Induced CD4$^+$Foxp3$^+$ Regulatory T Cells in Immune Tolerance

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Abstract

Regulatory T lymphocytes are essential to maintain homeostasis of the immune system, limiting the magnitude of effector responses and allowing the establishment of immunological tolerance. Two main types of regulatory T cells have been identified—natural and induced (or adaptive)—and both play significant roles in tuning down effector immune responses. Adaptive CD4$^+$Foxp3$^+$ regulatory T (iTreg) cells develop outside the thymus under a variety of conditions. These include not only antigen presentation under subimmunogenic or noninflammatory conditions, but also chronic inflammation and infections. We speculate that the different origin of iTreg cells (noninflammatory versus inflammatory) results in distinct properties, including their stability. iTreg cells are also generated during homeostasis of the gut and in cancer, although some cancers also favor expansion of natural regulatory T (nTreg) cells. Here we review how iTreg cells develop and how they participate in immunological tolerance, contrasting, when possible, iTreg cells with nTreg cells.

Foxp3: forkhead box p3

Tconv cell: conventional T cell

iTreg cell: induced regulatory T cell

nTreg cell: natural regulatory T cell

TCR: T cell receptor

APC: antigen-presenting cell

OVA: ovalbumin

Teff cell: effector T cell

INTRODUCTION

Foxp3$^+$ regulatory T cells are essential components of the homeostasis of the immune system (1–3). Foxp3 is a forkhead box transcription factor whose expression is restricted to regulatory T (Treg) cells, although under some circumstances conventional T (Tconv) cells can transiently express Foxp3 (4). Null mutations in the X-linked Foxp3 gene cause devastating autoimmune diseases in humans and mice, showing that the suppressive function of Foxp3-expressing cells is largely nonredundant (5–7).

This review focuses on extrathymically derived adaptive (or induced) CD4$^+$Foxp3$^+$ regulatory T (iTreg) cells. We discuss their generation, how they can be phenotypically and functionally distinguished from thymus-derived natural Foxp3$^+$ regulatory T (nTreg) cells, and their contribution to immune tolerance. We also discuss the potential of using iTreg cells as a therapeutic approach. Other populations of adaptive regulatory T lymphocytes such as Tr1 and Th3 cells are not discussed in this review, even though these Foxp3$^-$ Treg cells were the "original" iTreg cells. Although we use the abbreviation iTreg throughout this article, the word "induced" is misleading, as thymic Foxp3$^+$ Treg cells are also induced. The word "adaptive" better describes the induction by antigen at peripheral sites, but the abbreviation iTreg has become routinely used by most authors and we see no benefit in creating a nomenclature controversy.

DIFFERENT SUBSETS OF TREG CELLS: THYMUS-DERIVED nTREG CELLS AND PERIPHERY-DERIVED iTREG CELLS

It was previously thought that Foxp3$^+$ Treg cells could only arise from the thymus through high-avidity interactions between MHC class II molecules and the T cell receptor (TCR). However, it is now widely appreciated that Foxp3$^+$ Treg cells can also develop in the periphery from mature conventional CD4$^+$ T cells under a variety of conditions (8–14). Thus, the Treg cell populations can be divided into two major groups: the thymus-derived Treg cells, known as nTreg cells, and those that are extrathymically derived, known as iTreg cells. Development of extrathymic Treg cells is favored in two broad categories. The better understood one is defined by T cell activation in the absence of inflammation (sometimes mentioned in this category as a subimmunogenic situation) (15). Examples of this category are (*a*) the use of non-depleting anti-CD4 antibodies, which weaken coreceptor engagement (10); (*b*) "clean" antigen deliveries by osmotic pumps (8) or intravenous injections; (*c*) antigen presentation by antigen-presenting cells (APCs) in the absence of maturation signals (12); and (*d*) antigen presentation in tolerogenic microenvironments such as the mucosa of the small intestine (11). One experiment that typifies this category was carried out with chicken ovalbumin (OVA) administration to OVA-specific TCR-transgenic mice crossed with recombination activating gene (RAG)-deficient and Foxp3-deficient mice. In these mice, oral administration of antigen does not induce Foxp3$^+$ iTreg cells because the mice are Foxp3 deficient. However, T cells in these mice did not become effector T cells, even though they upregulated CD69, indicating antigen exposure (14). Thus, under these noninflammatory conditions, T cell stimulation can generate iTreg cells but not effector T (Teff) cells. In the second category of iTreg cells, inflammatory conditions allow the quasi-simultaneous development of both iTreg cells and Teff cells in inflammatory sites, and, in fact, iTreg cells are generated at a lower rate than Teff cells (15) (**Table 1**). It looks increasingly apparent that iTreg cells generated under noninflammatory or inflammatory conditions have distinctive properties.

Transcriptional Signatures of nTreg and iTreg Cells

The different origins of nTreg and iTreg cells, in particular the TCR requirements in the thymus, highlight potential differences between

Table 1 Comparison between natural and induced regulatory T cells

Characteristic	Natural Treg cell	Induced Treg cell
Site of induction	Thymus	Secondary lymphoid organs/inflamed tissues
Costimulation requirement	CD28	CTLA-4
Cytokine requirement	IL-2, TGF-β?	TGF-β, IL-2, RA (potentiation)
Specificity	Self-antigens	Self- and foreign antigens
Common markers	Foxp3, CD25, GITR, CTLA-4	
Specific markers	Helios, Nrp1, PD-1, Swap70	Dapl1, Igfbp4
Mechanism of suppression	Cell contact–dependent	Cytokine-dependent?

iTreg and nTreg cells that may be relevant to their functions. In this section, we review recent insights into the developmental and functional differences between nTreg and iTreg cells.

Several groups have used microarray analyses to unveil the developmental and functional differences between nTreg and iTreg cells. Haribhai and coworkers (16) compared the gene expression of in vitro–generated iTreg cells by a well-established method of TCR stimulation in the presence of TGF-β and IL-2 (17–19) with total Treg cells extracted from healthy Foxp3-GFP reporter mice, a population composed mostly of nTreg cells. To exclude biased analysis from in vitro activation, nTreg and Tconv cells were also activated. iTreg and nTreg cells were shown to be genetically distinct even though a significant number of genes were expressed by both Treg subsets (16). Among the genes differentially expressed, *Ikzf2* (Helios) and *Nrp1* (Neuropilin-1) expression were upregulated by nTreg cells when compared with iTreg cells. Both genes belong to the Treg genetic signature described previously (20, 21).

Comparison of Treg cells from a number of locations and origins showed that each Treg type displayed singularities (22). Among the different Treg cells, the comparison groups included iTreg cells induced by low-dose antigen delivery via DEC-205 antibody targeting (12) and by homeostatic proliferation of Tconv cells after transfer into lymphopenic hosts (9). Interestingly, the profile of iTreg cells sorted from the transferred lymphopenic hosts resembled that of total Treg cells extracted from healthy mice, suggesting that they were exposed to similar environmental pressures. Even though the profiles of total Treg cells and in vivo–generated iTreg cells were quite similar, some differences were noted. Among these are *Ctla4* (upregulated in DEC-205 iTreg cells) and *Ebi3*, a component of IL-35, and *Klrg1* (upregulated in iTreg cells induced by homeostatic proliferation) (22). The importance of these differentially expressed genes on the function of the different Treg cell subsets requires further evaluation.

More recently, the gene expression profile of in vivo–generated iTreg cells was compared with nTreg cells in a model of autoimmune lymphoproliferative disease (23). Neonatal Foxp3-deficient mice transferred with a combination of nTreg and Tconv cells do not develop lethal lymphoproliferative disease. In these mice, a fraction of transferred Tconv cells become Foxp3$^+$ iTreg cells. The advantage of this experimental system is that both iTreg and nTreg cells were extracted from the same mice and, therefore, exposed to the same environment. The gene expression profiles of in vivo–generated iTreg and nTreg cells were very similar, with shared expression of genes belonging to the Treg cell signature established by previous studies (20, 21). However, some genes were differentially expressed; among them, *Il10* (interleukin-10) was upregulated in iTreg cells and *Gzmb* (granzyme B) in nTreg cells. It is tempting to speculate that these differences are related to distinct suppression

TGF-β: transforming growth factor-β

IL-2: interleukin-2

Nrp1: Neuropilin-1

IL-10: interleukin-10

CD25: IL-2 receptor α

mechanisms. In contrast, the gene expression profile of in vitro–generated iTreg cells was very different from in vivo–generated iTreg cells (23). In vitro, developing iTreg cells faced a limited set of cues (TCR signaling, TGF-β, and IL-2), whereas in vivo scenarios were more complex, especially the ones with ongoing inflammatory responses. Paradoxically, despite the gene expression differences, in vitro– and in vivo–generated iTreg cells were interchangeable regarding their suppressive effect.

Division of Labor Between nTreg and iTreg Cells

The relative contributions of nTreg and iTreg cells in establishing and maintaining immune tolerance were comprehensively addressed recently. It was important to determine whether nTreg and iTreg cells had overlapping or separate roles, or if they could even act in concert to achieve immune regulation. In the colitis model developed by Powrie and colleagues (24), transfer of naive $CD4^+CD45RB^{high}$ T cells into immunodeficient mice causes colitis, which is cured by the transfer of total $CD4^+CD25^+$ Treg cells. Using this colitis model, Haribhai et al. (16) showed that if the naive T cell population (which contains the precursors of the disease-causing cells) had been obtained from mice that could generate in vivo $Foxp3^+$ iTreg cells, transfer of nTreg cells could fully rescue mice from colitis because, in addition to the transferred nTreg cells, the naive cells gave rise to some iTreg cells in vivo. If, however, the naive T cells had been derived from Foxp3-deficient mice (thus unable to convert in vivo into $Foxp3^+$ iTreg cells), full protection could only be achieved when the nTreg cells were cotransferred with in vitro–generated iTreg cells. Thus, in this colitis model, both nTreg and iTreg cells were required for protection from disease, supporting a division of labor between nTreg and iTreg cells.

Subsequently, similar conclusions were reached using a lymphoproliferative disease model (23). The results from this study provided significant insights into the roles of iTreg and nTreg cells (25). First, rescue from lymphoproliferative disease caused by Foxp3 deficiency (therefore a deficiency in both nTreg and iTreg cells) was unequivocally dependent on both nTreg and iTreg cells because selective depletion of iTreg cells, leaving the nTreg cell compartment intact, led to incomplete rescue. Second, differences in TCR repertoires (i.e., specificities) of nTreg and iTreg cells, but not in their gene expression profile, were possibly related to the functional complementarity (23).

The protective capacity of in vitro–generated iTreg cells had also been noted in some previous studies (18, 26, 27), although others found it to be lacking (21). A recent report sheds light on the issue of the suppressive properties of in vitro–generated iTreg cells. iTreg cells induced in vitro from nonobese diabetic (NOD) mice Tconv cells were deficient in suppressive properties, but the same induction procedure in other mouse strains yielded functional Treg cells (28). At the genetic level, the NOD deficiency correlated with a cluster of genes that included *Lrrc32* (GARP), *Ctla4*, and *Cd73*, which were underexpressed in NOD. Interestingly, a reduction of GARP expression was found in Treg cells from pancreas-draining lymph nodes but not from the spleen (28). One can speculate that a localized deficiency in iTreg cell function could contribute to disease development in NOD mice, which, incidentally, could explain why global Treg cell studies in NOD mice do not show overwhelming defects.

Can We Distinguish nTreg from iTreg Cells?

Ikzf2 (Helios), a transcription factor of the Ikaros family, is one of the genes belonging to the Treg cell signature (20, 21). Recently, Helios was found to be expressed by the majority of $CD4^+Foxp3^+$ T cells in human peripheral blood mononuclear cells (PBMCs) and by thymic and splenic Treg cells extracted from healthy mice, but not by iTreg cells (29). Contrary to Foxp3, which is indispensable for Treg cell function, Helios does not affect the suppressive ability of nTreg cells, as Helios-deficient

Treg cells could still suppress proliferation of Teff cells in vitro. In contrast, a brief study concluded that the method of activating T cells was a decisive factor in Helios expression on iTreg cells, rather than in nTreg or iTreg cell origin (30). In this case, the authors used T cells from TCR–transgenic RAG-deficient mice (which lack nTreg cells) and differentiated them to iTreg cells in vitro using TGF-β and IL-2 and two different activation stimuli, APCs plus cognate antigen or plate-bound anti-CD3 and anti-CD28. They observed that 50–60% of iTreg cells differentiated in the presence of APCs plus antigen expressed Helios, whereas few or no iTreg cells expressed Helios when generated by anti-CD3 and anti-CD28 stimulation. A subsequent study also supports the view that Helios expression cannot be used to distinguish nTreg from iTreg cells, as Helios expression could be transiently induced during T cell activation and proliferation, regardless of the T cell population that was analyzed (31). These results suggest that under certain circumstances iTreg cells can express Helios. It is likely that an even more complex situation can be found in vivo regarding the different environments in which iTreg cells may be generated. In any case, it appears that in unimmunized animals nTreg, but not iTreg, cells express Helios. One problem of using Helios as a marker is its intracellular localization, which makes it impossible to examine the fate of Helios-expressing cells without destroying the Treg cells or creating nonphysiological viral-driven expression systems. Thus, the generation of a reporter mouse for Helios will facilitate the clarification of the origin and role of Helios-positive and Helios-negative Treg cells.

Taking advantage of pure iTreg cells generated in vivo by oral tolerance induction at mucosal sites (11, 14), our group compared the gene expression profiles of Foxp3$^+$ iTreg cells generated under noninflammatory conditions in vivo with that of total Foxp3$^+$ Treg cells extracted from wild-type mice, which are mostly composed of nTreg cells. In vivo–generated iTreg cells of mucosal origin expressed lower levels of *Nrp1*, *Plagl1*, *Swap70*, and *Ikzf2* mRNA as well as high levels of *Igfbp4* and *Dapl1*. Among these, *Nrp1* encodes a surface protein with proposed implications for Treg function (32).

Previous analysis of gene expression profiles of total Treg cells compared with Tconv cells has revealed Nrp1 as a surface molecule preferentially expressed by Treg cells (21, 33). Moreover, retroviral transduction to induce ectopic expression of Foxp3 on Tconv cells led to expression of Nrp1 (21, 32, 33), suggesting that Foxp3 controls Nrp1 expression. Nrp1 was originally described as a receptor for class III semaphorins and for the heparin-binding forms of vascular endothelial growth factor expressed by subpopulations of neuronal and endothelial cells known to be involved in axon guidance and angiogenesis, respectively (34). Recently, it has been proposed that Nrp1 is highly expressed by some cancer cells and can also act as a coreceptor for TGF-β, promoting the activation of its latent form (35).

The comparison between oral antigen-generated iTreg cells and total Treg cells places Nrp1 as the first surface marker that distinguishes nTreg from iTreg cells of mucosal origin in healthy mice. The great majority of iTreg cells originating through oral antigen administration or other subimmunogenic procedures do not express Nrp1, whereas thymic Foxp3$^+$ Treg cells do (J. Weiss, M. Gobert, A.M. Bilate, M. Curroto de Lafaille, J.J. Lafaille, et al., unpublished observations).

Regardless of its usefulness as a marker, Nrp1's role, if any, in the suppressive function of Treg cells remains unclear. Conditional knockout of Nrp1 on all T cells resulted in no defect in Treg cell generation and no overt spontaneous inflammatory disease (36). In contrast, another report indicates that mice deficient in Nrp1 exclusively on CD4$^+$ T cells developed more severe experimental autoimmune encephalomyelitis (EAE), and Nrp1-deficient Treg cells could not rescue the mice from disease (37). Further studies are now required to determine precisely the functional role of Nrp1 on nTreg cells. It also remains to be investigated whether Nrp1$^+$ and Nrp1$^-$ Treg cells operate

EAE: experimental autoimmune encephalomyelitis

through different or similar mechanisms of suppression.

Taken together, all the gene expression studies lead to the conclusion that iTreg cells retain the expression of some genes that are more typical of Tconv than nTreg cells. In fact, no gene was found to be exclusively expressed by iTreg cells.

The use of high-throughput techniques such as genome-wide analysis will further contribute to the integration of signaling pathways and networks with iTreg cell differentiation and function. It will be interesting to determine which genes are related to a possible differential function between iTreg and nTreg cells or to differentiation of iTreg versus Teff cells.

nTreg and iTreg Cells in Cancer

It has long been noted in animal and human cancer that many tumors contain high proportions of Foxp3$^+$ Treg cells (38–41). In most cases, Treg cells contribute to the establishment of tumors by impairing the antitumor immune response, as anti-CD25-mediated blockade of Treg cell function improved survival and reduced metastases (42, 43), whereas addition of CD4$^+$CD25$^+$ Treg cells suppressed concomitant antitumor immunity (44). However, in human colorectal cancer, a high Treg cell count is associated with a favorable prognosis (45). This special situation can perhaps be correlated with the large concentration of bacteria in the lower gut and the strong proinflammatory environment it creates in the absence of Treg cells.

The iTreg or nTreg cell origin of tumor-infiltrating lymphocytes is controversial. Some lines of experimentation support the in situ generation of iTreg cells (46, 47), whereas others favor a differential migration and/or expansion of nTreg cells (48, 49). In fact, all possibilities (iTreg cell generation and enhanced nTreg cell recruitment and proliferation) can occur simultaneously (50).

An early experiment using transplanted tumors showed extensive proliferation of Treg cells in the tumor bed and draining lymph nodes, but not in the spleen. This proliferation was dependent on TGF-β signaling by T cells (51). However, in these experiments it was difficult to distinguish between the proliferation of existing nTreg cells and the tumor-induced conversion of Tconv to iTreg cells, which can be accompanied by proliferation (9).

Another study postulates that preexisting human Treg cells (likely nTreg cells) migrated into the tumors due to attraction by CCL22 present in the tumors (48). As these were human Treg cells, the chemokine experiments were carried out in transwell plates and were confirmed by injection of human tumor cells and human T cells into NOD-SCID mice. Anti-CCL22 antibody treatment of the mice reduced the recovery of human Treg cells in the tumors. These are difficult experiments to interpret, as many human-mouse ligand-receptor pairs are required to be engaged for the extravasation and migration of leukocytes. Recently, CCL22 has been shown to attract mouse Treg cells to the pancreatic islets, thus protecting the animals from type 1 diabetes (52). In any case, the data of Zou's laboratory (48) provide another potential explanation for the enrichment in Treg cells in tumors: The tumor may provide an environment in which iTreg cells are generated, in which nTreg cells proliferate, or into which nTreg cells are attracted.

The idea that tumor Treg cells are expanded nTreg cells was also proposed upon analysis of T cell repertoires. In this study, TCR repertoires of Treg and Tconv cells within tumors were largely distinct (49); the authors speculate that, had the Treg cell population in the tumor derived from Tconv cells (which is the definition of iTreg cells), then the TCR repertoire overlap would have been higher. However, some TCR repertoire studies, including this one, lack sufficient TCR representation. In fact, the major caveat of the study is the low number of TCR sequences analyzed. A few hundred sequences in a given location (e.g., tumor Treg cells versus tumor Tconv cells) give too narrow a representation of the TCR repertoire, even in a restricted environment such as a tumor. Despite the limited value of

the aforementioned experiments to distinguish nTreg and iTreg cells in the tumors, this work does reaffirm that Treg cell expansion and/or conversion in tumors is not oligoclonal. Thus, although TCR repertoire analysis could be used to make inferences regarding the origin of different cell types, the number of sequences must be very large, and the α and β chain pairing issues must be considered as well.

Another approach to distinguish nTreg from iTreg cells in the tumors made use of the Ikaros family member Helios, which, as discussed in the above section, is expressed by nTreg cells (29). Due to the fact that Treg cells from peripheral blood of patients with human renal cell carcinoma (RCC) expressed Helios, it was concluded that these Treg cells, which are expanded in RCC patients, were more likely to be nTreg cells (53). However, as discussed above, the use of Helios expression alone to establish the origin of Treg cells does not appear to be a sufficiently stringent criterion.

Other groups concluded that tumor Treg cells were iTreg cells. In one key study, thymectomized and anti-CD25-treated tumor-bearing mice developed a population of T cells that were phenotypically and functionally indistinguishable from Treg cells; because of the experimental design, these Treg cells were considered iTreg cells (46). However, a problem with the design is that several groups showed that the anti-CD25 antibody PC61 does not completely deplete Treg cells (54, 55); it functionally inactivates them temporarily, but a substantial percentage of Treg cells remain alive. The experiment did not therefore rigorously exclude the possibility that nTreg cells survived and expanded.

Another important study shows that mouse prostate tumor cells (and conditioned media) could induce Foxp3 expression in $CD4^+CD25^-$ cells in a TGF-β-dependent manner (47). In vivo, neutralization of TGF-β reduced the number of $CD4^+CD25^+$ cells in the tumor and decreased the tumor burden. Although this result was taken as an indication that tumors induced iTreg cell differentiation, TGF-β was shown not only to induce iTreg cells (18, 19), but also to expand preexisting Treg cells (nTreg cells) (56, 57).

An elegant series of experiments carried out with tumors expressing influenza hemagglutinin (HA) and with HA-specific TCR-transgenic T cells allelically marked allowed the conclusion that nTreg and iTreg cells contributed independently to the pool of Treg cells that induced tolerance in tumor-bearing mice (50). The presence of nTreg cells did not impact the generation of iTreg cells in a major way; iTreg cell generation was determined by the tumor microenvironment.

In conclusion, the controversy regarding the relative roles of nTreg and iTreg cells in tumor immunity and tolerance persists, although significant insight has been gained regarding differences in Treg cell populations that can be ascribed to the different tumor environments. The controversy will only be solved when adequate surface markers for nTreg and iTreg cells become available, as discussed elsewhere in this review.

HA: influenza hemagglutinin

HOW ARE iTREG CELLS INDUCED IN VIVO?

Although in vitro differentiation of Tconv cells into Foxp3-expressing cells requires a simple protocol (18), questions remain regarding how iTreg cells are induced and maintained in the periphery. This section reviews the in vivo scenarios in which iTreg cell generation has been observed and the impact of iTreg cell generation on immune responses (**Table 2**).

Mucosal Tolerance and Generation of iTreg Cells

More than 100 years ago, Besredka, and later Wells and Osborne, observed that guinea pigs fed dietary antigens could become tolerant upon subsequent challenge with the given antigen (58). These observations were extended by Chase (59), who characterized the phenomenon of oral tolerance to a fed antigen as a process of immunological unresponsiveness upon challenge with that antigen (see sidebar entitled

Table 2 In vivo scenarios in which iTreg cell generation has been observed

Scenario	Outcome
Suboptimal antigen stimulation (e.g., constant ingestion of proteins)	Oral tolerance
Autoimmune disease (e.g., EAE), allergic disease (e.g., asthma)	Downmodulates exacerbated effector responses, enabling the establishment of a chronic stage; prevents death by acute inflammation
Infection by parasites (e.g., *Leishmania*)	Decreases clearance of pathogen; leads to chronic infection
Cancer	Impairs tumor immunity and prevents tumor rejection
Allotransplant	Prevents allograft rejection

Oral Tolerance). A key experiment carried out in the 1970s revealed that mice that received T cell–enriched, but not T cell–depleted, splenocytes from OVA-fed mice were rendered tolerant to OVA but not to an unrelated antigen (58). The importance of the mucosal route to generate tolerance was emphasized when antigen administration by intravenous or intraperitoneal route was not as effective as the oral route (60, 61).

Since then, much attention has been placed on the mechanisms underlying oral tolerance induction. Among these are deletion and/or anergy of antigen-specific T cells (62–64), immune deviation (65, 66), and suppression by Treg cells (67, 68). Feeding OVA to OVA-specific TCR (DO11.10)-transgenic mice led to expansion of $CD4^+CD25^+$ T cells. These expanded Treg cells, when transferred to wild-type recipients, were able to suppress immune response in vivo (67). Oral tolerance experiments were also carried out after transfer of D011.10-transgenic T cells into wild-type mice (69). Thus, oral tolerance leads to expansion and/or generation of Treg cells. However, questions remained as to whether Treg cells were induced de novo from conventional $CD4^+Foxp3^-$ T cells or expanded from the existing pool of $Foxp3^+$ nTreg cells, and also whether iTreg cell induction was possible in the absence of nTreg cells. To address these issues, it is necessary to have mice that lack nTreg cells while harboring Tconv cells. To obtain such mice, our laboratory used the now customary method of crossing TCR-transgenic mice with RAG-deficient mice (70). We could clearly demonstrate that oral administration of OVA to OVA-specific TCR-transgenic RAG-deficient mice led to de novo induction of OVA-specific $Foxp3^+$ iTreg cells from $CD4^+Foxp3^-$ Tconv cells. These iTreg cells could efficiently suppress the main features of asthma (11). Subsequently, we crossed the D011.10 RAG-deficient mice [also carrying B cell receptor (BCR) knockin heavy and light chains specific for HA] with Foxp3-deficient scurfy mice, and demonstrated that oral tolerance could not be induced in the absence of iTreg cells (14).

In the experiments described above, one could selectively preclude the development of nTreg cells, allowing (or not) iTreg cell generation. Recently, Rudensky's laboratory generated mice deficient for three conserved noncoding DNA sequence (CNS) elements at the *Foxp3* locus (71). Although CNS3-deficient mice had a drastic reduction in thymic nTreg cells, the peripheral Treg cell compartment

DO11.10: OVA-specific TCR

BCR: B cell receptor

ORAL TOLERANCE

Oral tolerance was described in the beginning of the twentieth century as a static process of immunological nonresponsiveness to dietary antigens. It is now widely accepted as an active process of regulation of immune responses to fed antigens upon challenge with the same antigen by parenteral route.

appeared to be intact. However, CNS1-deficient mice had normal numbers of thymic Treg cells but defective generation of iTreg cells. Contrary to what was observed in CNS3 knockout mice, overt disseminated lymphoproliferative disease was not seen in CNS1 knockout mice (71), suggesting that iTreg cells are not essential to maintain immune homeostasis. However, one can anticipate that CNS1 knockout mice would display impaired regulation of responses to foreign antigens, such as allergens and infections.

Role of Intestinal Microbiota in Tolerance Induction

The microbiota of the gut is very diverse, containing, at a minimum, over 100 different species of bacteria that make up to 10^{14} total bacteria in the colon (72, 73). The gut-associated lymphoid tissue (GALT) is the largest immune organ present in the body; it harbors many different subsets of lymphocytes, including Treg cells (74). Therefore, different bacteria species may differently stimulate the numerous lymphocytes that reside within the gut (75–77).

As mentioned above, antigen administration by mucosal route is more efficient in inducing tolerance than are systemic routes such as intravenous and intraperitoneal. The question that follows is why the mucosae seem to be ideal places for the establishment of immune tolerance. Several reports suggest that the commensal gut microbiota plays an important role in shaping the immune system (73), including the generation of an appropriate environment for the development of oral tolerance to food allergens (78). Studies with mice devoid of the commensal microbiota [e.g., germ-free (GF) mice] have provided significant insight into the importance of the intestinal microbiota. GF mice were less susceptible to the development of oral tolerance to OVA than were mice reared in specific pathogen–free (SPF) facilities (79–82). However, in another study, oral tolerance could be induced in GF mice to the same extent as in SPF mice (83). In vitro data suggest that Treg cells from GF mice are less suppressive than Treg cells from SPF mice (84), but this was not confirmed in another study (85). The discrepancies between the different reports could be related to the "absolute" GF nature of animal colonies believed to be GF and, for the SPF mice used to compare with GF mice, the composition of the intestinal microbiota in different SPF facilities (76). An additional consideration of experiments carried out with GF mice is that the mice may have altered immune responses due to altered lymphoid structures and lymphocyte composition, such as a reduced number of Peyer's patches (86) and intraepithelial lymphocytes (87, 88) as well as decreased frequency of $Foxp3^+$ Treg cells in the colon (89) and of Th17 cells in the small intestine (76).

One way to address the effect of the microbiota on Treg cell biology is to colonize GF mice with a single species or specific groups of commensal microorganisms. The first of such studies has recently been reported. The bacteria *Bacteroides fragilis* may induce iTreg cell generation in the intestines of otherwise GF mice (90). *B. fragilis*–induced Treg cells produced IL-10 and were able to protect against colitis. In a follow-up study, the same lab proposed that polysaccharide A, present in the cell wall of *B. fragilis*, directly triggers TLR2 signaling on Treg cells, allowing efficient colonization by this bacteria and suppression of Th17 cell responses in the gut (91). A caveat of the aforementioned studies is that *B. fragilis* is not part of the microbiota of SPF mice.

More recently, an elegant report showed that the gut commensal bacteria *Clostridium* specifically promote Treg cell accumulation in the colon of mice in a TGF-β-dependent fashion (89). These Treg cells stimulated by *Clostridium* seemed to have arisen from Tconv cells, given that a significant fraction of Treg cells found in the colon of otherwise GF mice colonized with *Clostridium* do not express the transcription factor Helios, discussed above in this review. Atarashi's data (89) were further corroborated by Lathrop's recent report (92) showing that at least some of the TCR from colonic Treg cells are specific for colonic

Gut microbiota: commensal microorganisms that inhabit the intestinal tract of all mammals without causing harm to the host; some species of commensal bacteria present in the gut may directly promote generation of induced regulatory T cells

MBP: myelin basic protein

bacteria and also express low levels of Helios. Although these experiments strongly suggested that bacterium-specific Treg cells were iTreg cells, their thymic origin could not be excluded. Forced expression of these colonic bacterium–specific TCR in immature thymocytes did not result in significant thymic nTreg cell development, demonstrating that colonic Treg cells arise in the periphery from Tconv cells. It would be interesting to determine whether Foxp3$^-$ Tconv cells, when transferred to GF RAG-deficient mice, could convert into Foxp3$^+$ iTreg cells upon colonization with *Clostridium*. The molecular basis of *Clostridium*-iTreg cell induction is not known. Interestingly, a deficiency in MyD88, Rip2, or Card9, pathways known to be activated by bacterial products, had no impact on Treg cell expansion in the colon upon *Clostridium* colonization.

Colonization of mice with a specific, yet complex, microbiota called altered Schaedler flora (ASF) can induce de novo generation of iTreg cells and promote expansion of nTreg cells in the colon but not in peripheral lymphoid organs (93). ASF-induced Treg cells, like most Treg cells found in the colon of ASF-colonized mice, lacked Helios expression (a proposed marker of nTreg cells, discussed above). In addition, cell transfer experiments showed that a proportion of CD4$^+$Foxp3$^-$ cells became Foxp3$^+$ Treg cells in the colon of mice colonized with ASF (93). Treg cell induction/expansion in GF mice colonized with ASF was partially dependent on TLR signaling as MyD88/TICAM-1 (also known as Trif) doubly deficient mice had decreased frequency of Treg cells in the colon. However, some Treg cells could still develop in the absence of these pathways, suggesting that TLR-independent signaling is also involved in colonic Treg cell generation and/or expansion.

iTreg Cells and Inflammation

The generation of iTreg cells has been observed in a variety of inflammatory disease models, such as asthma, arthritis, colitis, diabetes, and EAE, and also in allotransplantation. Inflammation induced by immunization with antigen emulsified in adjuvants or by transfer of pathogenic Teff cells may also promote, to some extent, the conversion of naive Tconv into iTreg cells. Infections by some pathogens can also promote the generation of iTreg cells. This section reviews the main inflammatory situations in which iTreg cell generation has been observed (or not) and the consequences of developing such iTreg cells for disease outcome (see **Figure 1**).

Our laboratory has developed two mouse models of inflammation; in these models, disease occurs because of the absence of nTreg cells, allowing us to study the impact of iTreg cell generation on the course of spontaneous EAE and chronic asthma. The basis of both experimental systems is a strict allelic exclusion of TCR chains encoded by endogenous TCR loci (through cross with RAG-deficient or TCR$\alpha\beta$-deficient mice).

We immunized RAG-deficient OVA-specific TCR-transgenic mice and HA-specific BCR knockin mice with the cross-linked cognate antigens adsorbed in Alum adjuvant. A single immunization led to a hyper IgE response (94). Subsequent challenges via nasal route resulted in lung inflammation typical of asthma, with the concomitant induction of a significant number of iTreg cells (14). These iTreg cells, generated in the activated environment, were not present at an early enough stage to prevent asthma, but they had a beneficial role in the reduction of the degree of chronic inflammation, the level of IgE, and the appearance of tertiary lymphoid structures in the lungs (14). Most likely, inflammatory cytokines secreted by Teff cells and activated APCs prevented full Treg cell activity. For example, IL-6 prevents Treg cell differentiation/expansion in inflammatory settings (95), and blockade of IL-6 induces Treg cell expansion in an asthma model (96).

iTreg cells are also generated during inflammation in the context of autoimmune diseases. Spontaneous EAE occurs because mice lack nTreg cells but harbor myelin basic protein (MBP)–specific Tconv cells. The disease

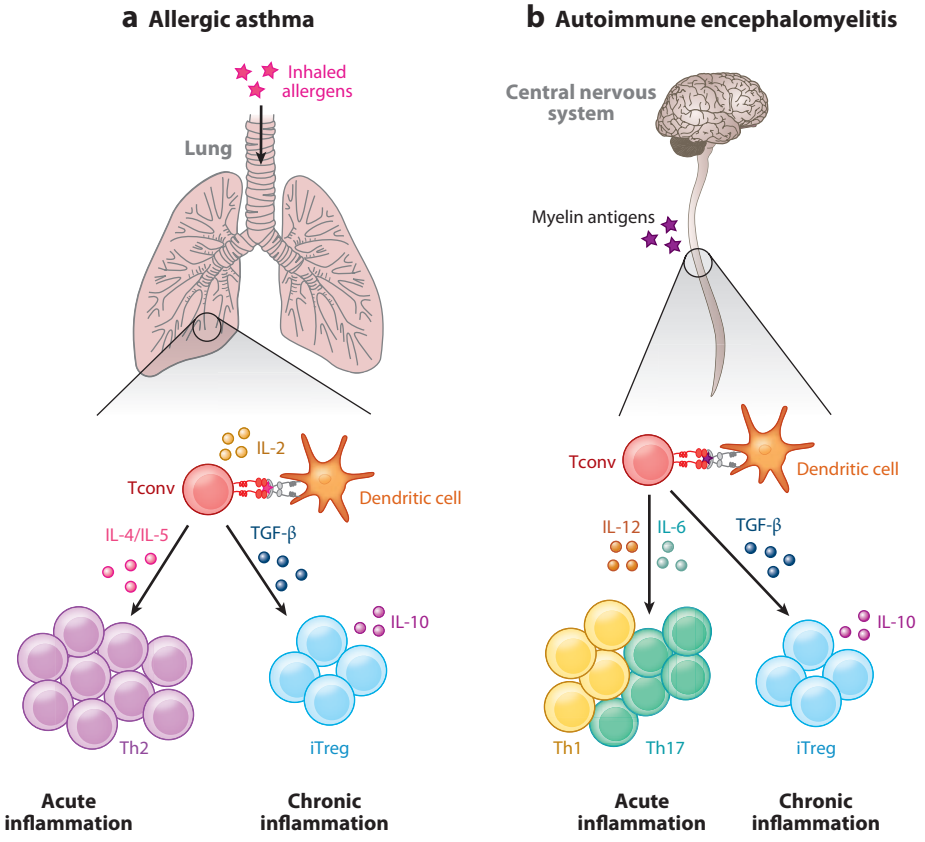

Figure 1

iTreg cells generated during inflammation help limit disease progression. During the course of inflammation caused by (*a*) allergic (e.g., asthma) or (*b*) autoimmune (e.g., EAE) disease, antigen-specific iTreg cells are generated later on and help control damage caused by Th1, Th17, and Th2 cells.

displays three stages: subclinical, with a rapidly developing inflammation in the central nervous system but no neurologic signs; acute, with a clinically progressing disease; and chronic, a lengthy phase in which mice remain relatively stable (97). Soon after disease onset, MBP-specific Foxp3$^+$ Treg cells can be readily found in the central nervous system, at much higher frequencies than in secondary lymphoid organs. When the generation of iTreg cells was prevented through the cross with Foxp3-deficient mice, disease onset was not accelerated significantly, but once mice displayed first signs of disease, they rapidly progressed to death, whereas Foxp3-sufficient mice, though also sick, mostly survived and established a chronic stage. Similarly, using a RAG$^+$ TCR-transgenic model for spontaneous arthritis (K/BxN) crossed to Foxp3-deficient scurfy mice, a faster onset of disease was observed, and disease affected joints that were normally not affected (98). Owing to the use of RAG$^+$ TCR-transgenic K/BxN mice, which have nTreg cells, the observed effects of Foxp3$^+$ cells could not be ascribed to either iTreg or nTreg cells.

Interestingly, a study using an EAE model induced by myelin oligodendrocyte glycoprotein (MOG) immunization with complete Freund's adjuvant (CFA) and pertussis toxin (PT) failed to observe conversion of naive T cells into iTreg cells (99). In this report, the mice already had a normal complement of

nTreg and iTreg cells prior to immunization. Moreover, myelin antigens in the context of CFA emulsions and PT generate Treg cells in the thymus (100), a result likely to incite further mechanistic studies. Unlike the CFA-created environment, the inflamed central nervous system milieu of spontaneous EAE favors the generation of iTreg cells, as it probably occurs in other natural inflammatory conditions.

Generation of iTreg cells has also been observed in other inflammatory contexts. In a model of lymphoproliferative disease in mice carrying a Foxp3 deficiency, the conversion rate of naive T cells into Foxp3$^+$ iTreg cells was estimated to be 10–15% (23). In the colitis model, some labs observed iTreg cell generation whereas others did not. In one report, a significant fraction of naive T cells transferred to RAG-deficient mice converted into Foxp3$^+$ iTreg cells [about 10% of total CD4$^+$ cells recovered from mesenteric lymph nodes (mLN)] (16). Similar to the asthma experiments described above, even though these colitis-induced iTreg cells were not sufficient to prevent disease, the disease outcome was improved compared with the situation in which colitis was induced by naive T cells unable to convert into iTreg cells. Once again, in this context, Foxp3$^+$ iTreg cells were generated concomitantly with Teff cells and were probably outcompeted by the latter. In contrast, an early report describes lack of conversion of Foxp3$^+$ iTreg cells from the naive T cell pool transferred into irradiated RAG-deficient hosts (101). This is surprising because homeostatic proliferation has now become one of the established methods to generate Foxp3$^+$ iTreg cells in vivo. Differences in the microbiota, the irradiation of the hosts, or even the sensitivity of the Foxp3 reporter strains could explain the discrepancies.

iTreg Cells and Infections

A variety of chronic infections by parasites, bacteria, and viruses have been associated with an increased frequency of Treg cells at inflammatory sites and/or at draining lymph nodes. The generation of iTreg cells during infection may be facilitated by high amounts of TGF-β secreted by several cell types at the infected site (102); some pathogens can also secrete a TGF-β homolog (103) that may directly contribute to iTreg cell generation. Treg cells generated during infections, similar to the autoimmune and allergy contexts described above, may enable the establishment of a chronic phase, limiting immune-mediated tissue damage but also impairing complete clearance of the pathogen.

Mice infected with *Leishmania major* show an increased accumulation of Treg cells in the dermis. Cell transfer experiments using CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ cells firmly established that although the CD25$^-$ fraction mediates sterile immunity to *L. major*, the CD25$^+$ fraction leads to a chronic infection, in an IL-10-dependent manner (104). However, sterile immunity is accompanied by impaired immunity to reinfection, indicating that the balance between Teff and Treg cells is tightly regulated. The situation is similar in other parasitic infections. For instance, mice infected with the nematodes *Heligmosomoides polygyrus* and *Brugia malayi* display increased numbers of Foxp3$^+$ Treg cells, possibly iTreg cells, that can efficiently suppress proliferation of Teff cells (13, 105–107). Both parasites are able to establish long-lasting chronic infection in immunocompetent hosts. In bacterial infections, iTreg cells may also play an important role. Pathogen-specific Foxp3$^+$ Treg cells have been identified in infections by *Listeria monocytogenes* (108) and *Helicobacter pylori* (109). At least in the case of *H. pylori* infection, the presence of Treg cells correlated with a reduced response to *H. pylori* antigens in vitro, a phenomenon that could contribute to the lack *H. pylori* clearance and establishment of chronic infections.

Infections by several viruses can also induce Treg cell expansion and/or de novo generation that could be detrimental or beneficial (102). Mucosal infection by herpes simplex virus 2 (HSV-2) induces accumulation of Treg cells in draining lymph nodes and genitalia. In this model, in vivo ablation of Treg cells resulted in accelerated disease and decreased virus clearance (110). These outcomes were attributed

to the impaired migration of plasmacytoid dendritic cells (DCs), natural killer (NK) cells, and Teff cells to the site of infection, suggesting that, perhaps paradoxically, Treg cells contribute to pathogen elimination by promoting efficient recruitment of other immune cells. Similarly, acute infection by respiratory syncytial virus (RSV) induced accumulation of Treg cells in the draining mediastinal lymph nodes and lungs, and depletion of Treg cells before infection resulted not only in exacerbated disease severity but also in delayed virus clearance (111). Disease severity could be attributed to an increased infiltration of virus-specific $CD8^+$ Teff cells that produce high amounts of IFN-γ and TNF-α, whereas delayed virus clearance could be the result of a slow migration of Teff cells. The paradigm of HSV-2 and RSV is less clear in hepatitis B (HBV) or C (HCV) infections. Although increased Treg cell numbers have been observed in human carriers of HBV or HCV (112–114), their role in immunopathology is less clear. Reports suggest that Treg cells could either contribute to liver damage or reduce it. There is, however, agreement that during HBV or HCV infection, Treg cells inhibit Teff cell response, and therefore impair virus clearance (115, 116).

In some infection models, iTreg cell generation has not been observed. Microenvironments in some acute infections may not favor the conversion of iTreg cells. This could be due to secretion of inflammatory mediators such as the cytokines IL-6 and IL-12, which inhibit Treg cell conversion and expansion (95, 100, 117). For instance, after acute infection by OVA-expressing *L. monocytogenes*, OVA-specific naive T cells expanded but did not convert into iTreg cells (118). In the context of *L. major* infection, $CD4^+CD25^-$ T cells also did not convert into $Foxp3^+$ iTreg cells, but instead $CD4^+CD25^+$ nTreg cells expanded and accumulated in the draining lymph node (119). Another study shows that wild-type mice orally infected with a high inoculum of *Toxoplasma gondii* displayed a significant decrease in Treg cells in peripheral lymphoid organs and in the gut, and die within 10 days post infection (120). Furthermore, even in conditions that promoted iTreg cell generation (e.g., oral tolerance by OVA feeding), iTreg cells were not generated; instead, OVA-specific T cells transferred to infected mice orally treated with OVA converted into Th1 cells due to the highly inflamed environment provoked by the acute infection. Collectively, these data indicate that iTreg cell generation during infection-induced inflammation is a highly regulated process, and we are only now beginning to understand the interplay between the many opposing forces.

DC: dendritic cell

ROLE OF ANTIGEN-PRESENTING CELLS IN iTREG CELL GENERATION

Generation of iTreg cells depends on productive antigen presentation by APCs in a microenvironment rich in TGF-β and IL-2. Attention has been placed on the role of DCs in the generation of iTreg cells (see **Figure 2**). DCs comprise a heterogeneous group of cells with heterogeneous abilities of antigen processing and presentation (121). Moreover, DCs that are present in different tissues are conditioned by the local milieu and may acquire singular properties that could enhance or impair their capacity to influence iTreg cell conversion. The complex relationship between DCs and Treg cells has recently been demonstrated: Depletion of DCs led to decreased $Foxp3^+$ Treg cells and increased Th1 and Th17 cell responses (122). Conditional deletion of MHC class II molecules on DCs also led to decreased frequency of Treg cells, suggesting that antigen presentation by DCs was essential for Treg cell homeostasis.

Even though most APC types at steady state are capable of inducing iTreg cells, DCs excel in this process; induction of iTreg cells was more efficiently accomplished by splenic DCs than by DC-depleted APCs (123). It has been suggested that antigen presentation by immature DCs leads to iTreg cell conversion, whereas mature DCs promote Th1 or Th17 cell conversion (124, 125); activation-induced changes

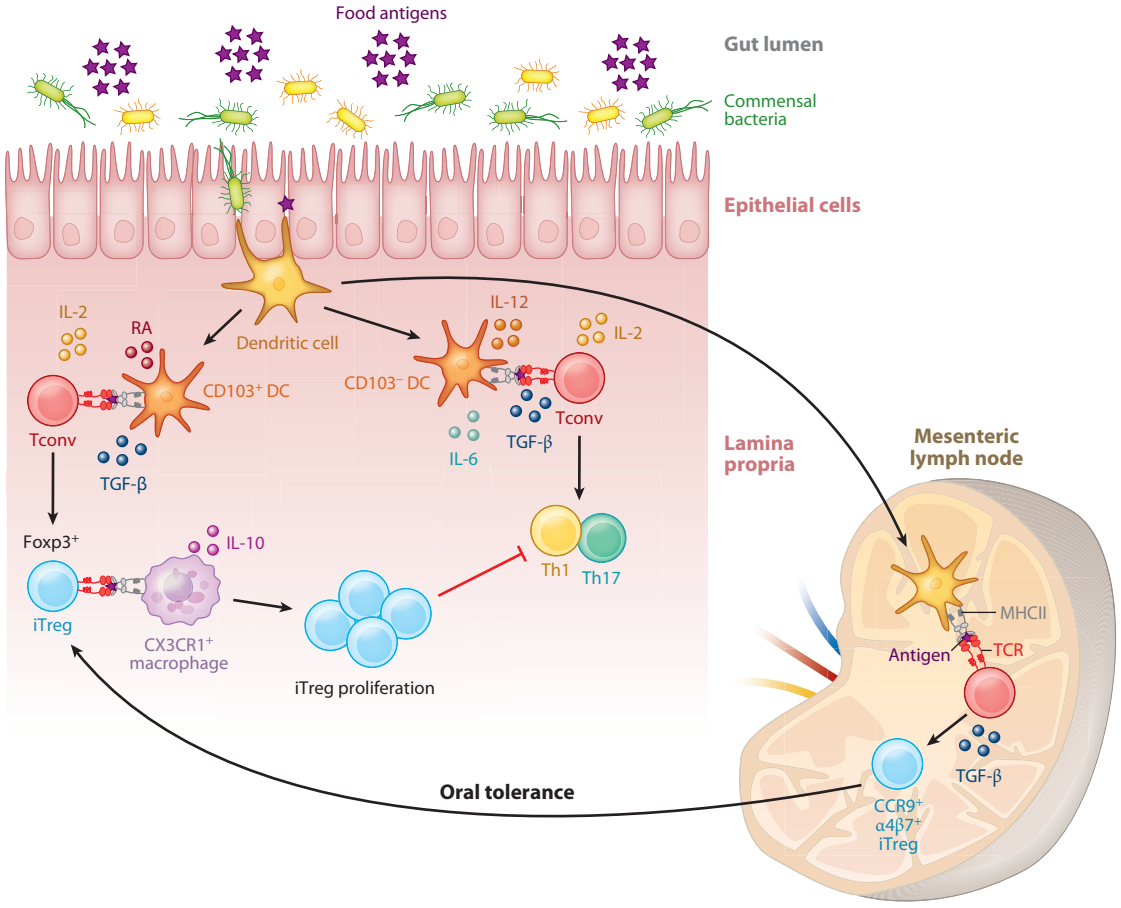

Figure 2

Establishment of oral tolerance by induced Treg cells and special dendritic cells (DCs). The gut mucosa is constantly exposed to an array of antigens, including antigens from the diet and from the intestinal microbiota. DCs present in the lamina propria sample antigens from the lumen and present them to conventional T cells (Tconv cells) present in the lamina propria and mesenteric lymph nodes. A special subset of DCs expressing CD103 produce retinoic acid (RA) and, together with TGF-β, promote the conversion of Tconv cells into induced Treg (iTreg) cells, whereas $CD103^-$ DCs promote conversion of Tconv cells into effector T cells such as Th1 and Th17 cells. iTreg cell proliferation is further promoted by a subset of macrophages present in the lamina propria that express the chemokine receptor CX3CR1 and produce IL-10. iTreg cells induced in mesenteric lymph nodes express gut-homing receptors such as the chemokine receptor CCR9 and the integrin α4β7, which allow their migration to the lamina propria. iTreg cells promote tolerance by keeping in check exacerbated Th1 and Th17 cell responses.

on DCs may, therefore, directly affect their ability to promote iTreg cell conversion. However, another study reports that both immature and mature DCs are equivalent in their capacity to induce iTreg cells in the presence of TGF-β (123). Besides maturation stimuli triggered by different TLR ligands and cytokines, intrinsic features of DCs seem to influence iTreg cell conversion. For instance, splenic $CD8^+$ DCs but not $CD8^-$ DCs induced iTreg cell differentiation in the absence of exogenous TGF-β (126), suggesting that the ability of $CD8^+$ DCs to promote iTreg cell generation is related to endogenous TGF-β production. $CD8^+$ DCs are the major DC subset that expresses the C-type lectin receptors DEC-205 and DNGR-1 (also known as Clec9). Interestingly, low-dose antigen targeting through DEC-205 or

DNGR-1 led to iTreg cell conversion in the absence of proliferation (12, 127). Therefore, the combination of subimmunogenic stimulation and endocytic receptor–mediated antigen processing seems to favor iTreg cell generation.

The environment at the gut mucosa is particularly permissive for the generation of iTreg cells (128). As discussed above, the GALT appears to be the ideal place for the establishment of tolerance. Cell transfer experiments using congenic markers and RAG-deficient hosts showed that a fraction of polyclonal $CD4^+Foxp3^-$ T cells converted into $Foxp3^+$ iTreg cells and accumulated in the small intestine lamina propria (SI LP) (129). OVA treatment by the oral route also induced conversion of OVA TCR-specific naive T cells into iTreg cells, and the SI LP harbored the highest frequency of these converted iTreg cells (129). Thus, DCs present in the gut, conditioned by the microenvironment, may have special abilities to generate iTreg cells (see **Figure 2**). Indeed, $CD103^+$ DCs extracted from the SI LP and from mLN excel in converting $CD4^+$ naive T cells into $Foxp3^+$ iTreg cells in the absence of exogenous TGF-β (129, 130). The enhanced ability of gut and mLN $CD103^+$ DCs in promoting iTreg cell generation is related to the production of the vitamin A metabolite retinoic acid (RA) by this DC subset. In contrast, $CD103^-$ DCs produce proinflammatory cytokines (130). Besides $CD103^+$ DCs, SI LP macrophages can also convert naive T cells into $Foxp3^+$ iTreg cells, whereas SI LP $CD11b^+CD11c^+$ conventional DCs promote Th17 cell differentiation; conversion is also dependent on RA and TGF-β (131).

RA was shown to be a key player in tilting the balance toward iTreg cell differentiation in vitro (128). Although the presence of exogenous RA potentiated TGF-β-induced iTreg cell differentiation in vitro, antagonizing RA receptor signaling led to Th17 cell differentiation (128). Besides favoring iTreg cell conversion, RA produced by DCs also induced the expression of gut-homing receptors such as the integrin α4β7 and the chemokine receptor CCR9 on lymphocytes (132, 133). However, in vivo studies on the role of RA in iTreg cell differentiation point in a different direction. New evidence suggests that, in vivo, RA boosts effector responses by activating a proinflammatory cascade in DCs (134–136). Mucosal responses to infection as well as vaccination were severely impaired in mice made deficient in RA either by being fed a vitamin A–deficient diet or by genetic ablation of RA receptor (RAR) α (134, 138). In a skin transplantation model, blockade of RARα impaired not only T cell migration into the allograft but also effector function (measured by secretion of IFN-γ and IL-17) and allograft rejection (135). Lastly, RA, together with IL-15, induced DC activation in a c-Jun N-terminal kinase (JNK)–dependent manner. These activated DCs secreted the proinflammatory cytokines IL-12 and IL-23 and were no longer able to convert naive T cells into $Foxp3^+$ iTreg cells, but instead promoted Th1 cell differentiation (136). Furthermore, IL-23 was proposed to act on T cells to inhibit Foxp3 expression (137).

One can hypothesize that the dual effect of RAR signaling on immune responses is a consequence of environmental pressures to which the gut is exposed daily. Under noninflammatory conditions, RAR signaling augments iTreg cell differentiation, which helps to maintain in check what would otherwise be exacerbated immune responses against commensal bacteria and food antigens. In contrast, in conditions created by pathogens that cause DC activation, RAR signaling, possibly together with other inflammatory stimuli, triggers effector responses.

The GALT is essential for the establishment of oral tolerance, and DCs derived from this location can efficiently induce iTreg cells. Within 48 h of antigen administration via the oral route, dividing T cells can be found in mLN and can transfer tolerance to a third party (68). Moreover, oral tolerance is abrogated in mice lacking mLN but is intact in mice lacking Peyer's patches (139), showing the crucial role of mLN on oral tolerance. However, it was not clear whether iTreg cells were generated in mLN, where migratory $CD103^+$ DCs from the gut drain, or converted in situ in the gut

lamina propria. A recent report showed that, indeed, iTreg cells are induced in mLN, but an expansion phase in the SI LP is indispensable for establishment of mucosal tolerance (140). This expansion was promoted by CX3CR1$^+$ macrophages present in the SI LP, unveiling a role not only for DCs but also for macrophages in the process of oral tolerance induction.

CELL METABOLISM AND iTREG CELL GENERATION

A connection has been proposed between cell metabolism and Treg cell differentiation (141). The process of differentiation (and activation) of T cells is a very metabolically active one, comparable only to cancer cells. To generate enough energy during activation/differentiation, CD4$^+$ T cells use the glycolytic pathway. In this context, the mammalian target of rapamycin (mTOR) plays a fundamental role. Indeed, rapamycin is used as an immunosuppressant drug to control transplant rejection. However, the mechanism of rapamycin action was not completely understood. Initially, it was suggested that rapamycin inhibited proliferation of Teff cells and/or promoted their death. Another possibility was that it promoted iTreg cell induction/expansion or a combination of effects on Teff and Treg cells. A skin allograft model showed that rapamycin induced the conversion of CD4$^+$Foxp3$^-$ T cells into allospecific Foxp3$^+$ iTreg cells that were able to prevent skin graft rejection (142); there was also selective death of Teff cells. Whether Teff cell death was directly induced by rapamycin or was influenced by iTreg cells was not assessed. The effects of rapamycin on T cells were also evaluated in conditions other than transplants. Cell transfer experiments of OVA-specific T cells into wild-type mice demonstrated that iTreg cell induction is augmented when recipients are treated with rapamycin, regardless of the dose of antigen the animals receive (143). Taken together, these data suggest that inhibition of mTOR signaling promotes iTreg cell conversion and expansion. To directly evaluate the role of mTOR signaling in T cell differentiation, Powell's lab (144) generated conditional mTOR knockout mice with CD4-Cre. In vitro, mTOR-deficient T cells failed to differentiate into Th1, Th2, and Th17 cells. In vivo, mice carrying mTOR-deficient T cells failed to respond to vaccinia virus infection, an infection that triggers a strong Th1 cell–type response. In addition, the authors observed that T cell stimulation in the absence of mTOR resulted in the accumulation of Foxp3$^+$ Treg cells (144). Conversion occurred in the absence of exogenous TGF-β, but the process was nevertheless dependent on TGF-β, as its blockade resulted in decreased frequency of Foxp3$^+$ Treg cells. Corroborating these findings, rapamycin was shown to increase the sensitivity of T cells to TGF-β, and thus could promote iTreg generation even in the presence of low amounts of TGF-β (145). In addition, inhibition of the phosphatidylinositol 3-kinase (PI3K)–protein kinase B (Akt) pathway also enhanced Treg differentiation, but in a TGF-β-independent manner. In this in vitro system, rapamycin could induce epigenetic modifications in the Foxp3 promoter that resulted in enhanced Foxp3 activity (146).

Another metabolic sensor, hypoxia-inducible factor-1 (HIF-1), has also been implicated in the balance between Th17 and Treg cell differentiation programs. In vitro, hypoxic conditions favored Th17 cell differentiation and inhibited Foxp3 iTreg cell differentiation (147, 148). In vivo, HIF-1-deficient mice harbored a significantly higher frequency of Treg cells than did wild-type mice and were resistant to EAE induction. Moreover, rapamycin inhibited the expression of HIF-1α in Th17 cells, indicating that mTOR pathway is necessary for HIF-1α activity (148). Collectively, these findings suggest that T cell differentiation programs respond actively to metabolic cues. A better understanding of these signals could lead to the design of new therapeutic approaches.

iTREG CELLS IN CLINICAL SETTINGS

Clinical trials using iTreg cells for therapeutic purposes are in the initial but promising steps

(149–151). This section reviews the recent advances of Treg cell therapy, focusing on new perspectives for the use of iTreg cells to treat diseases.

The major approach utilized thus far has been to expand ex vivo populations of preexisting Treg cells (of which most are nTreg cells) from human peripheral blood under good manufacturing practices and to give them back to patients (152–154). The results are promising: Xenogeneic models of graft-versus-host disease (GVHD) showed that ex vivo–expanded nTreg cells could prevent GVHD, although high ratios of Treg to Teff cells were necessary. Recently, expansion of Treg cells from umbilical cord blood was also described, and these cells were safe in a phase I trial of 23 patients (40).

Although Treg cell therapy holds great promise, it also raises some concerns. One major concern is that during the expansion of a total Treg cell population, some of the T cells convert back into Teff cells and could exert pathogenic effector functions. Because it has been reported that a fraction of ex vivo–expanded Treg cells lose Foxp3 expression, which is related to a lower suppressive function, it will be important to determine what factors influence Treg cell instability in Treg cell cultures. Perhaps the improvement of the starting Treg cell populations to be expanded will prove to be crucial. In murine systems, nTreg cells are less plastic than iTreg cells. Therefore, for human therapy, it may be beneficial to start Treg cell cultures with pure nTreg cells. Another caveat of the therapeutic use of Treg cells is the contamination by Teff cells that express the same surface markers as nTreg cells (155). In addition, a fraction of human Teff cells transiently expresses Foxp3 upon activation, but these cells are not suppressive.

Protocols for ex vivo expansion of human nTreg cells are laborious and expensive, as multiple rounds of expansion are necessary from the 1–3% present in peripheral blood. Therefore, some groups have focused on generating iTreg cells from the pool of circulating Tconv cells, which are much more abundant. To optimize the conditions for iTreg cell generation, several groups started to include rapamycin in the generation/expansion protocols because this drug has been shown to specifically prevent Teff cell expansion while allowing Treg cell proliferation (144, 156, 157). Generation of iTreg cells from a pool of naive T cells from human peripheral blood has proven to be very efficient; the total numbers of cells obtained was 50 times higher than the standard protocols. Moreover, iTreg cell populations generated and expanded in the presence of TGF-β, and rapamycin contained fewer cells that produce the prototypical effector cytokines IFN-γ and IL-17. Suppression of xenogeneic GVHD by iTreg cells generated in the presence of rapamycin was comparable to nTreg cell suppression (158). These results provide a new ground for a phase I trial for GVHD treatment using in vitro–generated iTreg cells.

In mouse models, nTreg and iTreg cells are phenotypically different and display complementary suppressive activity (14–16, 23). As in the mouse, the pool of human circulating Treg cells likely contains both nTreg and iTreg cells, although given the clean environment of most animal facilities, the dominance of nTreg over iTreg cells is unlikely to be so evident in the blood of older humans. In the near future, it will be important to distinguish these two populations to achieve customized therapy that specifically targets nTreg or iTreg cells.

Lastly, at the early stages of development in this field, patients undergoing Treg cell therapy must be carefully monitored for negative impacts on the outcome of some infectious and malignant diseases.

PERSPECTIVES

One of the major issues still not completely solved in Treg cell biology is the lack of markers that precisely distinguish nTreg from iTreg cells. It appears that Foxp3$^+$ iTreg cells are quite different if they are generated under subimmunogenic conditions (oral tolerance, systemic antigen with no adjuvants, osmotic pumps, DEC-205 targeting) or under inflammatory conditions (EAE, chronic asthma,

arthritis, etc.), further complicating the identification of iTreg cells as an entity. Gene expression analyses have failed thus far to identify a single molecule that is expressed exclusively by iTreg cells and not by nTreg or Tconv cells.

Thus, the heterogeneity of Treg cell populations may not only reflect the origin (thymus versus periphery) of Treg cells, but also be the consequence of the milieu. For instance, different Treg cell subsets express different homing receptors depending on the environment they are exposed to, resulting in different properties (159). Within iTreg cells, their origin in inflammatory or noninflammatory environments may be a defining property, as indicated above.

Given the natural self-reactivity of nTreg cells, the issue of whether Treg cells can become effector cells (and vice versa) has received considerable attention and has generated controversy. Recent reviews addressed this subject (117, 160, 161). In the context of nTreg and iTreg cells, it is generally accepted that nTreg cells are more stable (less plastic) than iTreg cells. DNA methylation studies at the *Foxp3* locus have shown that nTreg cells are more demethylated (162, 163). The partial methylation of the *Foxp3* locus in iTreg cells may explain why it would be more likely to shut down Foxp3 expression in iTreg cells than in nTreg cells. Thus, the discrepancies regarding Treg cell plasticity could be partially explained by the relative proportion of nTreg and iTreg cells in each of the different studies.

Finally, as we fill some of the gaps in the Treg cell biology, new questions emerge: What is the relationship between the specificity of iTreg cells and the range of responses they can suppress? Do nTreg and iTreg cells recognize different epitopes? What is the impact of the TCR repertoire of iTreg and nTreg cells in the immune responses? Do iTreg and nTreg cells use the same or different mechanisms of suppression? To what extent do the mechanisms of suppression depend on the microenvironment? Addressing these questions in the near future will contribute to a more complete understanding of this T cell subset and enhance the potential for clinical applications.

SUMMARY POINTS

1. iTreg cells generated in the peripheral lymphoid organs contribute to the circulating pool of total Treg cells and may differently impact the regulation of the immune response.
2. nTreg and iTreg cells may be phenotypically distinct, have different specificities, and have complementary functions in vivo.
3. Mucosal tolerance depends on the generation of iTreg cells.
4. Intestinal commensal bacteria promote the generation of iTreg cells.
5. iTreg cells can be generated under inflammatory and noninflammatory (subimmunogenic) conditions; the biological properties of these subtypes of iTreg cells may be different.

DISCLOSURE STATEMENT

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