactive T cells into Th1 effector cells, as shown by a decrease in antigen-specific IFN-γ production [45]. For prevention of gastritis, Treg do not need to produce IL-4, IL-10, or TGF-β. Treg isolated from these cytokine knockout mice inhibit gastritis and the injection of mAb blocking these cytokines into mice receiving wildtype Treg does not interfere with protection [47–50]. With regard to CLTA-4, one study showed that blocking of Treg induced protection in gastritis while another observed no abrogation of tolerance upon CTLA-4 blockade. The role, if any, of CTLA-4 in the suppressive mechanism in vitro is also unclear [12, 48]. Mice treated with anti-GITR mAb develop gastritis [8]. It is unclear, and rather unlikely, that this treatment directly modulates Treg activity. Results from in vitro suppression assays show that (a) GITR−/− Treg suppress well and (b) the anti-GITR mAb costimulates Teff, which then resist suppression [50].

Other autoimmune diseases induced by d3Tx (prostatitis, oophoritis, and thyroiditis)

Most organs show inflammation and destruction after d3Tx or after injection of $CD4^{+}CD25^{-}$ cells into nude mice. The incidence of the involvement of different organs varies between strains. The following diseases have been described: gastritis, thyroiditis, oophoritis, prostatitis, sialoadenitis, glomerulonephritis, epididymitis, arthritis, dacryoadenitis, neuropathy, etc. [2, 29]. Nevertheless,

although more than one single organ can be affected in a particular mouse, it should be stressed that the disease is completely organ-specific with no evidence of systemic autoimmune disease. We shall focus on studies regarding the antigen specificity of Treg. Already in 1999, Seddon and Mason [51] demonstrated that $CD4^+CD45RC^-$ suppressive T cells from athyroid rats were unable to prevent thyroiditis, while they still prevented diabetes. In contrast, thymocytes prevented both diseases. Subsequent analysis of d3Tx animals revealed that Treg adoptively transferred from male mice were better at suppressing prostatitis than Treg from female mice, or from males without prostates [52]. Surprisingly, male Treg can suppress d3Tx-induced oophoritis as potently as female Treg [53]. The explanation for this discrepancy lies in the different postnatal onset of ovarian and prostate antigen expression. The ovarian-antigens mater and ZP3 are present from birth onwards. Therefore, male splenic Treg injected a few days after thymectomy recognize ovarian antigens in the host, expand ovarian antigen-specific Treg and, thus, suppress oophoritis which takes 6 weeks to develop. If female mice were ovariectomized at birth, thymectomized at day 3, and then received ovarian grafts at 3 weeks, 63% developed oophoritis. This was completely prevented by the injection of female but not male Treg on day 5. Given that male Treg encounter ovarian antigen in the periphery of ovariectomized mice only after ovarian graft transfer (3 weeks later), it is likely that expansion of ovarian antigen-specific Treg is insufficient for prevention of oophoritis [52, 53]. In contrast, the main prostate antigens EAPA1 and EAPA2 are expressed only after puberty, and this explains the lack of protection from prostatitis by adoptively transferred female Treg or Treg from prostatectomized males [52]. If EAPA1 and EAPA2 are already missing in the thymus, such as in $Aire^{-/-}$ mice, prostatitis develops spontaneously [52]. This demonstrates the need for EAPA1/2-specific Treg derived from the thymus for prevention of prostatitis. These results indicate that (a) expression of self-antigen in the thymus, and (b) further presence of this antigen in the periphery, are needed to retain sizable numbers of organ-specific Treg. However, it is not clear if the thymic and the peripheral antigen(s) that select Treg are identical.

Studies on the prevention of oophoritis also showed that the draining LN is the major site of Teff inhibition by Treg, as well as of accumulation of ovarian antigen-specific Treg. Treg reisolated from ovarian LN of protected d3Tx mice were much more potent in suppressing ovarian disease upon transfer into a second recipient than Treg from other LN or spleen. In contrast, inflammation of the lacrimal glands (dacryoadenitis) was less well inhibited by the Treg from ovarian LN than from other LN [53]. Thus, ovarian LN were enriched in Treg preventing oophoritis while Treg of other specificities were diminished.

Inflammatory bowel diseases

Inflammatory bowel diseases encompass Crohn's disease and ulcerative colitis and are chronic inflammatory diseases of the gastrointestinal tract. Patients and mice with colitis mount an aberrant response against the bacterial flora of the gut. While the development of inflammatory bowel diseases (IBD) in mice has not been attributed to one single common pathogen, it has been shown that *Helicobacter hepaticus* can induce colitis in models where Treg are absent or malfunctioning [54]. Many different murine models of colitis have been described, e.g., transfer of $CD45RB^{hi}$ cells to SCID or $RAG\text{-}2^{-/-}$ mice, tgε26 mice transplanted with bone marrow, as well as various cytokine knockout mice ($IL\text{-}10^{-/-}$, $IL\text{-}2^{-/-}$, and $TGF\text{-}\beta^{-/-}$) and $CTLA\text{-}4^{-/-}$ mice [54, 55]. A recent article describes a model for Crohn's disease in which expanded B cells block Treg function [56]. Because most of the paradigms of colitis prevention by Treg have been unraveled in the "$CD45RB^{hi}$ cell transfer system", we shall concentrate on these studies. Briefly, the injection of $CD45RB^{hi}$ naïve T cells into SCID or $RAG\text{-}2^{-/-}$ mice induces colitis. This is prevented by simultaneous transfer of $CD25^+$ cells (or $CD45RB^{low}$ cells that are enriched in $CD25^+$ cells) [54]. Importantly, infusion of Treg can also cure ongoing colitis [57, 58]. This cure is associated with a migration of injected Treg into the mesenteric LN and the colon, where they expand and suppress proliferation of Teff. In fact, 10 weeks after injection of Treg, when the colon architecture has normalized, the number of $Ki67^+$ proliferating cells has declined from 30 to 5% in both Teff and Treg [57, 59]. It has been suggested that CD103 paired with β7 integrin allows Treg to home to the gut or other inflamed sites [14, 15]. However, the use of cells from $CD103^{-/-}$ or integrin $\beta7^{-/-}$ mice showed that Treg do not need to express these molecules to prevent colitis [60, 61].

Studies evaluating the mechanism by which Treg suppress the formation of colitis indicate that many factors contribute to the inhibition of Teff activation. First, the fact that $IL\text{-}10^{-/-}$ and normal adult mice treated with anti-IL-10 mAb develop colitis in the presence of a normal flora indicates the importance of IL-10 for the suppression of inflammation [54]. However, if disease is induced by wildtype $CD45RB^{hi}$ cells, the suppressing Treg do not have to produce IL-10 themselves [62]. The second crucial cytokine for the prevention of colitis is TGF-β. $TGF\text{-}\beta^{-/-}$ mice develop colitis and anti-TGF-β mAb abrogates Treg-mediated suppression of colitis [54]. It is crucial that the colitogenic Teff respond to TGF-β, which could be produced by any host cell and are not necessarily derived from the Treg itself [63, 64] (for additional discussion see [54]). TGF-β is further needed for the survival and function of Treg [30, 31, 65]. Finally, as TGF-β is abundant in the

gut associated lymphoid tissue (GALT), it may locally convert Tcon into Treg and thereby shift the balance towards immune tolerance [23, 66, 67]. Regarding the family of costimulatory molecules, injection of anti-CTLA-4 mAb abrogates protection of colitis after the cotransfer of $CD45RB^{hi}$ cells together with $CD25^{+}$ cells [11, 68]. Regulatory T cells devoid of CD28 are still able to protect from colitis, while the absence of CD28 and ICOS abrogates their function [68, 69].

The antigen specificity of Treg in inflammatory bowel disease has not yet been established. Treg isolated from germ-free mice inhibit colitis [70, 71], and Treg from *H. hepaticus* uninfected donors are as efficient in preventing colitis as those from infected donors [54]. Teff are constantly reacting to the antigenic stimulation in the gut and when Treg are depleted, Teff start to produce inflammatory cytokines [72]. These data indicate that in the colitis model, Teff are not educated to stay anergic (no "infectious tolerance") nor do they convert into regulatory cells.

Experimental allergic/autoimmune encephalomyelitis

In contrast to many other autoimmune diseases, EAE is not observed after d3Tx [73]. Instead, it can be induced via immunization with neural self-antigens [myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MPB), and proteolipid protein (PLP)] in complete Freund's adjuvant in susceptible mice and rats. Pertussis toxin is often injected in addition to open the blood brain barrier (active EAE induction). Alternatively, mice develop EAE upon the injection of autoreactive T cells expanded with these central nervous system (CNS) antigens in vitro (passive EAE transfer). We refer to these two models as "induced EAE", which should be separated from "spontaneous EAE" models in TCR transgenic mice [73]. As both strength and modality of the triggered autoimmune response differ, the course of disease (monophasic/biphasic/relapsing–remitting) is also very different between the models. This in turn can influence both Treg function and Treg-independent tolerance mechanisms.

All mice bearing transgenic T cells with specificity for MBP spontaneously develop EAE when crossed with RAG-$1^{-/-}$ mice [73, 74]. These MBP-specific TCR-transgenic RAG-$1^{-/-}$ mice lack Treg in contrast to MPB-specific TCR-transgenic RAG-$1^{+/+}$ mice that contain low numbers of Treg and do not develop EAE [74]. This protection depends on the presence of the transgenic MBP-specific TCR in conjunction with endogenous TCR α-chains on Treg in the TCR-transgenic RAG-$1^{+/+}$ mice [74]. Such a need for endogenous TCR α-chains for the generation of protective Treg is also seen in the BDC–NOD diabetes model [75]. In contrast, transgenic Teff are present in the same animals and mediate autoimmune destruction. These data imply that the intrathymic Treg commitment of TCR-transgenic cells fails without recombination of endogenous TCR chains. In fact, selection of thymocytes into the Treg lineage occurs at a higher affinity than selection of Tcon [34]. Another interesting observation emanating from the study of MBP-specific TCR-transgenic mice is that $CD4^{+}CD25^{-}$ T cells of the same TCR-specificity also protected from EAE. It is possible that (a) $Foxp3^{+}$ Treg might be able to up- and downregulate CD25 or (b) other suppressive CD4 populations prevent EAE [73, 76].

When EAE is induced by active immunization or by passive transfer of autoantigen-reactive T cells, pretreatment of mice with polyclonal Treg ameliorates severity in MBP- [77], MOG- [36, 42, 78], and PLP-induced [79, 80] EAE. Conversely, depletion of Treg by anti-CD25 mAb (PC61) previous to EAE induction increases mortality and morbidity of PLP- or MOG-induced EAE [78, 79]. Moreover, EAE-induction with otherwise insufficient doses of PLP-peptide is possible when mice are depleted from Treg before EAE induction [81]. Furthermore, the mild and monophasic MOG–EAE turns into a more severe form of EAE when Treg are depleted before EAE-induction and a second relapse can be induced in Treg depleted B6 mice [78]. Antibody based depletion of Treg alone is not sufficient to induce EAE without any further immunization with self-antigen [82]. As a caution in judging Treg depletion experiments, it should also be mentioned here that (a) Treg depletion by anti-CD25 mAb may not be sufficient to deplete all $Foxp3^{+}$ Treg cells [81], and (b) CD25-expressing autoreactive Teff can also be diminished by anti-CD25 mAb treatment [76]. Collectively, frequencies of Treg are probably critical because lower numbers of antigen-specific Treg are found in strains which are more susceptible to PLP–EAE than in strains with resistance towards PLP–EAE [83].

Although the natural recovery from monophasic MOG–EAE is associated with an accumulation of $CD4^{+}CD25^{+}$ T cells in the CNS [78], little is known about the in vivo mechanism of EAE suppression by Treg. Current data support a model of antigen-specific Treg activation in the draining LN during the early phase of EAE [84], followed by accumulation of Treg in the CNS in later stages when CNS-inflammation declines [78, 81]. Treg isolated from CNS but not from LN are consistently reported as a major source of IL-10 in EAE [78, 80, 82]. IL-10-dependent suppression might explain bystander suppression in models where Teff and Treg express different Ag-specificity [80]. During recovery from PLP-induced EAE, Treg show enhanced TGF-β precursor peptide expression (LAP) and anti-TGF-β or anti-CD25 administration in the recovery phase leads to EAE relapse [85]. This implies that Treg and TGF-β are required for recovery from EAE. However, the possible cellular sources of TGF-β should be further investigated. In addition, the local targets for suppression, e.g., Teff (CD4 or CD8), B cells, DC or macrophages, need to be defined. Recent efforts to prove an effect of Treg on

expansion, migration, or differentiation of pathogenic T cells in PLP–EAE failed [81].

The role of antigen specificity for the suppressive function of Treg has also been addressed in various EAE models. On the one hand, as described for gastritis, polyclonal Treg have been shown to protect from EAE caused by MBP-specific TCR-transgenic Teff [74, 81]. On the other hand, PLP1-specific transgenic Treg could suppress EAE induced by PLP1 or CNS homogenate, but not EAE induced by MBP or MOG peptide unless they had been preactivated before injection [80]. Together, these data reinforce the in vitro finding that Treg must be activated by their corresponding antigen, but they can then suppress Teff of other specificities.

Because it has become clear that Treg can prevent EAE, many groups are trying to activate Treg or convert $CD4^+CD25^-$ Tcon into Treg with the help of drugs [86, 87]. The efficacy of these treatments is not yet clear. It has also not been firmly established if $Foxp3^-$ true Tcon can indeed be converted into Treg, e.g., MBP-specific TCR-transgenic Tcon in Rag-$1^{-/-}$ mice could not be induced to become suppressive [77]. Furthermore, although Treg have been reported to accumulate during EAE recovery in the brain [78], little data is available on the immunosuppressive potency of Treg to break ongoing EAE. The most promising experiment in this direction was the therapeutic expansion of Treg by superagonistic anti-CD28 mAb after onset of EAE, which was able to ameliorate the course of the disease [88]. Thus, even activated myelin-specific effector T cells in the CNS might be suppressed by potent Treg.

Diabetes in nonobese diabetic and TCR transgenic mice

A protective role of Treg for the prevention of diabetes was already seen when Sakaguchi and colleagues detected insulitis in some nude recipients of $CD4^+CD25^-$ T cells [2]. Subsequently, natural $CD4^+CD25^+$ Treg have been studied both in spontaneous (i.e., NOD mouse, BB rat) and inducible (i.e., Streptozocin induced diabetic rat) animal models of Insulin Dependent Diabetes Mellitus (IDDM). The most frequently used model to study Treg biology in IDDM is the nonobese diabetic (NOD) mouse and we shall limit our discussion to this model. NOD mice spontaneously develop T cell-dependent autoimmune diseases such as thyroiditis, sialadenitis, peripheral polyneuropathy in addition to IDDM, due to multiple immune (regulatory) defects (reviewed in [89, 90]. NOD mice develop a mild peri-insulitis around 4 weeks after birth which then changes to an aggressive massive insulitis with increased production of Th1 cytokines around 12 weeks of age [90]. Overt diabetes is more frequent among female (60–80%) than male (20–30%) NOD mice [89].

NOD.$CD28^{-/-}$ and NOD.$B7^{-/-}$ mice show accelerated induction of diabetes compared to NOD mice and further evaluation revealed a lack of Treg in these substrains. Disruption of the B7/CD28 pathway crucially affects both thymic development and peripheral homeostasis of Treg [32, 91]. Infusion of Treg from young wildtype NOD mice prevents disease in NOD.$CD28^{-/-}$ mice [90, 91]. NOD mice also exhibit multiple immune-regulatory defects and disequilibrium between Tcon and Treg in NOD has initially been suggested to precede excessive activation of islet antigen-specific Th1 cells [92]. However, other reports did not observe any numerical reduction of Treg in young NOD mice [90, 93], and Pop et al. [94] determined a rather late age-dependent decline of $Foxp3^+$ Treg around the onset of insulitis at 8–16 weeks.

Other means to accelerate the onset of diabetes include the transfer of diabetogenic Teff from prediabetic NOD mice to NOD.SCID, NOD.RAG-$1^{-/-}$, neonatal, or irradiated NOD mice. In all these instances, homeostatic proliferation of the transferred T cells is observed and may contribute to accelerated T cell activation and subsequent earlier rise of glucose levels. As Treg interfere with homeostatic expansion [39], it is possible that the prevention of diabetes in this model is a side effect of preventing homeostatic expansion. Likewise, acceleration of diabetes onset caused by neonatal thymectomy, cyclophosphamide, or sublethal irradiation could be due to such homeostatic effects or the depletion of Treg [90].

A lymphopenia-independent model is the BDC2.5 mouse. T cells of these mice express the TCR of a diabetogenic $CD4^+$, Th1-like T cell clone recognizing an unknown islet antigen presented by the NOD MHC II molecule I-A^{g7} [75]. In contrast to wildtype NOD mice, BDC2.5.NOD mice exhibit very synchronous disease, but mostly no overt diabetes. This protection is reverted when BDC2.5.NOD are crossed with RAG-$1^{-/-}$ mice (BDC2.5.NOD.RAG-$1^{-/-}$), which rapidly present full-blown diabetes [75]. As mentioned above for EAE, the deficiency to recombine endogenous TCR leads to the elimination of Treg development in TCR-transgenic RAG-$1^{-/-}$ mice. Injection of wildtype Treg protects BDC2.5.NOD.RAG-$1^{-/-}$ mice from diabetes. An elegant model that specifically ablates all Treg without creating completely artificial TCR-monospecific mice, are BDC2.5.NOD.$Foxp3^{-/-}$ mice [95]. Together with the availability of NOD.BDC2.5.Thy-1.1.Yeti mice [96], which allow detection of IFN-γ producing auto-aggressive Tcon in vivo, these murine diabetes models should allow new insights into the Treg biology in IDDM.

A major open question about Treg-mediated suppression concerns the location at which tolerance induction occurs in IDDM in vivo. NOD Treg transferred into NOD.$CD28^{-/-}$ mice preferentially accumulate in the pancreatic LN and islet regions of the pancreas [90]. Several reports have suggested that the majority of Treg are actively suppressing Teff function in the pancreas tissue, rather than in the pancreatic

LN [90, 95, 97]. Moreover, initial priming of Teff, as determined by measuring cytokine production, proliferation, chemokine, and costimulatory molecule expression of Teff in the pancreatic LN was not changed [90, 95]. The outcome of such experiments probably depends on the antigen specificity of the transferred cells and on the timing of cell transfers. BDC2.5 Teff only proliferate in the pancreatic LN where they recognize islet self-antigen. This proliferation is only marginally inhibited by wildtype NOD Treg. However, BDC2.5 Treg injected 2 days before BDC2.5 or 4.1 TCR-transgenic Teff completely inhibited the proliferation of both of these islet-specific Teff. Furthermore, IFN-γ production, as measured in vivo using BDC2.5 Yeti cells, was also suppressed. To detect IFN-γ-producing cells, directly ex vivo without artificial restimulation of Teff in vitro is a great advantage because in vitro restimulation could overcome inhibition. In summary, islet-specific Treg home to the pancreatic LN and efficiently prevent priming of autoreactive Tcon cells when Treg are present in the draining LN before arrival of Tcon [96]. Most importantly, two-photon laser-scanning microscopy of pancreatic LN showed stable DC–Treg cluster and no stable Treg–Tcon cluster, suggesting a DC-mediated Treg suppression rather than a direct effect of Treg on Tcon in vivo [96]. Such an inhibition of DC by Treg is very potent in the absence of DC-help via CD40L and completely suppressed diabetes in NOD.CD154$^{-/-}$ mice [98]. Targeting islet antigen to immature DC and, thus, increasing antigen-specific Treg has been shown to be a promising avenue for novel Treg-based strategies of diabetes prevention in the prediabetic organism [99].

In conclusion, many laboratories confirm that BDC2.5 Treg with their enriched anti-islet repertoire, as well as in vitro expanded islet antigen-enriched NOD Treg are more potent in suppressing diabetes than wildtype NOD Treg [95, 96, 100–102]. Such in vitro expanded islet-specific Treg may even be used to cure diabetes [102]. Furthermore, Treg isolated from the pancreatic LN are very potent in inhibiting diabetes while Treg from nonpancreatic LN are not [90, 92] and CD62L^{hi} CCR7^{+} NOD splenic Treg suppress diabetes much better than CD62L^{-} NOD splenic Treg [103]. It remains to be seen whether Treg use different mechanisms to suppress Teff activation in the pancreatic LN vs inflammation in the pancreas. Although it is not yet clear if suppression of diabetes by Treg proceeds via cytokines [90], systemic expression of IL-10 or expression of TGF-β in the pancreas have been shown to prevent or even cure diabetes most likely via expansion of Treg [65, 104, 105].

Regulatory T cells in systemic autoimmune disease (lupus and arthritis)

Antinuclear Ab and glomerulonephritis were detected in some of the nude mice that received CD4^{+}CD25^{-} cells, indicating that systemic autoimmune disease might also be controlled by regulatory cells [2]. Indeed, d3Tx of lupus prone New Zealand Mixed 2328 (NZM2328) mice leads to an acceleration of lupus glomerulonephritis, as well as to extrarenal autoimmune disease. It is interesting to note that most of these extrarenal diseases (prostatitis, thyroiditis, and dacryoadenitis) can be suppressed by the injection of purified CD25^{+} cells from young NZM2328 mice, while glomerulonephritis and sialoadenitis are not affected [106]. This is not due to a polyclonal suppression of B cells as hypergammaglobulinemia is not affected even though anti-dsDNA Ab titers are reduced. These data point to a selective defect of Treg for specific antigens rather than a global Treg defect. This should be considered when studying human autoimmune disease(s) where a polyclonal Treg defect seems to be the focus of most clinical research on Treg. Increased numbers of CD25^{+} cells have been found in CD95-deficient lupus prone mice and CD25 cells in CD95/CD95L double-deficient BALB/c *lpr/lpr gld/gld* mice all express Foxp3 [107–109]. Treg are able to suppress B cell activation directly, as well as indirectly via inhibition of T cell help [107–109]. Very recent data indicate that preactivated Treg also kill B cells in vitro, and the implications of this for autoimmune diseases and B cell responses need to be analyzed [110]. Moreover, the possibility that autoimmunity could also occur due to aberrant signaling in Teff, rendering them resistant to suppression by Treg as described for MRL/Mp lupus prone mice [111], should be considered.

One animal model for the study of arthritis is the immunization of mice with bovine or chicken collagen and complete Freund's adjuvant (collagen-induced arthritis). The severity and incidence of arthritis is increased when mice are depleted of Treg and reduced when mice are injected with high numbers of Treg [14, 112]. Such an ameliorating effect can still be discerned when Treg are injected after onset of early disease symptoms. Because the overall T cell and antibody response to collagen II is not altered in these mice, the decrease in symptoms is probably due to local suppression of inflammation in the joint. Indeed, Treg immigrated into the inflamed synovial tissue and the synovial fluid [113].

Principles of immunosuppression mediated by Treg

Amelioration vs prevention and cure of autoimmune disease

Most experimental settings analyzed so far tested Treg for their capacity to prevent autoimmune disease. In models of "spontaneous" development of autoimmunity such as in the d3Tx model or in TCR transgenic mice, Treg

completely prevent autoimmune disease, even though in some settings a mild nonaggressive inflammation can be found. In the case of immunization with self-antigen and adjuvant, Treg can often only ameliorate disease. The adjuvant leads to a strong activation of antigen-presenting cells and, thus, to a powerful stimulation of T cells, which can no longer efficiently be counteracted by Treg. Exciting recent data indicate that injection of Treg even cures ongoing autoimmune disease. This has been shown in models of colitis and diabetes [57, 101]. In particular, high scale in vitro expansion of Treg specific for tissue self-antigens and their injection into patients holds great promise.

Migration of Treg and localization of suppression

The site of Treg function in vivo could be the draining LN, the respective target organ or both. Current research has just begun to address this question and data are still controversial, which could also be due to the different models being analyzed. Treg migrate to the draining LN, as well as to the inflamed organ [45, 114]. In addition, tissue antigen-specific Treg accumulate preferentially in the draining LN compared to nondraining LN [53]. The efficacy of this migration depends on Treg subpopulations, with $CD62L^+$ cells migrating more to the draining LN and $CD103^+$ cells migrating more to the site of inflammation [15, 115]. While Treg express chemokine receptors (e.g., CCR4 and CCR8), the relative importance of these for their migration to LN, draining LN, and inflamed tissue needs further evaluation. Clearly, CCL22 derived from macrophages, DC, or microglia attracts $CCR4^+$ Treg. This has been shown in tumor and transplantation settings [79, 116].

Upon simultaneous injection of polyclonal Treg and antigen-specific Teff, the expansion of Teff in the draining LN and their migration to the target organ is not inhibited, while aggressive tissue destruction in the organ is stopped in gastritis, as well as in NOD mice [45, 90, 95, 97]. In contrast, Samy et al. [53] reported that Treg inhibit expansion, activation marker expression, and cytokine release by Teff in the ovarian LN of d3Tx mice. In this model, Treg are injected at day 5 of age, and the antigen-specific Treg had time to expand in the draining LN. Similarly, islet antigen-specific Treg injected before Teff completely inhibit Teff activation [96]. Concerning treatment of ongoing autoimmune disease, it would be preferable if Treg could inhibit Teff in the inflamed tissue as well as in the draining LN.

Target cells and mechanism of suppression

While early work has concentrated on suppression of $CD4^+$ cells, recent data show that in vitro activation of CD8 cells, B cells, natural killer (NK) cells, NKT cells, DC, and macrophages can be inhibited by Treg (see Table 1) [107, 117–121]. Because all these cell types interact during the immune response in vivo, it is difficult to determine which cells are affected by Treg in the various diseases. Nevertheless, using models with defined tissue-destructive cell populations, it has also become evident that CD8, B, NK cells, and other innate immune cells are also downmodulated by Treg in vivo [109, 114, 119, 122].

Inhibition of T cells in vitro requires direct cell contact between Treg and Teff. Upon activation, Treg secrete IL-10 and TGF-β. While most studies do not find a role for IL-10 in in vitro suppression assays, there is still debate if membrane-bound TGF-β on Treg could be important for Teff suppression [35]. Crosslinking GITR costimulates Teff and, therefore, abrogates inhibition in cocultures [50]. The importance of inhibitory cytokines in vivo depends very much on the system studied (Table 2) where three scenarios, as exemplified for IL-10, are possible. First, IL-10 is secreted by Treg [78, 80] and this IL-10 secretion is mandatory for suppression, e.g., in EAE [82]. Second, IL-10 is crucial for the inhibition of disease, but locally produced IL-10 can help $IL\text{-}10^{-/-}$ Treg to dampen inflammation, e.g., colitis [62]. Third, in gastritis suppression is independent of IL-10 [47, 48]. TGF-β is a very pleiotrophic inhibitory cytokine that is involved in the suppression of many cell types in vitro and in vivo (CD8 cells [122], B cells [107], NK cells [119], or other innate immune cells [114]). TGF-β is needed for protection from many autoimmune diseases, although again, at least in colitis, local production of TGF-β can help $TGF\text{-}\beta^{-/-}$ Treg to curb inflammation [63, 64]. The finding that colitis prevention is dependent on IL-10 and TGF-β, while gastritis is not [47, 48], could be due to the fact that (a) colitis is induced by the bacterial flora and due to TLR-signals which constitute a stronger immune activation compared to self-antigen, and/or (b) these cytokines are needed for the suppression of the innate immune system activated in colitis [123].

Activated Treg express granzyme B and kill B cell blasts in a perforin- and CD95L-independent mechanism via Granzyme release [110, 118]. Further studies should clarify if this occurs in vivo and if other cells could be killed by the same mechanism.

Although in vitro studies demonstrate direct inhibition of T cells, B cells, and NK cells by Treg, it is not clear if this also happens in vivo. Recent real time imaging in LN does not show a long interaction time between Teff and Treg. In contrast, a clear interaction between Treg and antigen-bearing DC can be noted [96]. As various in vitro and in vivo data observe a restrained maturation of DC in the presence of Treg [98, 124, 125], the possibility that Teff

Table 1 Cells targeted by Treg

Target cells	Mechanism	Disease	References
$CD4^+$ T helper cell	IL-10 ?TGF-β ?	Autoimmune diseases, cancer, infection etc.	See text
$CD8^+$ cytotoxic T cell	TGF-β	Cancer	[117, 122]
B cell	TGF-β, killing	Lupus erythematodes	[110]
NK cell	TGF-β	Cancer	[119]
NKT cell			[120]
DC	?	Diabetes	[96, 98]

activation in the presence of Treg is due to inadequate stimulation from antigen presenting cells should be further analyzed.

Antigen specificity of regulatory T cells

Initial studies of the Treg repertoire indicated that they are polyclonal like Tcon and no difference in the overall TCR repertoire could be defined. However, the examination of TCR-transgenic mice revealed that Tcon and Treg are selected at different TCR interaction strength [34]. Furthermore, Hsieh et al. [126] demonstrated that the TCR repertoire of Tcon and Treg is equally diverse but only partly overlapping. Thus, when a TCR-transgene in combination with RAG-deficiency or lack of TCR α-chains restricts T cells to only one single TCR specific for MBP or islet antigen, Treg cannot develop and EAE or diabetes ensues. Treg only develop in the presence of endogenous TCR α-chains and then prevent disease in transgenic mice [34, 74, 75, 127].

Prevention or cure of autoimmune disease has mostly been studied using polyclonal populations of Treg. It is interesting to note that polyclonal Treg protect from disease induced by monoclonal tissue specific Teff [45, 74]. Nevertheless, transgenic tissue-specific Treg (e.g., BDC2.5 TCR-transgenic, islet mimotope p31-specific or MBP-specific Treg) are more potent in suppressing diabetes or EAE in that fewer cells are needed for a similar protective effect compared to polyclonal Treg [74, 101, 102]. Especially if a fast onset diabetes model is used, like the transfer of BDC2.5 Teff cells into NOD.RAG-$1^{-/-}$ mice, only islet-specific Treg injected before Teff can potently abrogate Teff priming in the draining LN [96, 97]. Islet antigen-specific Treg are very potent in suppressing diabetes, while the destruction of salivary and thyroid glands in the recipient NOD mice is not affected [100]. Thus, different sets of Treg protect different organs, as already discussed in the oophoritis and prostatitis section [52, 53]. In vitro suppression experiments have shown that the suppression itself is antigen-nonspecific. However, it is important that Treg recognize antigen to become activated. Therefore, it is generally believed that bystander suppression occurs and that all Tcon in close vicinity to a suppressive Treg are inhibited. This could explain why Treg from mice lacking H/K-ATPase nevertheless prevent gastritis induced by H/K-ATPase-specific Teff, probably due to activation of Treg to other gastric antigens [46]. In absence of the target organ, e.g., athyroid rats or ovariectomized females, the respective Treg are missing from the repertoire and cannot protect new hosts against thyroiditis or oophoritis, while other autoimmune diseases are still prevented [51, 53].

Table 2 Function of Treg in various autoimmune diseases

Disease	Cytokine/molecule needed for disease protection/amelioration	Localization of suppression	Antigen specificity of Treg
Gastritis	Not IL-10 [47, 48]; not TGF-β [48, 49]; not CTLA-4 [48]; not GITR [50]; CTLA-4 [12]	Gastric LN; gastric mucosa	Polyclonal [3, 45, 46]; anti-H/K-ATPase (TXA23)
Oophoritis		Ovarian LN [53]	Polyclonal [52, 53]; MATER ZP3 [53]
Prostatitis			Polyclonal [52]; EAPA1, EAPA2 [52]
Colitis	IL-10 [54, 58, 62]; TGF-β [58, 63, 64, 123]; CTLA-4 [11, 58]	Mesenteric LN [57]; lamina propria [57]	
EAE	IL-10 [78, 82]; TGF-β [85]	Brain (late) [78]; draining LN (early) [42]	Polyclonal [74]; anti-MBP TCR with 2^{nd} TCR α-chain [74]
Diabetes in NOD mice	TGF-β [65, 94]; not TGF-β [90]; not IL-10 [90, 94]	Pancreatic LN [96]; pancreas [90, 95, 97]	Polyclonal <anti-islet specific (BDC2.5) [100–102]

Modulation of suppression

The in vitro inhibition assays revealed that the strength of the TCR and costimulatory signal received by the Teff determines if Treg can still suppress T cell activation. High doses of anti-CD3 or anti-CD28 mAb and further stimulation of GITR, ICOS, or OX40 on Teff reduces or even abrogates suppression [50, 97, 128]. Increased DC activation, e.g., via Toll-like receptors (TLR), or addition of IL-12 also overcomes suppression [129, 130]. On the other hand, there may be a direct effect of TLR on Treg. Treg express a variety of TLR on the mRNA level and for most TLR protein expression still needs to be confirmed [131]. TLR2 and TLR8 signals reduce and TLR5 signals enhance the suppressive function of Treg [131–134]. How these findings relate to Treg in mice and how they influence autoimmunity needs to be investigated.

It would be of great interest to selectively enhance the suppressive capacity of Treg, without stimulating Teff and DC. This will be crucial for the treatment of autoimmune diseases. One interesting approach is to expand and activate Treg in vivo with a superagonistic anti-CD28 Ab. Application of this Ab in rats leads to inhibition of EAE if given at the time of immunization, while injection of the mAb at onset or during disease leads to a milder EAE course [88]. With regard to applying this finding to humans, caution is necessary as the dose of antibody may be very critical and many aspects of the immune system could be activated. Further work has been published indicating that hormones (estrogen, vasoactive intestinal peptide), cortisol derivates, or substances that lead to DC tolerance also induce, expand, or activate immunosuppressive T cells. However, these findings need to be confirmed with stringent methods regarding Treg phenotype and function (e.g., flow cytometry for Foxp3 and quantitative suppression assays at various Treg dilutions using CFSE labeled Teff) before drawing firm conclusions.

Conclusion

During the past 10 years, $CD4^+CD25^+$ regulatory T cells have been extensively studied with regard to their inhibitory role in autoimmune disease, transplantation tolerance, immune reactions towards infection, and anticancer immune responses (see the other chapters in this issue). It is evident that Treg can reduce basically all immune responses. The final outcome depends on the balance between the strength of stimulatory and inhibitory signals acting on Tcon. How exactly this balance is established, the means by which it can be broken, and how it can be reestablished, needs further evaluation. In addition, the relationship between thymic derived natural $CD4^+CD25^+Foxp3^+$ Treg and other subpopulations induced in the periphery, e.g., peripherally generated $CD4^+CD25^+Foxp3^+$ cells, as well as T regulatory 1 (Tr1) [135] and T helper 3 (Th3) [136] cells needs clarification. Nevertheless, Treg are now being analyzed in human autoimmune disease, and efforts to find drugs that could boost their numbers or function are under way. However, for this field to be successful and for an efficient translation of basic research into clinical studies, it would be very helpful to find decisive cell surface markers and to understand the mechanism of Treg function. Also care should be taken to work with well-defined $CD4^+CD25^+Foxp3^+$ cells instead of $CD4^+CD25^+$ cells to ensure that future investigations build on the same cell population and thus avoid misleading data that may discredit the field. Finally, the next few years will show if the in vitro expansion of antigen-specific Treg can be achieved under GMP-conditions and if the injection of these cells can cure or ameliorate human autoimmune disease.

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