Immunity
Review

Natural and Adaptive Foxp3$^+$ Regulatory T Cells: More of the Same or a Division of Labor?

Maria A. Curotto de Lafaille[1,*] and Juan J. Lafaille[1,*]
[1]Molecular Pathogenesis Program, Kimmel Center for Biology and Medicine of the Skirball Institute, and Department of Pathology, New York University School of Medicine, 540 First Avenue, New York, NY 10016, USA
*Correspondence: maria.lafaille@med.nyu.edu (M.A.C.d.L.), juan.lafaille@med.nyu.edu (J.J.L.)
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Adaptive Foxp3$^+$CD4$^+$ regulatory T (iTreg) cells develop outside the thymus under subimmunogenic antigen presentation, during chronic inflammation, and during normal homeostasis of the gut. iTreg cells are essential in mucosal immune tolerance and in the control of severe chronic allergic inflammation, and most likely are one of the main barriers to the eradication of tumors. The Foxp3$^+$ iTreg cell repertoire is drawn from naive conventional CD4$^+$ T cells, whereas natural Treg (nTreg) cells are selected by high-avidity interactions in the thymus. The full extent of differences and similarities between iTreg and nTreg cells is yet to be defined. We speculate that iTreg cell development is driven by the need to maintain a noninflammatory environment in the gut, to suppress immune responses to environmental and food allergens, and to decrease chronic inflammation, whereas nTreg cells prevent autoimmunity and raise the activation threshold for all immune responses.

Introduction

The transcription factor Foxp3 is essential to establish a functional regulatory T (Treg) cell lineage (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003; Sakaguchi et al., 2008; Zheng and Rudensky, 2007). Throughout this review, we will use the nomenclature "natural Treg (nTreg) cells" for thymic-derived Foxp3$^+$ Treg cells and "adaptive Treg (iTreg) cells" for peripherally generated Foxp3$^+$ T cells. Foxp3$^-$ iTreg cells such as Tr1 and Th3 cells will not be discussed.

Numerous studies have proposed and demonstrated that nTreg cells are generated in the thymus through MHC class II-dependent T cell receptor (TCR) interactions resulting in high-avidity selection (Apostolou et al., 2002; Bensinger et al., 2001; Fontenot et al., 2005b; Jordan et al., 2001; Larkin et al., 2008; Modigliani et al., 1996; Sakaguchi, 2001), although additional selection mechanisms may take place (van Santen et al., 2004). In recent years it became evident that Foxp3$^+$ Treg cells could also be generated outside the thymus under a variety of conditions (Figure 1). In this review we will discuss the current knowledge of adaptive iTreg generation and function, the areas that need further development to understand and distinguish iTreg cells and nTreg cells, and the proposed specific roles of nTreg cells and iTreg cells on tolerance and inflammation.

Differentiation of Foxp3$^+$ Regulatory T Cells outside the Thymus

A comparison between IL-2-deficient Treg cells (which are CD25 negative, but, surprisingly, effective at preventing autoimmune disease) with CD25-deficient T cells (which, in contrast to IL-2-deficient cells, are ineffective suppressors) led us to propose a model whereby acquisition of CD25 expression in cells that were previously CD25 negative was a requisite for the functionality of Treg cells (Furtado et al., 2002). Thus, CD25-negative cells from IL-2-deficient mice converted to CD25 positive in the presence of IL-2 produced by effector T cells, and, in the process, gained Treg cell function. Importantly, when the key role of Foxp3 in Treg cell biology was demonstrated, nonregulatory T cells were shown to be able to acquire Foxp3 expression and regulatory function (Apostolou and von Boehmer, 2004; Cobbold et al., 2004; Curotto de Lafaille et al., 2004).

Some of the early evidence of peripheral conversion of naive conventional CD4$^+$ cells into Foxp3$^+$ T cells originated from adoptive transfer experiments in which polyclonal CD4$^+$CD25$^-$ naive T cells were injected into lymphopenic mice or mice containing a monoclonal T cell repertoire devoid of nTreg cells (Curotto de Lafaille et al., 2004; Furtado et al., 2002). In such conditions, in which homeostatic proliferation of the donor lymphocytes took place, part of the donor population became CD25$^+$CTLA-4$^+$GITR$^+$ and acquired Foxp3 expression and suppressive activity. Given the experimental design, it is possible that these CD25$^+$Foxp3$^+$ Treg cells originated from CD25$^-$Foxp3$^+$ cells present in the initial population. This is not the case when TCR transgenic Foxp3$^+$ T cells were generated, because kinetic and other considerations indicate that they are derived from Foxp3$^-$ cells. In addition, Knoechel et al. (2005) studied the conversion of naive T cells into Foxp3$^+$ Treg cells during peripheral expansion by using an experimental system in which antigen-specific naive CD4$^+$ T cells were transferred into lymphocyte-deficient RAG-deficient mice that expressed the antigen as a systemic secreted protein. Under these conditions, massive T cell activation resulted in acute graft versus host (GvH)-like disease followed by a recovery phase associated with de novo generation of Treg cells. iTreg cell generation did not require the thymus but was dependent on IL-2.

iTreg cell induction by foreign antigens has been described by several groups. von Boehmer and colleagues showed the generation of Foxp3$^+$ iTreg cells in mice treated with minute antigen doses by osmotic pump delivery (Apostolou and von Boehmer, 2004), and after delivery of antigen crosslinked to DEC-205 antibody in the absence of costimulatory signals, a process that directs the antigen to DEC-205$^+$ dendritic cells (Kretschmer

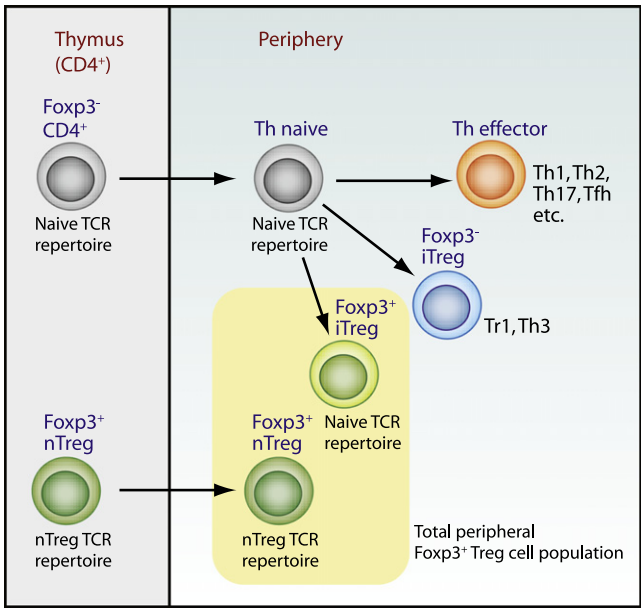

Figure 1. Thymic and Peripheral Generation of Foxp3$^+$ Treg Cells
Natural Treg (nTreg) cells differentiate in the thymus and migrate to peripheral tissues. Adaptive Foxp3$^+$ Treg (iTreg) cells differentiate in secondary lymphoid organs and tissues. The peripheral population of Foxp3$^+$ Treg cells comprises both nTreg and iTreg cells. It is very likely that nTreg and Foxp3$^+$ iTreg cells differ in their TCR repertoire because iTreg cells are derived from mature peripheral naive CD4$^+$ cells.

et al., 2005). By utilizing a similar principle, Waldmann and coworkers described the conversion of transplant-specific naive CD4$^+$ T cells into Foxp3$^+$ Treg cells in mice made tolerant by treatment with nondepleting CD4 antibodies (Cobbold et al., 2004). Our laboratory showed that ovalbumin (OVA)-specific Foxp3$^+$ Treg cells appeared in mesenteric lymph nodes (LNs) of mice administered the antigen chicken ovalbumin by oral route, a treatment that induces oral tolerance (Mucida et al., 2005). The use of the mucosal delivery of antigen, in particular the oral route, as a mean to generate Foxp3$^+$ iTreg cells has now become a major method of iTreg cell generation in vivo because of its simplicity, the effectiveness of these Treg cells in vitro and in vivo assays, and the fact that concomitant effector T cell generation is inefficient. Interestingly, we also showed that OVA-specific Treg cells were induced, alongside a much larger number of T helper 2 (Th2) cells, in spleens and LNs of naive mice after intraperitoneal immunization with OVA in the adjuvant alum (Mucida et al., 2005).

Protocols have been developed to differentiate Foxp3$^+$ Treg cells in vitro from naive conventional T cells in culture settings, particularly after the description that TGF-β induces Foxp3 expression in TCR-activated naive CD4 cells (Chen et al., 2003; Fantini et al., 2004). Thus, it is clear that naive CD4$^+$ T cells selected as Foxp3$^-$ in the thymus have full potential to become Foxp3$^+$ Treg cells in vivo and in vitro.

Mechanisms of iTreg Cell Differentiation: TGF-β, IL-2, and Costimulation

Although nTreg cells develop in a highly controlled thymic microenvironment (Josefowicz and Rudensky, 2009, in this issue of *Immunity*), Foxp3$^+$ iTreg cells differentiate under more varied conditions (Box 1). For example, iTreg cells appear in the mesenteric LNs during induction of oral tolerance (Coombes et al., 2007; Mucida et al., 2005), they may continuously differentiate in the lamina propria of the gut in response to microbiota and food antigens (Sun et al., 2007); they are also generated in chronically inflamed tissues (Curotto de Lafaille et al., 2008), tumor (Liu et al., 2007), and transplanted tissues (Cobbold et al., 2004). Our understanding of the different microenvironments of iTreg cell development in vivo is still incomplete. However, the minimal program for Foxp3$^+$ iTreg cell development has been defined: it requires TCR stimulation and the cytokines TGF-β and IL-2, for both in vitro and in vivo generated iTreg cells.

Addition of TGF-β to TCR-stimulated naive CD4$^+$ T cells induced transcription of Foxp3, acquisition of anergic and suppressive activity in vitro, and the ability to suppress inflammation in an experimental asthma model (Chen et al., 2003). TGF-β induced Foxp3 expression in cultures of plate-bound anti-CD3 and CD28-stimulated naive T cells (Chen et al., 2003); thus, antigen-presenting cells (APCs) were not required for in vitro conversion. The mechanism by which TGF-β induces transcription of Foxp3 involves cooperation of the transcription factors STAT3 and NFAT at a Foxp3 gene enhancer element (Fantini et al., 2004; Josefowicz and Rudensky, 2009). Consistent with the in vitro findings, in vivo neutralization of TGF-β impaired oral tolerance (Faria and Weiner, 2005; Miller et al., 1992) and inhibited the differentiation of antigen-specific Foxp3$^+$ iTreg cells (Mucida et al., 2005). TGF-β neutralization also blocked the iTreg cell-dependent tolerance to male grafts in an experimental model in which iTreg cells were induced by a nondepleting CD4 antibody (Cobbold et al., 2004).

In contrast to the essential role of TGF-β in the differentiation of Foxp3$^+$ iTreg cells, the role of TGF-β in the generation of nTreg cells is less clear. Studies with T cell-specific deletion of TGF-βRII receptor reported that TGF-β was not involved in thymic nTreg cell development (Fahlen et al., 2005; Li et al., 2006; Marie et al., 2006); furthermore, young TGF-β1-deficient mice have normal number of thymic nTreg cells. However, a recent study offered a different interpretation, based upon the fact that between postnatal days 3 and 5 there is a severe deficiency in nTreg cell generation in mice that have a conditional (lck-Cre driven) deletion of TGF-βRI (Liu et al., 2008). The authors attributed the late surge of nTreg cells in TGF-βRI-deficient mice to a heightened responsiveness of these T cells to IL-2 and showed that double deficiency in TGF-βRI and IL-2 abrogated nTreg generation (Liu et al., 2008). Nevertheless, it is clear that IL-2 by itself is not necessary for Foxp3$^+$ nTreg cell generation. We showed near normal amounts of Foxp3 mRNA in single-positive CD4$^+$CD25$^+$ thymocytes from young IL-2-deficient mice, indicating that IL-2 was not required for thymic development of Foxp3$^+$ Treg cells (Curotto de Lafaille et al., 2004). Two other studies used IL-2-deficient and IL-2Rα (CD25)-deficient mice to probe the role of IL-2 in Treg cell biology and also concluded that IL-2 was dispensable for the generation of nTreg cells in the thymus (D'Cruz and Klein, 2005; Fontenot et al., 2005a). Finally, neutralization of IL-2 with antibodies resulted in a strong reduction of Foxp3 expression

Box 1. Differences between nTreg and iTreg Cells

		nTreg cell	iTreg cell
Generation:	Tissue	Thymus	GALT, spleen, lymph node, inflamed tissue
	Costimulation requirement	CD28	CTLA-4
	Cytokine requirement	TGF-β (?) IL-2 or IL-15	TGF-β IL-2
Specificity		Self (?)	Allergens, commensal microbiota, neoantigens (tumor), alloantigens, self (inflammation)

in the spleen but a nonsignificant difference in the thymus (Setoguchi et al., 2005).

In the thymus of IL-2-deficient mice, the absence IL-2 is likely to be compensated by IL-15, because CD122-deficient mice, which have defective IL-2 and IL-15 signaling, have a profound reduction in Treg cell development (Burchill et al., 2007; Soper et al., 2007). Similar Treg cell defect was reported in IL-2 and IL-15 double-deficient mice (Burchill et al., 2007). These results are consistent with earlier work showing the amelioration of the lymphoproliferative syndrome of CD122-deficient mice with transgenic expression of CD122 driven by the lck proximal promoter, a condition that restored the generation of Treg cells (Malek et al., 2002). The results are also consistent with the expression of a constitutively active form of STAT5B, a molecule involved in both IL-2 and IL-15 signaling (Burchill et al., 2008). It thus appears that during development of cells poised to become nTreg cells because of the strength of their TCR signals, IL-2 or IL-15 can direct a TCR-independent developmental step (Lio and Hsieh, 2008). In the periphery, however, the role of IL-2 cannot be replaced by IL-15.

As indicated above, IL-2 appears to be essential for iTreg cell generation and/or homeostasis. In cultures of naive $CD4^+$ T cells stimulated with anti-CD3 and TGF-β, IL-2 was required to release the TGF-β-mediated proliferation inhibition (Chen et al., 2003). Experiments utilizing IL-2 neutralization and IL-2-deficient T cells demonstrated that IL-2 is required in vitro for TGF-β induction of Foxp3 transcription and suppressor activity (Zheng et al., 2007). IL-2, but not other common-γ chain signaling cytokines, could replace the requirement for CD28 costimulation for the induction of Foxp3 by anti-CD3 and TGF-β (Davidson et al., 2007). In vitro differentiated iTreg cells did not require IL-2 to maintain Foxp3 expression after transfer into in RAG-deficient recipient mice (Davidson et al., 2007). IL-2 signaling activates STAT5. STAT5 binds to the Foxp3 gene and may cooperate with STAT3 for Foxp3 induction (Burchill et al., 2007; Li and Flavell, 2008). Thus, as may be the case for TGF-β, the requirement for IL-2 also differs between thymic-derived nTreg cells and peripheral iTreg cells.

Another important difference between nTreg and iTreg cell generation relates to CTLA-4. Expression of high amounts of Foxp3 and acquisition of suppressor activity by naive cells activated with TGF-β in vitro required upregulation of CTLA-4. In contrast, CTLA-4 is not necessary for the development of nTreg cells in the thymus (Zheng et al., 2006). Consistently, B7 expression in host cells was required for the conversion of $CD4^+CD25^-$ adoptively transferred T cells into $Foxp3^+CD25^+$ cells (Liang et al., 2005). In contrast to CTLA-4, stimulation through CD28 was shown to inhibit TGF-β-induced Treg cell differentiation in vitro (Benson et al., 2007). However, in the thymus of CD28-deficient mice, the number of nTreg cells is reduced to about one third of the wild-type number (Salomon et al., 2000; Tai et al., 2005).

Dendritic Cells, Retinoic Acid, and iTreg Cell Generation in the Gut

In addition to the minimal cytokine conditions for iTreg cell development, other microenvironmental factors may promote or impair iTreg cell induction. Cytokines that induce the differentiation of other T helper cell types (e.g., Th1, Th2, Th9, and Th17 cells) antagonize $Foxp3^+$ Treg cell differentiation. This topic is reviewed in this issue of *Immunity* by Zhou et al. (2009).

Inhibition of TGF-β-induced Treg cell development by inflammatory cytokines or high amount of costimulation in cell culture could be suppressed by retinoic acid (RA) (Benson et al., 2007; Hill et al., 2008; Mucida et al., 2007). $CD103^+$ dendritic cells (DCs) isolated from the small intestine and the mesenteric LN produce both TGF-β and RA; these DCs efficiently mediate the differentiation of naive T cells into $Foxp3^+$ Treg cells (Coombes et al., 2007; Sun et al., 2007). Expression of the integrin CD103 is itself regulated by TGF-β (Lim et al., 1998).

Although we know of a number of key players involved in the induction of $Foxp3^+$ iTreg cells in the gut and mesenteric LN, less is known about Treg cell induction in other lymphoid organs and tissues. $CD103^+$ DCs, which mediate Treg cell conversion in the gut, are abundant in the lamina propria (LP) and in the mesenteric LN, but are less frequent in the spleen. Recently, Yamazaki and collaborators described $CD8^+DEC205^+$ spleen DCs that produce TGF-β and are able to induce naive T cell differentiation into $Foxp3^+$ Treg cells. In contrast, $CD8^-CD205^-$DC inhibitory receptor-2 $(DCIR2)^+$ spleen DCs can induce Foxp3 expression only if exogenous TGF-β is provided (Yamazaki et al., 2008). The fact that $CD8^+CD205^+$ splenic DCs induce iTreg cells is consistent with the previously described observation that

administration of antigen coupled with DEC205 (CD205) induces iTreg cells (Kretschmer et al., 2005).

Retinoic acid boosts the percentage of in vitro conversion into Foxp3$^+$ iTreg cells when TGF-β plus IL-2 is not sufficient to promote high yield (Benson et al., 2007; Hill et al., 2008). However, some reports show a very high frequency (more than 90%) of Foxp3$^+$ iTreg cells without addition of RA (DiPaolo et al., 2007). The reasons for the variation among different laboratories of the frequency of Foxp3$^+$ iTreg cells are not clear. The main suspect is the different batches of serum, which contain variable amounts of TGF-β and RA.

Perhaps the most compelling view of Foxp3$^+$ iTreg cells is that they are the result of coevolution of the adaptive immune system and the commensal bacteria and food antigens in the gut. One could speculate that continuous Treg cell differentiation in the gut may be required to dampen immune reactions to gut antigens. Several groups compared germ-free (GF) mice with mice from specific pathogen-free (SPF) facilities to determine how commensal bacteria affected the frequency and function of Treg cells in the gut and other tissues. Because no markers have yet been identified to differentiate nTreg from iTreg cells, the analysis could only address the whole Treg cell population in various tissues. The analysis of Treg cell populations in various studies have provided discordant results, with some studies showing a deficit in Treg cell function in GF mice (Ishikawa et al., 2008; Ostman et al., 2006; Strauch et al., 2005) and others showing normal numbers and function (Gad et al., 2004; Min et al., 2007). Perhaps some of the conflicting analysis stems from the fact that the components of the intestinal microbiota may differ among various SPF animal facilities (Ivanov et al., 2008). The discrepancies could also be attributed to differences in the GF colonies (paradoxically, there are different degrees of "germ-freeness"!), and the type of functional analysis carried out in each case. The gut is a complex environment in which there is induction of Th17 and Treg cells. The interplay between Th17 and Treg cells has been widely documented and is reviewed in this issue by Zhou et al. (2009).

Ivanov et al. (2008) found that the proportion of Foxp3$^+$ Treg cells was increased in the lamina propria of the small intestine of GF mice compared with SPF mice, whereas the proportion of Th17 cells was greatly reduced. Interestingly, treatment of mice for several weeks with the antibiotic vancomycin, which reduced the population of cytophaga-flavobacter-bacteroides, led to decrease in Th17 cells and an increase in Foxp3 Treg cells (Ivanov et al., 2008). Similarly, Atarashi and colleagues found that in GF mice and in mice treated with vancomycin plus metronidazole, Th17 cell frequency decreased in the small intestine, whereas Treg cell frequency increased. In the LP of the large intestine, however, Treg cell numbers also decreased in GF mice and antibiotic-treated mice (Atarashi et al., 2008). In summary, it appears that the microbiota impacts the Treg cell population of animals, but it is yet unclear whether or not there is a direct effect of the microbiota on the induction of iTreg cells.

Microrganisms activate the innate immune system through a variety of pattern-recognition receptors (PRRs), such as the membrane Toll-like receptors (TLR), the cytosolic nucleotide-binding domain, and leucine-rich repeat containing (NLR) molecules and RIG-I-like receptors (RLRs) (Kawai and Akira, 2009; Palm and Medzhitov, 2009). Animals defective in selected PRRs have been analyzed in regard to the status of their Treg cell populations. Importantly, one must bear in mind that a Treg cell deficiency in PRR-deficient animals does not imply that the microbiota is exerting a direct effect on Treg cell differentiation, because endogenous ligands have been described for PRRs and, even if the microbiota is involved, it may be in an indirect way.

Among the PRR-deficient mice that have been tested, MyD88- and TLR2-deficient mice had reduced Treg cell numbers, whereas TLR4-deficient mice were not affected (Sutmuller et al., 2006; van Maren et al., 2008). In contrast, TLR9-deficient mice had increased frequency of Foxp3$^+$ Treg cells in intestinal tissues and decreased frequency of IL-17- and IFN-γ-producing cells (Hall et al., 2008). Thus, PRR effects on Treg cells are diverse and can be opposite, like the cases of TLR2 and TLR9. It is not yet known whether natural and adaptive Treg cells are equally affected by signaling through these receptors. In addition, local and systemic effects are likely to play a role.

Commensal bacteria are necessary to create the right gut environment for the development of oral tolerance to food allergens (Prioult and Nagler-Anderson, 2005). GF mice were less susceptible to develop oral tolerance to OVA than were mice reared in SPF facilities (Ishikawa et al., 2008; Moreau and Gaboriau-Routhiau, 1996; Rask et al., 2005; Sudo et al., 1997). TLR4-deficient mice but not wild-type mice were highly susceptible to food allergy (Bashir et al., 2004). Coadministration of the TLR9 ligand CpG prevented allergy and promoted a Th1 cell response. In summary, commensal microbiota may contribute to the induction and/or expansion of Foxp3$^+$ Treg cells in the gut by directly or indirectly engaging TLR2. The inability of TLR4-deficient mice to become tolerant to allergic sensitization through the gut appears to be related to deficient Th1 cell induction rather than to deficient Treg cells (Bashir et al., 2004). Finally, TLR9 engagement may affect Treg cell numbers by switching the Treg cell-Th17 cell balance toward Th17 cell differentiation in TGF-β-rich environments of the gut.

iTreg Cells and Infectious Tolerance

An interesting corollary of the induction of Treg cells under subimmunogenic conditions is that Treg cells themselves contribute to the creation of such subimmunogenic conditions. Thus, a Treg cell-dominated environment could result in the generation of more iTreg cells, recruited from the pool of conventional T cells. Infectious tolerance, initially described by Gershon and Kondo as the dominant transfer of tolerance by spleen cells from tolerant mice to secondary recipients (Gershon and Kondo, 1971), was later redefined by Waldmann's laboratory. In Waldmann's experiments, transplantation tolerance was induced by short-term treatment with nondepleting CD4 and CD8 antibodies. Adoptively transferred CD4$^+$ T cells from tolerant mice not only prevented transplant rejection in naive recipients but also conferred to the recipients' CD4 cells the ability to suppress transplant rejection (Qin et al., 1993). Similarly, thymus epithelium induced a population of regulatory T cells that, upon transfer into a secondary recipient, was able to recruit recipient-derived T cells into a tolerance-inducing function (Modigliani et al., 1996). Mechanistically, activated Foxp3$^+$ Treg cells, but not resting Treg cells, expressed TGF-β coupled to latency-associated peptide LAP. Activated Treg cells induced the differentiation

of naive cells to Foxp3$^+$ Treg cells in a TGF-β-dependent cell contact-dependent manner (Andersson et al., 2008).

Given these facts, it is unclear why infectious tolerance mechanisms do not recruit a larger fraction of the conventional T cell pool into the iTreg cell compartment. For example, in a myelin basic protein (MBP)-specific TCR transgenic system lacking nTreg cells, transfer of polyclonal nTreg cells could prevent the development of spontaneous autoimmune encephalomyelitis (EAE), but even as these nTreg cells are at work protecting the recipients from EAE, they do not transfer their regulatory function to recipients' MBP-specific T cells. Antibody-mediated elimination of the transferred nTreg cells resulted in the rapid onset of EAE, indicating that nTreg cell-induced conversion of MBP-specific Treg cells into Foxp3$^+$ iTreg cells did not take place or was minimal (Hori et al., 2002). It is thus apparent that infectious tolerance operates under a set of special conditions that go beyond the creation of subimmunogenic conditions.

There is no doubt that we still have an incomplete understanding on the different conditions that lead to iTreg cell differentiation in vivo, the inductive microenvironments, the APCs that are involved, and the neutralization of specific inhibitory factors.

Differences between nTreg and Foxp3$^+$ iTreg Cells

A number of studies have provided data that shed light on the differences between nTreg and iTreg cells as well as the differences between iTreg cells generated in different ways. Within iTreg cells, the differences between in vitro and in vivo generated iTreg cells are not fully understood. There has been some disagreement on whether in vitro generated Foxp3$^+$ iTreg cells are as good suppressor cells as natural Treg cells (Aricha et al., 2008; Chen et al., 2003; DiPaolo et al., 2007; Hill et al., 2007; Horwitz et al., 2008; Selvaraj and Geiger, 2008), but there is consensus that in vivo generated iTreg cells are effective suppressors (Apostolou and von Boehmer, 2004; Cobbold et al., 2004; Curotto de Lafaille et al., 2008; Knoechel et al., 2005; Mucida et al., 2005).

Studies on methylation of CpG motifs in the Foxp3 locus of natural Foxp3$^+$ Treg cells identified complete demethylation within an evolutionary conserved region upstream of exon 1, named TSDR (Treg cell-specific demethylated region). TSDR demethylation is unique to Foxp3$^+$ regulatory T cells. In contrast to nTreg cells, in vitro generated iTreg cells displayed only partial demethylation in spite of high Foxp3 expression. These in vitro generated iTreg cells, but not nTreg cells, lost both Foxp3 expression and suppressive activity when restimulated in the absence of TGF-β (Floess et al., 2007; Huehn et al., 2009). A marked difference in the methylation state of TSDR between in vitro and in vivo generated iTreg cells has been reported. Like nTreg cells, but unlike in vitro generated iTreg cells, iTreg cells generated in vivo by administration of antigen coupled with DEC205 antibodies displayed efficient TSDR demethylation (Polansky et al., 2008). A potential problem that needs to be addressed in future studies is the timing of TSDR demethylation. Although in vitro generated iTreg cells were analyzed for TSDR demethylation only 6 days after induction with TGF-β (Floess et al., 2007), the in vivo generated iTreg cells were analyzed 3 weeks after administration of antigen (Polansky et al., 2008). These differences in the time of analysis could be significant. Consistent with this view, Floess et al. (2007) showed that in the thymus, TSDR demethylation in CD4$^+$SP Foxp3$^+$ nTreg cells is not yet complete. Thus it may take some time before TSDR demethylation is completed in both iTreg and nTreg cells.

Gene profiling studies have defined a subset of genes expressed in natural Foxp3$^+$ Treg cells defined as the Treg cell signature. Part of the Treg cell signature corresponds to genes that are induced or repressed in Treg cells independently of Foxp3 expression, with the other gene subset being downstream of Foxp3. Hill et al. (2007) compared the expression of the Treg cell signature genes in Treg cells generated in a number of different conditions, including in vitro generated iTreg cells. Of relevance to the discussion here, the authors found that in vitro generated Foxp3$^+$ iTreg cells lacked part of the Treg cell signature genes, despite their high expression of Foxp3 (Hill et al., 2007). A caveat of the analysis is that the iTreg cells analyzed in the above study displayed poor suppression activity in vitro and in vivo. Further comparison of in vitro generated Foxp3$^+$ iTreg and nTreg cell by expression microarray was recently reported (Haribhai et al., 2009). However, the gene expression profile of in vivo generated iTreg cells has not yet been reported. Furthermore, there are no consensus nTreg and iTreg cells markers that can be used to study the properties of each type separately.

Foxp3$^+$ iTreg cells, by definition, have the TCR repertoire of naive conventional T cells. This conventional TCR repertoire is different from Foxp3$^+$ Treg cells (Hsieh et al., 2004, 2006; Pacholczyk et al., 2006; Wong et al., 2007b). An overlap between the two TCR repertoires has been highlighted in all reports, although there was variation among the studies regarding the similarity index, calculated as lower than 25% (Hsieh et al., 2004), between 10% and 20% (Pacholczyk et al., 2006), or 42% (Wong et al., 2007b). The repertoire overlap could be explained by the presence of iTreg cells, which will have a conventional T cell repertoire but will be analyzed in the Foxp3$^+$ group mixed with nTreg cells. The repertoire overlap could also be interpreted as indicating that, although TCR signaling is important in determining the commitment along the Foxp3$^+$ pathway, other factors are also important, or that there is a stochastic component to Treg cell selection.

A more recent study indicated that even though a relatively high percentage of the TCR sequences from conventional T cells were found in Treg cells and, likewise, a high percentage of the TCR sequences from Treg cells were found in the conventional T cells, the frequency of these "shared" sequences in the two T cell populations was dramatically different (Pacholczyk et al., 2007). Overall, the studies point to the fact that the repertoire of Treg cells and conventional T cells is different. As indicated above, it is tempting to speculate that the Treg cell TCR sequences shared with conventional T cells correspond to iTreg cells. In fact, because the repertoire overlap between Treg cells and conventional T cells was small, this could be interpreted as indicating that the contribution of iTreg cells to the total Treg cell pool is small. If the frequency of iTreg cells among total Treg cells were high, the repertoires of conventional T cells and Treg cells would be more overlapping. One problem with this conclusion is that it is based upon nonimmunized mice housed under clean, specific-pathogen-free conditions. As discussed below in this review, it has become apparent that chronic antigenic exposure represents one of the main sources of iTreg

cells, and therefore it is expected that mice with limited exposure to infections and antigenic stimulation would also have a limited iTreg cell contribution. Some of the established differences between Foxp3$^+$ nTreg and iTreg cells are summarized in Box 1.

iTreg Cells in Mucosal Tolerance and Chronic Inflammatory Responses

Mucosal tolerance to environmental and food antigens is essential to prevent exacerbated immune reactions that can cause allergic diseases and chronic inflammation, and it has long being recognized that the gut is an important site for the induction of tolerance (Faria and Weiner, 2005). Currently, there is no experimental system that allows the study of nTreg cells in the absence of iTreg cells, because every Foxp3$^+$ population is likely to contain an iTreg cell component. However, the converse study is possible. TCR transgenic RAG-deficient mice have monoclonal T cell repertoires and were shown to lack nTreg cells initially through their enhanced susceptibility to spontaneous autoimmune disease (Lafaille et al., 1994). When Foxp3 staining became available, it was confirmed that these mice lack Foxp3$^+$ T cells (Kretschmer et al., 2005; Shen et al., 2005). The lack of nTreg cells in TCR transgenic RAG-deficient mice is consistent with the accepted models of high-affinity thymic selection of nTreg cells (i.e., the transgene-encoded T cell receptors do not encounter the appropriate high-affinity ligand in the thymus), although there are alternative explanations. In TCR transgenic RAG-deficient mice, iTreg cells can be generated, as described above, in the absence of nTreg cells. By using one such experimental system (T-Bmc) in which the T cells recognize OVA, we determined that administration of OVA in the drinking water led to generation of OVA-specific Foxp3$^+$ iTreg cells (Mucida et al., 2005). These cells were responsible for oral tolerance, because crossing of the T-Bmc mice with Foxp3-deficient mice abrogated oral tolerance, even though other tolerance mechanisms were intact in these mice (Curotto de Lafaille et al., 2008). In spite of being deficient in nTreg cells and unable to generate Foxp3$^+$ iTreg cells, Foxp3-deficient T-Bmc mice are healthy throughout life if antigen is not administered, because all T and B cells recognize foreign antigens.

OVA-specific iTreg cells were also induced in wild-type mice with polyclonal repertoires of conventional T and Treg cells, via bone marrow reconstitution of BALB/c mice with a small proportion of OVA-specific T cell progenitors (Curotto de Lafaille et al., 2008). Thus, in the absence of nTreg cells, induction of Foxp3$^+$ iTreg cells is essential to establish tolerance and avoid allergic sensitization.

In the same mouse system, OVA-specific iTreg cells were also induced by immunization with OVA in alum, a protocol used to induce experimental allergic asthma. In this case, however, Foxp3$^+$ iTreg cells were generated concomitantly with a much more robust effector Th2 cell response. These immunization-induced iTreg cells did not prevent the primary IgE response and acute eosinophilic inflammation in the lung. However, iTreg cell numbers rapidly built up in the lungs of immunized mice after airway challenge and became prominent after repeated antigen exposure. In a chronic model of allergic inflammation, iTreg cells, in the absence of nTreg cells, were essential to reduce the severe inflammation and lung remodeling, suppress neolymphoid development in the lung, and prevent the dissemination of Th2 cells to distant LN and the skin (Curotto de Lafaille et al., 2008). Thus, in tolerogenic conditions, antigen-specific Foxp3$^+$ Treg cells are induced but Th effector cells are not. These Treg cells efficiently block the development of effector Th cells and prevent inflammation upon subsequent immunization. In contrast, immunization-induced Treg cells that develop concomitant with effector T cells do not prevent acute inflammation but expand on inflammation. They function to suppress severe inflammation and the spreading of Th cell effectors to distant LN and tissues.

Treg Cells and Infections

With few exceptions, chronic infections have been associated with increased Foxp3$^+$ Treg cell numbers. Induction and/or expansion of Treg cells during infections could be responsible for a failure to clear the infection, with consequent establishment of a chronic phase in which Treg cells are involved in limiting immune-mediated tissue damage. The role of Treg cells during infections has been recently reviewed (Demengeot et al., 2006; Wohlfert and Belkaid, 2008). The presence of Treg cells during infections is an important barrier for vaccination and treatment strategies. Important for the purpose of this review, the accumulation of Foxp3$^+$ Treg cells during infections could be due to expansion of nTreg cells and/or to conversion of naive T cells to iTreg cells.

Many parasites establish persistent infections in the mammalian host that modulate the immune system in ways that allow long-term parasite survival (Wohlfert and Belkaid, 2008). An increase in the Foxp3$^+$ Treg cell population was demonstrated in several models of rodent filariasis (Maizels, 2007; McSorley et al., 2008). Interestingly, *Brugia malayi* secretes a homolog of mammalian TGF-β (Gomez-Escobar et al., 2000) that may be involved in Treg cell conversion. Further, increased Foxp3$^+$ Treg cells and IL-10-producing Tr1 cells may be responsible for the lower incidence of allergic disease in individuals infected with helminth parasites (Wills-Karp et al., 2001; Wilson and Maizels, 2004). Expansion of pathogen-specific Foxp3$^+$ Treg cells was also observed in bacterial infections, such as *Listeria monocytogenes* (Ertelt et al., 2009). *Helicobacter pylori* establish chronic infections that can be cured by antibiotic treatment. However, cured individuals are not immune to reinfection. A large increase in transcripts for Foxp3 and TGF-β was found in the gastric mucosa of *H. pylori*-infected individuals (Kandulski et al., 2008). Interestingly, memory peripheral blood T cells from infected and uninfected individuals proliferated to *H. pylori* antigens, but only in infected individuals did the depletion of Treg cells lead to enhanced proliferation, suggesting the existence of *H. pylori*-specific Treg cells (Lundgren et al., 2003).

In addition to parasitic and bacterial infections, chronic viral infections have been described with enhanced Treg cell activity (reviewed by Robertson and Hasenkrug, 2006). The involvement of Treg cells may be particularly important to investigate in chronic viral infections for which there are no vaccines and no cure, such as HIV and hepatitis C virus (HCV) (Alatrakchi and Koziel, 2009; Dolganiuc and Szabo, 2008; Li et al., 2008). HCV infection is the most common blood-borne infection in the USA. Chronic infections are associated with a weak virus-immune response, impaired DC function (Li et al., 2008), and increased Foxp3$^+$ Treg cells (Ebinuma et al., 2008; Ward et al., 2007). In

the case of HCV, about a third of infections are resolved, which indicates that it is possible to mount effective immunity. Clearly, induction of iTreg cells can be an impediment to successful vaccination. More needs to be known about the type of response that leads to resolution of infection and effective immunity.

iTreg Cells and Cancer

It has become evident that Foxp3$^+$ Treg cells are one of the main if not *the* main barrier to the implementation of cancer immuno therapies (Curiel, 2008; Zou, 2006). Elimination of Treg cell function with CD25 antibodies resulted in survival of experimental animals that otherwise would succumb to cancer (Onizuka et al., 1999; Shimizu et al., 1999). Tumor tissues promote the conversion of naive T cells into Foxp3$^+$ Treg cells and the accumulation of Treg cells in tumor sites, thereby impairing the development of effector responses (Liu et al., 2007; Nishikawa et al., 2003). Thus, Foxp3$^+$ iTreg cells have a key function in obstructing tumor immunosurveillance (Liu et al., 2007; Zhou and Levitsky, 2007).

Absence of iTreg Cell Generation

In some experimental conditions where generation of iTreg cells would have been expected, investigators reported lack of conversion of naive polyclonal or antigen-specific CD4$^+$ T cells into Foxp3$^+$ Treg cells. For example, when CD4$^+$CD25$^-$RFP(Foxp3)$^-$ cells were transferred into RAG-deficient mice, the animals developed inflammatory bowel disease (IBD) with no conversion of transferred cells to RFP$^+$(Foxp3$^+$) Treg cells during 6 weeks of observation (Wan and Flavell, 2005). In addition, at least two reports failed to identify substantial iTreg cell generation during infections. In mice infected with OVA-expressing *Listeria monocytogenes*, OVA-specific naive T cells expanded but did not convert to Foxp3$^+$ (Fontenot et al., 2005b). Similarly, the Treg cells accumulated in lymph nodes that drain *Leishmania*-infected tissues were derived from a population of CD4$^+$CD25$^+$ Treg cells, and not from cotransferred CD4$^+$CD25$^-$ T cells (Suffia et al., 2006).

There are also examples of lack of conversion in experimental autoimmunity systems. When total CD4$^+$ T cells or Foxp3-negative T cells from Foxp3-GFP reporter mice were injected into MOG-immunized mice, GFP$^+$ Treg cells accumulated in the central nervous system only when total cells but not when GFP-negative cells were injected, thus attributing the accumulation of Foxp3$^+$ T cells to expansion of nTreg cells (Korn et al., 2007). Finally, by using a TCR transgenic system, BDC2.5, that recognized a pancreatic antigen, Wong et al. (2007a) compared the endogenous TCRα chain sequences expressed by Foxp3$^-$ and Foxp3$^+$ T cells and found signature CDR3 TCRα sequences. Comparison of these signature sequences in the thymus and periphery indicated that exposure to the pancreatic self-antigen in the periphery did not cause the signature Foxp3$^-$ sequences to appear in the Foxp3$^+$ compartment. The authors proposed that little or no peripheral conversion of Foxp3$^-$ into Foxp3$^+$ cells occurred upon encounter with antigen in this model (Wong et al., 2007a).

In some cases, it is possible to speculate that the lack of detection of Foxp3$^+$ iTreg cells was due to low sensitivity of the readouts, or to potential expression of the antigen in the thymus (in the case of the BDC2.5 experiment), or even to iTreg cells present in the original CD25$^+$ cell transfer (in the case of the *Leishmania* experiment). However, it is very unlikely that such technical problems would affect all the experiments described above. Much more likely is that there was little or no iTreg cell generation, reflecting the fact that we do not know the full assortment of conditions that result in iTreg cell generation or lack thereof, and how this process is regulated by tissue environment, antigen specificity, and pre-existing Treg cells.

Synergistic Functions for nTreg Cells and iTreg Cells

A consensus exists for the role of nTreg cells in preventing autoimmunity and preventing exaggerated immune responses. Experimental data now support the importance of Foxp3$^+$ iTreg cells in mucosal tolerance and in inflammatory responses to foreign antigens, in the suppression of responses to neo-antigens in tumors and transplants. However, the extent of the contribution of iTreg and nTreg cells in normal animals has been difficult to evaluate because of the lack of suitable surface markers or reporter mice distinguishing iTreg and nTreg cell populations.

The interplay between nTreg cells and iTreg cells in different immune responses is beginning to be addressed. Haribhai and colleagues used a transfer model of colitis to compare the function of nTreg cells, in vitro induced iTreg cells, and in vivo induced iTreg cells (Haribhai et al., 2009). As expected, transfer of naive Foxp3$^-$CD4$^+$ cells into RAG-deficient mice induced colitis. If the naive T cells were unable to become iTreg cells in vivo because of a Foxp3 mutation, the disease progressed faster, indicating that in vivo differentiated Treg cells conferred some protection, although the specificities of the effector T cells could be different in Foxp3-sufficient and -deficient mice (Hsieh et al., 2006). nTreg cells were better at controlling disease than in vitro induced iTreg cells when transferred into mice that had received naive cells capable of generating in vivo iTreg cells (i.e., from Foxp3-sufficient donors), but, importantly, only the combination of nTreg and in vitro generated iTreg cells could protect mice that had received naive cells that were unable to generate in vivo iTreg cells (i.e., from Foxp3-deficient donors). If the naive T cell population had derived from mice that could generate in vivo iTreg cells, then full protection from colitis could be afforded by nTreg cells alone. Thus, in this colitis model, both natural and induced (in vitro induced or in situ induced) Treg cells were required for protection from disease. One must add that in this and most other cases, the nTreg cell population is, in reality, a total population containing a majority of nTreg cells and some in vivo generated iTreg cells. Because these Treg cells originate from clean, nonimmunized animals, the contribution of iTreg cells to the total Foxp3$^+$ Treg cell pool is likely to be small, as indicated by the TCR repertoire analysis discussed above.

Altogether, the results discussed above indicate a similarity of function between iTreg cells generated in vitro and in vivo. Of relevance, a divergence of function between nTreg cells and iTreg cells in the regulation of the same disease was uncovered. Thus, it is possible that in many instances, nTreg and iTreg cells synergize to achieve optimal regulation. It is quite likely that there is a division of labor between nTreg cells and iTreg cells, something that could have been predicted based upon the different TCR repertoires of both Treg cell populations (Figure 1).

Concluding Remarks

Although their existence was controversial for some time, it is now absolutely clear that iTreg cells develop under a variety of

conditions, quite possibly during the normal homeostasis of the gut (Box 1). For what we know at this time, iTreg cells play essential roles in immune tolerance and in the control of severe chronic allergic inflammation, are responsible for preventing the clearance of parasites and other microorganisms, and are one of the main barriers to the immune eradication of tumors. Although iTreg cells seem tailored to respond to foreign antigens and neoantigens (such as tumor antigens), it is likely that they are also generated in response to self-antigens and synergize with nTreg cells in the control of autoimmune inflammation.

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