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Regulatory T cells and inhibitory cytokines in autoimmunity

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Summary of recent advances

Foxp3⁺ regulatory T cells (T_{regs}) contribute significantly to the maintenance of peripheral tolerance, but they ultimately fail in autoimmune diseases. The events that lead to T_{reg} failure in controlling autoreactive effector T cells (T_{effs}) during autoimmunity are not completely understood. In this review, we discuss possible mechanisms for this subversion as they relate to type 1 diabetes (T1D) and multiple sclerosis (MS). Recent studies emphasize (i) the role of inflammatory cytokines, such as IL-6, in inhibiting or subverting T_{reg} function, (ii) the issue of T_{reg} plasticity, (iii) the possible resistance of autoimmune T cells to T_{reg}-mediated control, and (iv) T_{reg}-associated inhibitory cytokines TGFβ, IL-10 and IL-35 in facilitating T_{reg} suppressive activity and promoting T_{reg} generation. These recent advances place a large emphasis on the local tissue specific inflammatory environment as it relates to T_{reg} function and disease development.

Introduction

Autoimmunity ensues when central and/or peripheral tolerance barriers are overcome, thereby allowing the activation of self-reactive T cells, which induce tissue destruction. Most self-reactive T cells are deleted in the thymus, a process referred to as central tolerance. The few remaining self-reactive T cells that enter the periphery are controlled by peripheral tolerance mechanisms, in which Foxp3⁺ T_{regs} have emerged as the primary mediators [1]. Autoimmune disease develops when this last tolerance barrier is compromised. It has been shown that many individuals with no autoimmune manifestations harbor self-reactive T cells [2]. Thus, the continual battle between self-reactive T cells and suppressive T_{regs} is critical in determining whether autoimmunity commences. Many autoimmune diseases manifest themselves in an oscillating fashion, which is likely due to the aforementioned struggle between the regulatory and inflammatory arms of the immune system, suggesting that T_{regs} are still actively involved in regulating this self-reactive response. There are three mechanisms that might contribute to a breakdown in T_{reg} control: (a) T_{reg} numbers are reduced and/or T_{regs} are dysfunctional due to inherent deficiencies in autoimmune susceptible individuals, (b) T_{reg} suppressive function is inhibited, diverted or converted by the chronic inflammation that occurs in autoimmunity, and/or (c) self-reactive effector T cells (T_{eff}) become unusually aggressive and are refractory to regulation by otherwise functional T_{regs} because they either overwhelm regulatory control or express molecules that render them resistant. Here we will discuss how these possibilities might contribute to this loss of T_{reg} control, illustrated by two key autoimmune diseases Type 1 Diabetes (T1D) and Multiple Sclerosis (MS), with occasional reference to other autoimmune or inflammatory diseases.

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Even though multiple mechanisms of T_{reg} -mediated suppression have been identified, the relative importance of each mechanism *in vivo* remains unclear [3]. Current dogma, based mostly on *in vitro* experiments, suggests that T_{reg} function is contact dependent. However, a recent study illustrates that this may be inaccurate as T_{regs} that are optimally stimulated by contact with T_{eff} can mediate potent third party suppression via soluble factors *in vitro* [4]. These data, together with significant support from *in vivo* observations, suggest that inhibitory cytokines such as TGF β , IL-10, and IL-35 are major contributors to T_{reg} function [3,5]. Consequently, this review will also focus on the contribution of these three inhibitory cytokines in mediating (or failing to mediate) T_{reg} function in autoimmune disease.

Why do T_{regs} fail to suppress during autoimmune disease?

Under normal conditions, T_{regs} effectively inhibit excessive inflammation and autoimmune manifestations [6]. However, in autoimmune diseases T_{regs} fail to control the initial inflammatory insult and subsequent disease progression due to cell-intrinsic and/or cell-extrinsic factors. Studies performed in a variety of autoimmune models suggest that T_{regs} are still actively and continuously involved in inflammatory regulation after disease onset [7,8], although this needs to be more definitively assessed. When naturally occurring T_{regs} are absent in Foxp3-deficient islet antigen-specific BDC2.5 TCR transgenic NOD mice, disease onset is significantly earlier [7]. Interestingly, the rate of islet infiltration in BDC2.5 mice is similar in the presence or absence of T_{regs} , however, the infiltrating cells cause immediate damage in their absence [7]. In experimental autoimmune encephalomyelitis (EAE), an induced murine model of MS, Foxp3⁺ T_{regs} accumulate in the central nervous system (CNS) as the disease progresses [9] and in some instances aid in natural recovery from EAE [8]. Depletion of CD25⁺ T cells prior to disease induction reduces recovery rate while their depletion after recovery obliterates their protection against secondary autoimmune disease induction [10]. Although T_{regs} have some control over autoimmune inflammation, there may be several reasons why they ultimately fail to prevent disease progression.

Inherent defect in T_{reg} number or function

A possible explanation for T_{reg} failure is due to a reduction in their number and/or function in autoimmune predisposed individuals. This may not necessarily manifest systemically, and may be restricted to the site of autoimmune insult. Some studies have reported an age-related defect in NOD T_{reg} function [11], while others were unable to detect any major deficiency [12]. In both studies T_{regs} were identified and isolated based on CD25 expression, which may have resulted in contamination with activated T_{eff} thereby altering data interpretation. A more recent study took advantage of NOD Foxp3-GFP reporter mice to cleanly separate the two populations and detected no intrinsic defects in NOD T_{reg} numbers or suppressive capabilities [13]. There is some evidence that T_{reg} function in human patients with an early onset of diabetes might be compromised [14]. However, human studies have to rely on CD25 as a marker for T_{reg} separation, and thus are not entirely conclusive. More recent studies were able to exclude contaminating T_{eff} from T_{reg} analysis based on CD127 (IL7R α) expression that has restricted expression on activated T cells and is absent from T_{regs} [15,16]. Although these studies are preliminary, at least in the early diagnosed T1D patients T_{reg} numbers appear to be decreased [16].

T_{reg} defects at the site of inflammation

Another possibility for T_{reg} failure is that the chronic inflammation and consequent molecular milieu at the site of autoimmune destruction could have a negative effect on T_{reg} function. This could be manifested as a reduction in T_{reg} inhibitory function, apoptosis, or conversion into a pro-inflammatory T cell lineage. Recently, the fate of Foxp3⁺ T_{regs} over the course of diabetes development in NOD mice was followed. T_{reg} function at the site of inflammation was found

to be diminished due to decreased levels of IL-2, a critical T_{reg} growth factor [17]. These tissue-infiltrating T_{regs} were more prone to apoptosis and thus failed to control autoimmune responses. This study underlines the dependence of T_{reg} function on local IL-2 production and the inflammatory milieu.

T_{reg} stability and plasticity during autoimmune response has recently become a hot issue, especially since administration of *in vitro* differentiated T_{regs} is being considered for human therapy. Recent studies addressing the stability of Foxp3⁺ T cells have shown that both lymphopenic and inflammatory environments can result in the loss of Foxp3 expression in T_{regs} [18–20]. The apparent plasticity of T_{reg} lineage might result in the instability of this population during chronic autoinflammatory responses and lead to loss of T_{reg} function in an autoimmune environment.

Clearly, T_{regs} possess other modes of action in addition to the three inhibitory cytokines discussed here [3,21]. There is still some controversy over the relative importance of soluble factors versus cell contact-dependent inhibition in mediating T_{reg} function. Substantial *in vivo* data, together with a recent study indicate that cytokines contribute significantly to T_{reg} function when cell contact is absent [4]. However, to unequivocally show the importance of cytokines in mediating T_{reg} function one would have to assess inhibition in their absence. The extent of redundancy within these three inhibitory cytokines remains unclear. At first glance, it seems that there is significant overlap between the functions of the three T_{reg} inhibitory cytokines (Figure 1). Both IL-10 and TGF β can inhibit Th1 type responses, both are able to convert T cells into a regulatory population, albeit with quite distinct phenotypes, and all three cytokines seem to play a role in the gut homeostasis. However, recent studies suggest that different transcriptional ‘programs’ are utilized by T_{regs} depending on the Th subset they are attempting to suppress which may likewise lead to the utilization of distinct suppressive functions [22,23]. Interestingly, T_{regs} appear to utilize similar transcription factors as the T helper cells they are suppressing, raising the possibility of a co-evolutionary progression. IRF4 deficiency in T_{regs} resulted in selective ablation of IL-10 transcription, partial decrease in EBI3 expression, and no effect on TGF β . These findings suggest that T_{regs} may respond with a distinct inhibitory cytokine profile depending on the inflammatory milieu [22]. Thus, it is possible that further examples of T_{reg} functional plasticity may emerge. Therefore, it is possible that although all three cytokines are utilized by T_{regs} in the same tissue they may control different cellular populations or that different cytokines may be more important in different organs, in a manner that cannot be fully gleaned from the studies conducted thus far. Given the brevity of cellular targets and unique microenvironments within which T_{regs} have to operate, it would not be surprising if there is limited functional redundancy for these cytokines, as has been suggested for IL-10 [24]. It will also be important to assess the effect of losing one inhibitory mechanism on the remaining functional landscape as it is conceivable that T_{regs} may make adjustments to compensate for such a loss given the functional plasticity already described.

T_{eff} resistance to inhibition

A third possibility is that highly activated self-reactive T_{effs} become resistant to T_{reg} -mediated control. Studies performed in both spontaneous T1D and induced EAE models report normal T_{reg} function during disease, but show that the T_{effs} are hyper activated and do not capitulate to suppression by T_{regs} [7,9,13,25,26]. NOD T_{reg} function, as measured by *in vitro* assays, was reported to be fairly comparable to B6 T_{regs} . In contrast, NOD T_{effs} exhibited an increased ability to proliferate and were unresponsive to T_{reg} -mediated suppression [13]. Similarly, T_{effs} from human T1D patients are more refractory to inhibition by T_{regs} than T_{effs} from healthy controls [27].

Mounting evidence shows that inhibitory actions of T_{reg} -associated cytokines can be subverted in highly inflammatory environments. In particular, IL-6 has been shown to have a negative effect on T_{reg} function [28], and in the CNS of EAE mice a typically inhibitory cytokine TGF β synergizes with IL-6 to induce pro-inflammatory Th17 cells [29,30]. Moreover, when directly compared to Th1 and Th2 cells, Th17 cells were more likely to overcome the inhibition by T_{regs} [31]. These data have raised the possibility that Th17 cells are more resistant to regulatory control than other T helper subsets. The molecular basis for this resistance remains obscure.

Thus it is possible that T_{reg} function is normal during autoimmune disease, but the chronic inflammatory environment at the tissue site of autoimmune damage counteracts and overwhelms the suppressive T_{reg} activity. While the issue of T_{reg} fitness during autoimmunity remains a hot topic of discussion, it is possible that multiple pathways lead to the breakdown of peripheral tolerance. It is clear, however, that T_{regs} function has to be considered and assessed in a context of the inflammatory environment where they operate rather than in the periphery.

Inhibitory cytokines facilitate T_{reg} -mediated control of T_{effs} and promote the induction of induced T_{regs}

As discussed in the previous section, the local inflammatory milieu at the site of autoimmune destruction can have a significant effect on T_{reg} function. Cytokines secreted by T_{regs} , T_{effs} and other cellular infiltrates contribute to the generation of this environment. Inhibitory cytokines, such as TGF β and IL-10, can directly inhibit cells that participate in this autoimmune destruction and/or can mediate the generation of induced T regulatory cells (iT_{regs}) [3]. More recently IL-35 has also been shown to directly inhibit T cell proliferation [4,32], although its ability to affect other cell populations or its capacity to mediate/influence the induction of iT_{regs} has yet to be established.

Inhibitory cytokines regulate T_{effs}

The *in vivo* role of T_{reg} -derived cytokines in modulating autoimmune diseases has been clearly established. Mice deficient in TGF β succumb to spontaneous autoimmune disease at 4–5 weeks of age [33,34], while the IL-10 knock out animals are more susceptible to induced autoimmune disease [35]. In TGF β deficient mice, the dysregulated inflammatory response is alleviated after the depletion of CD4 $^{+}$ and CD8 $^{+}$ T cells [36,37]. Although, T_{regs} are not the only source of inhibitory cytokines, it has become more evident that T_{reg} secreted cytokines contribute significantly to T_{reg} function *in vivo*.

TGF β has been implicated in the function of T_{regs} in T1D and inflammatory bowel disease (IBD) mouse models [38,39]. Depletion of TGF β with neutralizing antibodies results in a decrease in both mouse and human T_{reg} function [39–41]. T cell transfer and bone marrow chimeric studies revealed that TGF β has both cell-intrinsic and cell-extrinsic regulatory effects [42,43]. These data show that TGF β helps to preserve peripheral tolerance by induction of iT_{regs} and maintenance of nT_{regs} in the periphery.

IL-10 is a homodimeric cytokine with a wide range of inhibitory actions. IL-10 is produced by a variety of cells, including monocytes and T cells, and can exert its effects on both myeloid and lymphoid cells [35]. Its regulatory activity is mediated largely through its effects on APCs [44]. Interestingly, IL-10 and IL-10 receptor-deficient mice, unlike their TGF β counterparts, do not develop spontaneous autoimmune symptoms which only becomes apparent following inflammatory insult. In the absence of IL-10, mice develop colitis in response to some intestinal microorganisms [45,46]. In other murine autoimmune models, such as EAE, mice lacking

IL-10 exhibit an exacerbated form of the disease [35]. An increase in IL-10 production by $\text{Foxp3}^+ \text{T}_{\text{regs}}$ is associated with the recovery phase of EAE [8], and transfer of *in vitro* generated iT_{regs} or exogenously purified natural T_{regs} was shown to prevent the induction of EAE through the production of IL-10 [47]. IL-10 plays a significant role in intestinal homeostasis. Although, IL-10-deficient T_{regs} are not devoid of regulatory activity, their function is particularly affected in controlling already established gut inflammation [48], and mice with T_{reg} -specific deletion of IL-10 are susceptible to colitis induced by commensal flora [24]. Similarly to $\text{TGF}\beta$, IL-10 can also mediate the generation of an induced regulatory T population, Tr1, that is characterized by secretion of IL-10 [49].

IL-35 is a recently discovered heterodimeric cytokine that is produced by $\text{Foxp3}^+ \text{T}_{\text{regs}}$ and contributes to their suppressive function [32]. IL-35 is composed of two chains, IL-12 α (p35) and EBI3 that it shares with two other heterodimeric cytokines IL-12 and IL-27, respectively. IL-35 is required for maximal regulatory function *in vivo* as T_{regs} deficient in either chain are unable to control homeostatic T cell expansion and IBD [32]. Given that IL-35 emerged more recently, we still have a limited understanding of its biological activity which has also been complicated by the lack of appropriate tools for its functional dissection. Recombinant IL-35 has proven particularly challenging to generate and purify, compared with IL-12 and IL-27. It is tempting to speculate that the apparent poor stability of IL-35 might underlie important physiological features of this cytokine, such as limited potency over short-range. Alternatively, DCs that secrete IL-12 or IL-27 may be precluded from generating IL-35, due to preferential pairing of IL-12 and IL-27.

It is clearly important to determine the bioactivity of IL-35. Whether IL-35 can inhibit all T helper subsets and other cellular populations, such as B cells, macrophages or DCs, or has more selective targets remains to be determined. Significant insight is likely to be gained from the determination of the IL-35 receptor and its expression pattern. It is tempting to speculate that since IL-35 represents a novel pairing of the IL-12 cytokine chains, its receptor will also be a new combination of the known family receptor chains [50]. Alternatively, the receptor might be composed of novel subunits. If it is the former, a key question will be how cells can translate similar signaling pathways into completely different downstream functional consequences, i.e. stimulatory signaling induced by IL-12 and IL-27 vs inhibitory signal delivered by IL-35. Interestingly, precedence does exist for a similar signaling cascade mediating quite different functional outcomes, as exemplified by the IL-6 and IL-10 receptor which both use STAT3 [51]. Lastly, an open question is whether IL-35 can mediate the generation of an induced regulatory T cell population. This is clearly a feature of the other two inhibitory cytokines IL-10 and $\text{TGF}\beta$ and thus it remains plausible that IL-35 might have a similar capacity. $\text{TGF}\beta$ -induced iT_{regs} (Th3) and IL-10-induced Tr1 clearly have very distinct transcriptional and functional profiles [3,52] and so it is possible that any IL-35-induced regulatory populations may also be quite distinct.

Inhibitory cytokines promote infectious tolerance

It is clear that $\text{Foxp3}^+ \text{T}_{\text{regs}}$ play a major role in most autoimmune diseases. What remains unknown is the relative contribution of natural, thymus-derived T_{regs} (nT_{regs}) versus iT_{regs} in mediating control of autoimmunity. Furthermore, recent studies suggest that the importance of iT_{regs} in controlling autoimmunity may vary depending on the site of tissue inflammation [4,32].

Recent studies have shown that T_{regs} and T_{reg} -derived cytokines have long lasting tolerance effects *in vivo* [4,32]. Even though diabetes onset can be prevented by T_{reg} transfer into NOD mice, the endogenous $\text{Foxp3}^+ \text{T}_{\text{reg}}$ population dominates at the site of the original autoimmune response in the pancreas while the transferred population is significantly reduced [38]. In a mouse model of IBD, while $\text{TGF}\beta$ is essential for downregulating the autoimmune response,

T_{reg} -derived $TGF\beta$ was not critical for protection [39,42]. This suggests that the main function of $TGF\beta$ may be to mediate infectious tolerance (ie. generation of iT_{regs}) which may not necessarily be T_{reg} -derived [53]. It is possible that in order to achieve infectious tolerance several simultaneous signals are required in addition to $TGF\beta$. These can be other inhibitory cytokines, such as IL-10, or cell surface molecules that require cell contact for ligation [54].

Recent findings suggest that different organs or tissues are more suitable or permissive to T_{reg} differentiation due to their cellular and/or molecular composition. Mucosal $CD103^+$ dendritic cells are exceptionally adept at inducing T_{regs} through production of $TGF\beta$ and the Vitamin A metabolite retinoic acid (RA) [55,56]. RA counteracts the effects of inflammatory cytokines and aides in skewing T cells towards a regulatory phenotype [57–59]. $CD8^+CD205^+$ dendritic cells in the spleen can also mediate iT_{reg} development in part through production of $TGF\beta$ [60]. The CNS, however, does not seem to favor the generation of $Foxp3^+ iT_{regs}$ [9]. This is in part due to T_{eff} -derived IL-6, which in combination with $TGF\beta$ induces Th17 cells rather than $Foxp3^+ iT_{regs}$ [29]. DC phenotype is initially induced by microbial interactions leading to the induction of either inflammatory or inhibitory/toleragenic DCs [61]. It is conceivable that the complete Freund's adjuvant used to induce EAE, which contains mycobacterial cell wall components, results in the generation of DCs that are suboptimal for iT_{reg} generation. In support to this, mice immunized with myelin peptide in incomplete Freund's adjuvant that lacks *Mycobacterium tuberculosis* exhibited a decrease in IL-6 production and an increase in antigen specific $Foxp3^+$ T cells in the spleen. However, there was only a modest increase in iT_{regs} and whether these were present in the CNS is unknown [29]. Taken together, these studies suggest that the stimulus received by DCs may be more critical than the particular site or organ that is affected in determining iT_{reg} development. Thus, under alternate stimulatory conditions the CNS might be an acceptable location for iT_{reg} differentiation.

Concluding remarks

Recent advances have placed significant emphasis on dissecting T_{reg} function in autoimmunity and assessing their therapeutic utility. The local inflammatory environment, consisting of specialized APCs, metabolic components, growth factors and inhibitory and/or inflammatory cytokines, can significantly alter the efficacy and generation of T_{regs} . Given the complexity of T_{reg} function *in vivo*, several key questions remain before we fully understand molecular and cellular defects that occur in autoimmunity, as they pertain to T_{regs} , and how this knowledge might be utilized in a clinical setting.

1. How does inflammation at different tissue sites affect T_{reg} plasticity and how can we counteract the negative effect of inflammatory cytokines on T_{reg} stability? Although we know the minimal requirements for *in vitro* induction of iT_{regs} ($TGF\beta$, IL-2, TCR stimulation), we have yet to determine what signals are required for stabilization of the *Foxp3* locus. This is likely to be key for the therapeutic use of $Foxp3^+ iT_{reg}$. Furthermore, it is still unclear what molecular events mediate nT_{reg} plasticity and how this might be prevented.
2. Which autoimmune diseases require antigen specificity for successful T_{reg} therapy and what should those antigen targets be? The therapeutic use of *in vitro* induced populations of regulatory cells, both $TGF\beta$ -induced iT_{regs} and IL-10-induced Tr1 cells, is an attractive possibility as one can generate antigen-specific regulatory populations that may effectively home to, and be activated in, the diseased site [49]. For instance, it has recently been shown that T cell migration to the islets is a cell autonomous event suggesting that islet antigen-specific iT_{regs} could be effective mediators of tolerogenic therapy even though there is still debate over the key initiating antigen in T1D [62]. Oral antigen administration results in induction of

antigen specific T_{regs} and is a potentially promising therapeutic approach; however, further clinical trials are warranted before the success of the treatment can be clearly assessed [49,63,64]. However, for many autoimmune diseases the choice of the target antigen remains obscure and its importance ill-defined.

3. How can we improve the efficacy and safety of cytokine treatments and achieve effective local delivery? Cytokine therapy is possibly one of the most challenging therapies to administer due to pleiotropic activities and potential toxicities. Thus, it is possible that future therapies might be combinatorial and may also include modifications to target cytokines to specific sites and/or modifications to enhance bioactivity or stability. Targeted cytokine delivery, although challenging, could result in improved treatment efficacy and reduced side effects. In one study local administration of IL-10 into the gastrointestinal tract of IBD patients was achieved with the help of genetically modified *Lactococcus lactis* [65]. Alternatively, gelatin nanoparticles fused with the IL-10 gene has been used to effect local delivery of IL-10 [66]. While much remains to be learnt about IL-35, thus far it appears to be functionally monotropic with seemingly no activating or inflammatory activities [32]. If verified, this focused functionality would be therapeutically desirable as it may have limited *in vivo* toxicities.

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Figure 1.
Redundancy and specificity of T_{reg} inhibitory cytokine function.