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Review

Plasticity of CD4$^+$ T Cell Lineage Differentiation

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The differentiation of naive CD4$^+$ T cells into lineages with distinct effector functions has been considered to be an irreversible event. T helper type 1 (Th1) cells stably express IFN-γ, whereas Th2 cells express IL-4. The discovery and investigation of two other CD4$^+$ T cell subsets, induced regulatory T (iTreg) cells and Th17 cells, has led to a rethinking of the notion that helper T cell subsets represent irreversibly differentiated endpoints. Accumulating evidence suggests that CD4$^+$ T cells, particularly iTreg and Th17 cells, are more plastic than previously appreciated. It appears that expression of Foxp3 by iTreg cells or IL-17 by Th17 cells may not be stable and that there is a great degree of flexibility in their differentiation options. Here, we will discuss recent findings that demonstrate the plasticity of CD4$^+$ T cell differentiation and the biological implications of this flexibility.

Introduction

Host detection of infectious microbes triggers a chain of events that results in recruitment and differentiation of T lymphocytes with functions suited to contain the offending organism. The initial effector T cell response is often followed by a shift to regulatory T (Treg) cells needed to limit potential collateral tissue damage. The appropriate balance of effector and Treg cells can be achieved by influx of predifferentiated T cells, by local induction of distinct effector and/or regulatory T cell profiles, or by redirection of the differentiation program of the T cells, which requires plasticity in the regulation of transcriptional targets. The latter process, only recently appreciated to have a substantial contribution in vivo, may provide the host with the flexibility to shift resources, as appropriate, toward the clearance of specific microbes. In this overview, we will discuss some of the recent findings suggesting that CD4$^+$ T cells often have the capacity to redirect their functional programs, thus affecting the balance between regulatory T cells and cytokine-producing effector T cells and the cytokine profile of the effector cells.

The T Helper Cell Subset Paradigm

Efficient host defense against invading pathogenic microorganisms is achieved through coordination of complex signaling networks that link the innate and adaptive immune systems. Upon interaction with cognate antigen presented by antigen-presenting cells such as dendritic cells (DCs), CD4$^+$ T cells can differentiate into a variety of effector subsets, including classical Th1 cells and Th2 cells, the more recently defined Th17 cells, follicular helper T (Tfh) cells, and induced regulatory T (iTreg) cells. The differentiation decision is governed predominantly by the cytokines in the microenvironment and, to some extent, by the strength of the interaction of the T cell antigen receptor with antigen (Boyton and Altmann, 2002). Th1 cells are characterized by their production of IFN-γ and are involved in cellular immunity against intracellular microorganisms. IL-12 produced by innate immune cells as well as IFN-γ produced by both NK cells and T cells polarize cells toward the Th1 cell differentiation program through action of the signal transducer and activator of transcription 4 (Stat4), Stat1, and T box transcription factor T-bet. Th2 cells produce IL-4, IL-5, and IL-13 and are required for humoral immunity to control helminths and other extracellular pathogens. Th2 cell differentiation requires the action of GATA3 downstream of IL-4 and Stat6. Th17 cells produce IL-17A (hereafter referred to as IL-17), IL-17F, and IL-22 and play important roles in clearance of extracellular bacteria and fungi, especially at mucosal surfaces. Th17 cell differentiation requires retinoid-related orphan receptor (ROR)γt, a transcription factor that is induced by TGF-β in combination with the proinflammatory cytokines IL-6, IL-21, and IL-23, all of which activate Stat3 phosphorylation (Chen et al., 2007). Tfh cells are a subset of helper T cells that regulate the maturation of B cell responses. Differentiation of these cells requires the cytokine IL-21 (Vogelzang et al., 2008; Nurieva et al., 2008) and may be dependent on the transcription factor Bcl-6 (Fazilleau et al., 2009).

Tight regulation of effector T cell responses is required for effective control of infections and avoidance of autoimmune and immunopathological diseases. Aberrant Th1 and Th17 cell responses play critical roles in organ-specific autoimmunity, whereas Th2 cells are culprits in allergy and asthma. Treg cells have essential roles in maintenance of immune homeostasis, regulating these effector T cell responses and thus preventing their potentially pathogenic effects through a variety of mechanisms (Vignali et al., 2008; Shevach, 2009, this issue of *Immunity*). Treg cells are characterized by the expression of the forkhead transcription factor Foxp3. Foxp3 plays a critical role in specifying and maintaining the functional program of Treg cells, but the signals upstream of Foxp3 remain unclear (Hori et al., 2003; Fontenot et al., 2003; Khattri et al., 2003; Fontenot et al., 2005). Two major categories of Foxp3$^+$ Treg cells have been identified: the naturally occurring CD4$^+$ CD25$^+$ Treg (nTreg) cells that arise in the thymus and the TGF-β-induced iTreg cells produced in the periphery (Curotto de Lafaille and Lafaille, 2009; Josefowicz and Rudensky, 2009, both in this issue). Both types of Treg cells may participate in maintenance of peripheral tolerance and prevention of autoimmunity, although their individual contributions have not yet been closely examined in vivo (Chen et al., 2003).

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Treg cells clearly have functions in the suppression of Th1 and Th2 cell immune responses because mice lacking them exhibit overwhelming autoimmune disease characterized by excessive production of Th1 and Th2 cell cytokines (Kanangat et al., 1996; Kim et al., 2007). Whether Treg cells also suppress Th17 cell responses is less clear. One potential piece of evidence comes from mice that lack microRNA processing from the CD4$^+$CD8$^+$ stage onward in T cell development. In these mice, spontaneous differentiation of Th1 and Th17 cells occurs and is associated with the loss of Treg cell function (Chong et al., 2008). However, it has become apparent that the differentiation of Th17 cells may actually be linked to the differentiation of iTreg cells (Zhou et al., 2008a). The differentiation of both iTreg and Th17 cells requires TGF-β. In response to TCR stimulation in the presence of TGF-β, naive CD4$^+$ T cells can differentiate into either Th17 or iTreg cells depending on the overall cytokine milieu. The balance between Th17 cells and Treg cells may be influenced by the ability of nTreg cells to produce TGF-β, which, in the presence of proinflammatory cytokines, can induce naive CD4$^+$ T cells to differentiate into Th17 cells (Veldhoen et al., 2006a).

Commitment of CD4$^+$ T cells to distinct effector lineages in the course of cytokine polarization and multiple rounds of stimulation was proposed to involve stable programs of gene expression that correlate with epigenetic changes at loci encoding cytokine genes (Murphy et al., 1996; Bird et al., 1998; Ansel et al., 2003). As a result, cells maintain distinct cytokine profiles even in conditions that promote other effector lineages (Murphy et al., 1996). This view has undergone considerable re-evaluation in the wake of recent findings based on closer scrutiny of Th17 and Treg cell differentiation and function. The role of plasticity in CD4$^+$ T cell differentiation will be discussed in the sections that follow.

Relationship between Th17 and Th1-Th2 Cells

The notion that effector T helper cell subsets represent distinct terminally differentiated lineages has been favored on the basis of a series of in vitro experiments demonstrating that, once naive cells have been polarized into Th1 or Th2 cells, these phenotypes could not be reversed even when transferred into new polarizing conditions (Murphy et al., 1996). In addition, under polarizing conditions, stable cytokine production was observed only after a set number of cell divisions; such a result could be interpreted as a requirement for establishment of a stable transcriptional program (Bird et al., 1998; Grogan et al., 2001). The requirement for specific "master regulator" transcription factors, T-bet and GATA3 for differentiation of Th1 and Th2 cells, respectively, has also reinforced the lineage model. Crossregulation during Th1-Th2 cell differentiation has been demonstrated in numerous studies. For example, the commitment to the Th1 cell lineage is associated with the expression of the β2 chain of the IL-12R complex, thus conferring IL-12 responsiveness. IL-12Rβ2 induction is dependent on T-bet activation through IFN-γ and Stat1 signaling (Mullen et al., 2001; Afkarian et al., 2002). The Th2 cell cytokine IL-4 appears to repress IL-12 signaling through inhibition of IL-12Rβ2 expression, thus antagonizing Th1 cell differentiation (Szabo et al., 1997). Conversely, Th1 cell cytokines repress Th2 cell differentiation through a feed-forward mechanism. IFN-γ induces T-bet, which in turn induces Runx3 expression. Runx3 then cooperates with T-bet to further promote IFN-γ production while silencing the *Il4* gene in Th1 cells by binding to the *Ifng* promoter and the *Il4* silencer, respectively (Djuretic et al., 2007; Naoe et al., 2007). These data have led to the notion that Th1 and Th2 cells constitute stable terminally differentiated lineages.

RORγt is the T cell-subset-specific transcription factor required for Th17 cell differentiation and is both necessary and sufficient for induction of IL-17 and other genes whose expression characterizes the Th17 effector cell subset. Crossregulation similar to that between Th1 and Th2 cells may also occur between Th17 and Th1 or Th2 cells. IFN-γ and IL-4 both inhibit induction of IL-17 (Harrington et al., 2005). Additionally, IL-27, a member of the heterodimeric IL-12 cytokine family, promotes Th1 cell differentiation by inducing T-bet and IL-12Rβ2 (Takeda et al., 2003; Lucas et al., 2003) and concurrently inhibits Th17 cell differentiation in a Stat1-dependent manner (Batten et al., 2006; Stumhofer et al., 2006). The genetic loss of T-bet strongly favors IL-17 expression both in CD4$^+$ and CD8$^+$ T cells (Harrington et al., 2005; Park et al., 2005; Mathur et al., 2006; Yang et al., 2008c; Intlekofer et al., 2008). Likewise, Growth factor independent 1 (Gfi-1) induced by IL-4 supports optimal Th2 cell differentiation (Zhu et al., 2002) and inhibits differentiation of either Th17 or iTreg cells (Zhu et al., 2009). Normally, Th2 cells are unable to express IL-17 when subjected to Th17 cell-polarizing conditions. However, Gfi-1 deficiency in Th2 cells leads to expression of IL-17, suggesting that Gfi-1 may play a crucial role in repressing IL-17 expression (Zhu et al., 2009). Thus it appears that Th1 and Th2 cell signals are potent inhibitors of Th17 cell differentiation. However, whether the converse occurs, i.e., inhibition of Th1 and Th2 cell differentiation by Th17 cell signals, remains to be determined. These findings have reinforced the notion that Th17 cells represent a unique T helper cell lineage different from Th1 or Th2 cells (Ivanov et al., 2006). The stability of the Th17 cell phenotype, once it is established, will be discussed further below.

Relationship between Th17 and iTreg Cells

TGF-β is profoundly important for establishment of immunological tolerance, at least in part because it is required for the differentiation of iTreg cells and for maintenance of nTreg cells after they emigrate from the thymus (Chen et al., 2003; Li et al., 2006; Marie et al., 2005). For this reason, it was surprising to learn that TGF-β is also essential for the differentiation of proinflammatory Th17 cells (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006a). Mice lacking TGF-β lack both Foxp3$^+$ Treg and Th17 cells and have overwhelming autoimmunity largely caused by uncontrolled Th1 cell activity (Li et al., 2006; Veldhoen et al., 2006b). These findings suggest an intimate link between the Treg and Th17 cell programs of differentiation, and indeed there is evidence for such a relationship from both in vitro and in vivo studies (Bettelli et al., 2006; Zhou et al., 2008a; Yang et al., 2008a; Koenen et al., 2008; Beriou et al., 2009). Exposure of antigen-activated naive CD4$^+$ T cells to TGF-β results in transcriptional upregulation of both Foxp3 and RORγt$^+$, transcription factors that direct distinct CD4$^+$ T cell differentiation programs (Zhou et al., 2008a). T cells that coexpress RORγt and Foxp3 have been identified in vivo in both mice and humans (Zhou et al., 2008a; Voo et al., 2009). Foxp3$^+$RORγt$^+$ cells from the small intestine produce less IL-17 compared to Foxp3$^-$RORγt$^+$ cells (Zhou et al., 2008a), whereas Foxp3 deficiency results in a marked

increase in IL-17 but not RORγt expression (Gavin et al., 2007). These observations suggest that Foxp3 may antagonize RORγt-induced IL-17 expression in a cell-intrinsic manner. The Foxp3$^+$RORγt$^+$ cells may also exist as a transient population that can give rise to either Treg or Th17 cells (Zhou et al., 2008a).

A plausible explanation for the reduced amount of IL-17 in cells that coexpress RORγt and Foxp3 is provided by the finding that these two transcription factors can interact with each other, perhaps in the context of larger nuclear complexes. A similar physical interaction was first demonstrated between Foxp3 and RORα, which is closely related to RORγt and is similarly sufficient for inducing IL-17 expression but has only a minor redundant role in Th17 cell differentiation (Du et al., 2008; Yang et al., 2008b; L.Z. and D.R.L., unpublished data). Whereas transduction of naive CD4$^+$ T cells with RORγt induces IL-17 expression, cotransduction with Foxp3 abrogates induction as long as Foxp3 contains its exon-2-encoded domain that is required for binding to RORγt (Zhou et al., 2008a). After in vitro induction of RORγt and Foxp3 by TGF-β, cells do not express IL-17 but have the dual potential to differentiate into either the Th17 or Treg cell lineage depending on the cytokine environment. In the presence of proinflammatory cytokines (IL-6, IL-21, or IL-23) and low concentrations of TGF-β, RORγt expression is further upregulated, whereas Foxp3 expression and function are inhibited. This relieves repression of RORγt activity by Foxp3 in favor of Th17 cell lineage specification. In contrast, in the absence of proinflammatory cytokines, high concentrations of TGF-β are optimal for Foxp3 expression and thus tip the balance toward Treg cell differentiation (Zhou et al., 2008a). This shift toward Treg cells is enhanced by IL-2 and retinoic acid (RA). Both inhibit Th17 cell differentiation by reducing RORγt expression and enhancing TGF-β-induced Foxp3 expression, thus influencing Th17-Treg cell specification (Laurence et al., 2007; Sun et al., 2007; Coombes et al., 2007; Mucida et al., 2007).

Even though Foxp3 inhibits expression of IL-17, Foxp3$^+$IL-17$^+$ CD4$^+$ T cells have been observed both in vitro, after polarization in the presence of TGF-β and IL-6, and in vivo in mice and humans (Voo et al., 2009; Yang et al., 2008a; Zhou et al., 2008a). Circulating human Foxp3$^+$IL-17$^+$ T cells have in vitro suppressive activity (Beriou et al., 2009; Voo et al., 2009), but it is not yet known whether they have the functions of conventional Th17 cells. Signals from proinflammatory cytokines appear to prevent Foxp3-mediated inhibition of RORγt activity (Zhou et al., 2008a), but may spare the regulatory activity of Treg cells.

Several transcription factors have been implicated in modulating the RORγt-Foxp3 balance during T helper cell differentiation. IRF4 is essential for Th17 cell differentiation both in vitro and in vivo (Brustle et al., 2007). Its absence in mice resulted in reduced RORγt expression, increased Foxp3 expression, loss of IL-17 production, and protection from experimental autoimmune encephalomyelitis (EAE). *Irf4* was also identified as a direct target of Foxp3, and its selective loss in Treg cells selectively impaired suppression of Th2 cell responses (Zheng et al., 2009). Runx1 was recently shown to form a complex with RORγt and to cooperate with it to promote Th17 cell differentiation (Zhang et al., 2008). In Treg cells, an interaction between Runx1 and Foxp3 was reported to be required for suppression of IL-2 and IFN-γ production, upregulation of Treg cell-associated molecules, and suppressive activity (Ono et al., 2007). Runx1 is also required for maintaining Foxp3 expression in nTreg cells and in TGF-β-induced iTreg cells (M.M.W.C. and D.R.L., unpublished data). Thus, functional plasticity in cells that coexpress RORγt and Foxp3 may be governed by the ability of Runx to cooperate with either transcription factor.

Stat3, a transcription factor common to the IL-6, IL-21, and IL-23 signaling pathways, is also essential for Th17 cell differentiation. Stat3 binds to the *Il17* locus to direct its transcription, presumably through cooperative activity with RORγt (Chen et al., 2006). TGF-β-induced Foxp3 expression is inhibited by proinflammatory cytokines in a Stat3-dependent manner. Forced expression of an active form of Stat3 enhanced IL-17 expression in the presence of TGF-β, presumably by upregulation of RORγt expression and suppression of Foxp3 expression (Zhou et al., 2007; Yang et al., 2007). It remains to be determined whether the coordinated activity of Stat3 and RORγt in induction of IL-17 is due to a physical interaction between these two factors and whether relief of Foxp3 inhibition by proinflammatory cytokines is mediated by perturbation of RORγt-Foxp3 interaction through the activity of Stat3.

Flexibility in the Differentiation of Foxp3$^+$ Cells

During the past two years, there have been multiple reports indicating that the differentiation program of Foxp3$^+$ Treg cells is not fixed. A series of in vitro studies and in vivo analyses of the intestine have shown that Treg cells have the propensity to differentiate into Th17 or Tfh cells. TCR-stimulated thymus-derived Foxp3$^+$ cells, which produce TGF-β, were shown to produce IL-17 after exposure to IL-6 (Xu et al., 2007). In contrast, culturing of the cells in Th1 cell polarization conditions did not alter their phenotype. By genetic fate mapping, using Foxp3-Cre mice to mark cells that expressed Foxp3 at some stage of development, at least one-quarter of small intestinal Th17 cells were found to have expressed Foxp3 during their ontogeny (Zhou et al., 2008a). It is unclear why some IL-17$^+$ cells but not others expressed Foxp3 during their differentiation. There may be stochastic activation of Foxp3 during the establishment of the Th17 cell program or, alternatively, Foxp3 expression may mark a distinct differentiation pathway. It remains to be determined whether there are functional differences between Th17 cells that differentiated in the presence or absence of Foxp3 expression.

It has been suggested that IL-6, produced by antigen-presenting cells as a result of Toll-like receptor engagement, blocks CD4$^+$CD25$^+$ Treg cell-suppressive activity (Pasare and Medzhitov, 2003). Another possible explanation is that IL-6 may convert Treg cells into proinflammatory cells. Indeed, it was recently reported that Treg cells can be converted to IL-17-expressing cells in a proinflammatory cytokine environment (Yang et al., 2008a; Osorio et al., 2008), but one study suggests that, unlike nTreg cells, iTreg cells induced by TGF-β and IL-2 are resistant to Th17 conversion by IL-6 (Zheng et al., 2008). It is unclear whether these discrepancies are due to in vitro phenomena. No evidence has been found for the reciprocal conversion of Treg cells from IL-17-expressing cells, consistent with a lack of active epigenetic modifications at the *Foxp3* locus in differentiated Th17 cells (discussed below) (Croxford et al., 2009; Wei et al., 2009). The physiological relevance of Treg cell conversion into Th17 cells has been unclear. Interestingly, conversion of Foxp3$^+$ Treg

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cells into IL-17$^+$ T cells was observed upon coculture with dendritic cells selectively activated by dectin-1, a C-type lectin receptor involved in fungal recognition (Osorio et al., 2008). Fungal infections have been linked to IL-17 responses (LeibundGut-Landmann et al., 2007). Thus, conversion of Treg cells into Th17 cells may help restrain infections with specific fungal pathogens such as *Candida albicans*. Treg cells were unexpectedly found to have a critical role in host protection from mucosal infection with Herpes simplex virus (Lund et al., 2008). The ability of Treg cells to be converted into effector cells in vivo may therefore also contribute to host protection after infection with viruses and other microbial pathogens.

Redifferentiation of Treg cells into effector T helper cells other than Th17 cells has also been reported. Although a fraction of highly purified nTreg cells can express IFN-γ and T-bet and maintain Foxp3 expression after culture in Th1 cell polarizing conditions (Wei et al., 2009), there is still no consensus on the ability of Treg cells to undergo this change of phenotype (Xu et al., 2007). It is unclear whether the Foxp3$^+$IFN-γ^+ cells retain suppressive activity. One setting in which Foxp3-expressing cells have clearly converted into Th1 or Th2 cell-phenotype cells is in mice with Treg cell-specific deletion of the RNaseIII enzyme Dicer that is required for microRNA biogenesis (Zhou et al., 2008b). In conditional Dicer-Foxp3-cre mice, genetic fate-mapping demonstrated that a substantial number of IL-4- and IFN-γ-expressing cells had differentiated from cells that previously had expressed Foxp3. Similarly, in mice that lacked Drosha, another RNaseIII enzyme required for microRNA biogenesis, in all T cells, there was spontaneous differentiation of Th1 and Th17 cell-phenotype cells, although it was not determined whether this was a result of Treg cell conversion (Chong et al., 2008). Thus, it appears that microRNAs may be required in Treg cells to provide stability and prevent conversion (Lu et al., 2009).

Foxp3$^+$ Treg cells as well as Th17 cells are most abundant at mucosal barriers. Specific commensal microbiota are required for differentiation or migration of Th17 cells to gut lamina propria (Ivanov et al., 2008), and these potentially inflammatory T cells are thought to be held in check by the local Treg cells. In light of their plasticity, Treg cells may also convert into inflammatory-cytokine-producing cells, especially in disease conditions. In the gut, commensal bacteria stimulate DCs to produce large quantities of proinflammatory cytokines through purinergic receptors or Toll-like receptors (Atarashi et al., 2008; Hall et al., 2008). It is conceivable that these proinflammatory cytokines might promote conversion of Treg cells into inflammatory effector T cells that could cause harmful inflammation. However, this is not the case, at least in the context of normal exposure to commensal flora. For example, the symbiont *Bacteroides fragilis*, via production of polysaccharide A (PSA), influences immune homeostasis and protects animals from experimental colitis by inhibiting Th17 cell differentiation and inducing IL-10 production (Mazmanian et al., 2005; Mazmanian et al., 2008). In addition, several recent studies have implicated RA-producing CD103$^+$ DC in the gut as inducers of Foxp3 expression (Mucida et al., 2007; Coombes et al., 2007; Sun et al., 2007). RA increased the proportion of Foxp3$^+$ cells induced by TGF-β in vitro and stabilized Foxp3 expression in vivo even upon adoptive transfer and under inflammatory conditions (Benson et al., 2007). In the gut, RA may thus play a role in stabilizing or reinforcing the Treg cell program, preventing microbiota-induced inflammation.

Most recently, Fagarasan and colleagues showed that Foxp3$^+$ Treg cells in Peyer's patches differentiate into Tfh cells, which then participate in germinal center formation and IgA synthesis in the gut. The differentiation of Foxp3$^+$ Treg cells into Tfh cells required the loss of Foxp3 expression and interaction with B cells through CD40 (Tsuji et al., 2009). Remarkably, only the Foxp3$^+$ T cell fraction had the capacity to give rise to Tfh cells, at least in the intestine. It is not yet known whether nTreg, iTreg cells, or both are precursors for these specialized T cells. Rudensky and colleagues recently showed that inactivation of IRF4 specifically in Foxp3$^+$ cells resulted in elevated Th2 cytokine production and enhanced formation of germinal centers (Zheng et al., 2009). Although differentiation of Tfh cells was not examined in that study, it is possible that increased Tfh cells could lead to those phenotypes. Consistent with this, it was recently shown that Tfh cells can be differentiated from IL-4$^+$ Th2 cells in response to helminth antigens (King and Mohrs, 2009; Zaretsky et al., 2009). It is possible that IRF4 is involved in restraining reprogramming of Treg cells toward the Tfh effector cell fate.

Taken together, the recent results suggest that the local microenvironment, through provision of specific cytokines and/or cell-cell (or cell-microorganism) interactions, directs multiple possible alternate fates that Foxp3$^+$ cells can adopt (Figure 1). In light of these findings, it will be important to consider potential complications for the therapeutic use of Treg cells in autoimmune diseases and organ transplantation. Treg cell therapy may thus be less beneficial or even harmful in established inflammatory conditions because their transfer into an established inflammatory lesion could lead to their reprogramming. It may be safer to administer Treg cells before disease onset or organ transplantation because the amounts of proinflammatory cytokines are expected to be low. Suppression of inflammatory cytokines (e.g., IL-6, IL-23, or IL-12) may also be required to prevent redifferentiation of Treg cells into cells with pathogenic potential (e.g., Th17 or Th1 cells).

Instability of the Th17 Cell Program

The notion that Th17 cells are derived from the Th1 cell lineage was discarded by the findings that these cells express RORγt and can develop in the absence of T-bet or Stat4 (Ivanov et al., 2006; Weaver et al., 2007). However, under homeostatic or inflammatory conditions, IL-17$^+$IFN-γ^+ double-producer cells are easily detected, suggesting that there may be some intricate relationship between the Th1 and Th17 cell differentiation programs. Indeed, several recent findings highlight the plasticity of Th17 cells (Figure 1). In vitro-generated Th17 cells lose IL-17 and IL-17F expression without constant exposure to IL-6 and TGF-β (Lexberg et al., 2008). Cells polarized in Th17 cell conditions for as long as 3 weeks still failed to maintain IL-17 and IL-17F expression and were converted to Th1 or Th2 cells in the presence of IL-12 or IL-4, respectively (Lexberg et al., 2008; Lee et al., 2009). Such cells also lost IL-17 expression and expressed IFN-γ in the presence IL-23, but this required Stat4, suggesting some partial signaling through the IL-12 receptor machinery. The presence of TGF-β alone was shown to sustain expression of IL-17 and IL-17F (Lee et al., 2009). However, it is worth noting that Th17 cells secrete IL-21, which may function

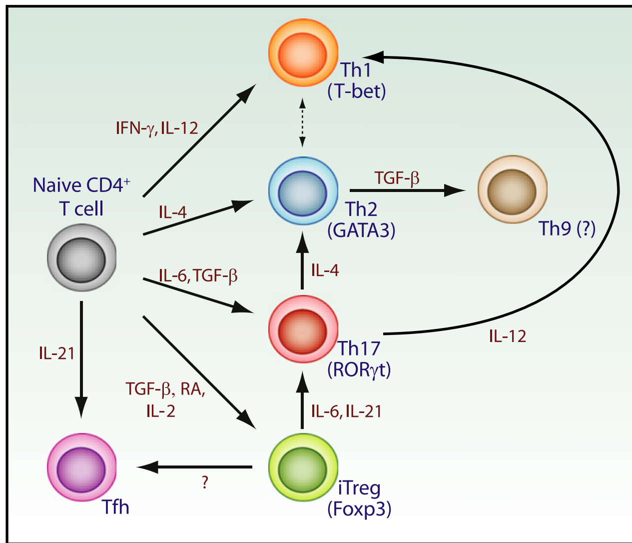

Figure 1. The Cytokine Milieu Determines CD4$^+$ T Cell Differentiation and Conversion
Upon encountering foreign antigens presented by antigen-presenting cells, naive CD4$^+$ T cells can differentiate into Th1, Th2, Th17, iTreg, and Tfh cells. These differentiation programs are controlled by cytokines produced by innate immune cells, such as IL-12 and IFN-γ, which are important for Th1 cell differentiation, and IL-4, which is crucial for Th2 cell differentiation. TGF-β together with IL-6 induces Th17 cell differentiation, whereas iTreg differentiation is induced by TGF-β, retinoic acid (RA), and IL-2. Tfh cell differentiation requires IL-21. Specific transcription factors that orchestrate the differentiation program of each T helper cell subset have been identified: T-bet for Th1 cells, GATA3 for Th2 cells, RORγt for Th17 cells, and Foxp3 for iTreg cells. The effector T cells had been thought to be terminally differentiated lineages, but it now appears that there is considerable plasticity allowing for conversion to other phenotypes. Although Th1 and Th2 cells display more stable phenotypes, iTreg cells and Th17 cells can readily switch to other T helper cell programs under certain cytokine conditions. For example, iTregs can become IL-17-producing cells upon stimulation of IL-6 and IL-21. Treg cells can also switch to Tfh cells, and this requires B cells and CD40-CD40L interaction. Th17 cells may also convert into IFN-γ-producing Th1 cells or IL-4-producing Th2 cells when stimulated by IL-12 or IL-4, respectively. Evidence also suggests that Th2 cells can switch to IL-9-producing cells in response to TGF-β, although it is unclear whether these "Th9" cells truly represent a distinct lineage.

together with TGF-β for maintenance of IL-17 expression in an autocrine manner. In contrast to in vitro-differentiated Th17 cells, in vivo memory CD4$^+$CD62L^{lo} Th17 cells appear more resistant to conversion (Lexberg et al., 2008). Thus, these results suggest that there may be epigenetic differences between in vitro- and in vivo-generated Th17 cells (discussed below).

Th17 cells (expressing an IL-17F-Thy1.1 transgene) are active in inducing colitis upon transfer into immunodeficient mice, but many of these cells convert to Th1 cells (Lee et al., 2009). Consistent with this, diabetogenic BDC2.5 CD4$^+$ T cells polarized in vitro to the Th17 cell phenotype lose IL-17 expression and express IFN-γ after adoptive transfer into NOD-SCID mice, ultimately causing β-cell destruction and diabetes (Bending et al., 2009). It will be important to determine whether passage through a Th17 cell stage of differentiation is important for the development of pathogenic Th1 cells in these and other disease models. It is also worthwhile to mention that the above in vivo studies involved transferring cells to lymphopenic hosts in which homeostatic proliferation may influence the plasticity of T helper cell differentiation.

Although Th17 cells make the signature cytokines IL-17, IL-17F, and IL-22, they may represent a heterogeneous population with dynamic phenotypic and/or functional properties that are influenced by different environmental cues. For example, high concentrations of TGF-β can inhibit IL-22 expression, leading to generation of IL-17$^+$IL-22$^-$ cells (Zheng et al., 2007). Th17 cells polarize in the presence of TGF-β and IL-6, but without IL-23, they appear to have curtailed pathogenic potential, despite expression of IL-17. These cells were shown to express IL-10, a cytokine that restrains inflammatory responses (and has recently been shown to be produced by all subsets of CD4$^+$ T cells). In the presence of IL-23, "protective" Th17 cells were converted into "pathogenic" Th17 cells that expressed proinflammatory cytokines but not IL-10 (McGeachy et al., 2007). The experiments described above, using in vitro polarization or adoptive transfer, are representative of current approaches for studying Th17 cell stability (Lee et al., 2009; Croxford et al., 2009; Wei et al., 2009; Bending et al., 2009). However, given our still limited understanding of in vitro Th17 cell differentiation, which varies greatly according to culture conditions (Veldhoen et al., 2009), and the divergence of transcriptional profiles of Th17 cells generated in vitro versus those present in vivo (L.Z. and D.R.L., unpublished data), there needs to be cautious interpretation of results.

The plasticity of Th17 cells has important biological implications. Th17 cells are abundant in the intestinal lamina propria of healthy animals. In view of an unstable Th17 cell phenotype, there is likely selective pressure for maintaining a Th17 cytokine profile within the gut environment. Commensal bacteria are essential for induction of Th17 cells that participate in the maintenance of epithelial integrity and clearance of extracellular pathogens (Atarashi et al., 2008; Hall et al., 2008; Ivanov et al., 2008). Selective antibiotic treatment inhibited intestinal lamina propria Th17 cell differentiation in mice (Ivanov et al., 2008; Atarashi et al., 2008). Accordingly, humans subjected to long-term and broad-spectrum antibiotic treatment can develop *Candida* infections, which are normally controlled by Th17 cell responses. Antibiotic-induced disruption of the normal intestinal microbiota can also lead to diarrhea caused by the anaerobic bacterium *Clostridium difficile* (DuPont et al., 2008), and this may be linked to an altered Th17-Treg cell balance as a result of clearance of Th17 cell-inducing commensal bacteria.

Recruitment of neutrophils after activation of Th17 cells results in clearance of microbes, but can also cause tissue destruction. Appropriate and timely decline of Th17 cell responses is therefore probably required to limit host tissue injury. This may be achieved by a shift toward production of IL-10 by Th17 cells. Steady-state Th17 cells may also have the capacity to adjust their cytokine profiles to combat diverse pathogens as needed. Thus, together with the de novo-differentiated Th1 cells, rapid transition from Th17 to Th1 cell responses may represent an efficient local host defense for controlling infections with intracellular microbes.

Epigenetic View of T Helper Cell Differentiation

The differentiation of T helper cells is intricately coupled to epigenetic changes in chromatin structure, histone and DNA modifications, and the expression of small noncoding RNAs (Ansel et al., 2003; Merkenschlager and Wilson, 2008; Wilson et al., 2009). Earlier analyses of epigenetic changes during T helper cell

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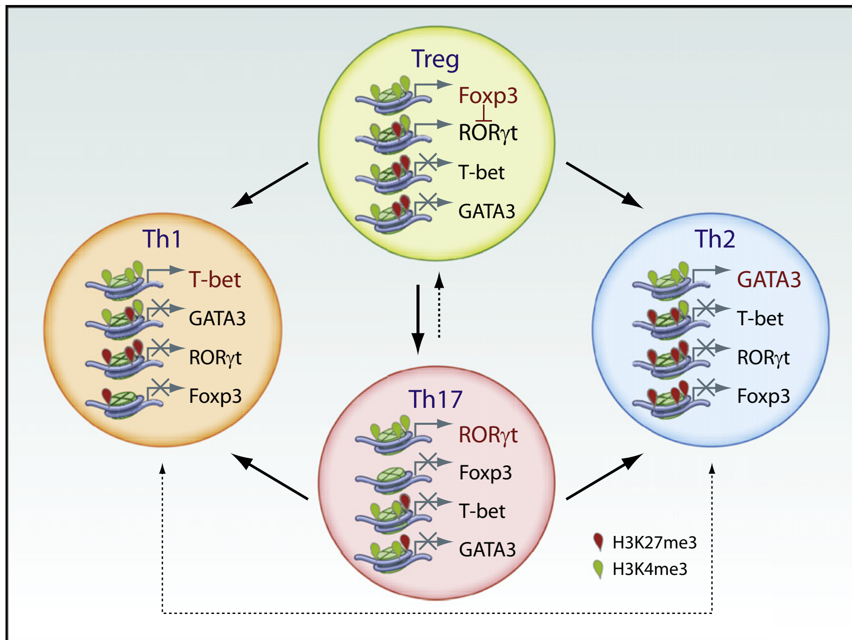

Figure 2. Epigenetic Status of Lineage-Specific Transcriptional Regulator Gene Loci in CD4+ T Cells
Recent findings suggest that T helper cell differentiation is more plastic than previously appreciated. Each CD4+ T cell subset can adopt alternate cytokine profiles in response to cytokine environmental changes. Among four subsets of T cells, Treg cells and Th17 cells display the highest propensity to switch to other phenotypes. The molecular mechanism underlying this plasticity may be related to poised, bivalent epigenetic states (i.e., permissive H3K4me3 plus repressive H3K27me3 marks) at the transcriptional regulator (e.g., T-bet and Gata3) gene loci. Consistent with permissive epigenetic marks at Foxp3 and RORγt gene loci, coexpression of Foxp3 and RORγt occurs in Treg cells, but RORγt activity is inhibited by Foxp3.

differentiation have largely focused on the gene loci encoding cytokines that mark Th1 or Th2 cell lineages, i.e., IFN-γ and IL-4, IL-5, and IL-13, respectively. Recent technological advances, e.g., chromatin immunoprecipitation (ChIP) followed by genomic tiling arrays (ChIP-chip) or by high-throughput sequencing (ChIP-Seq), have allowed genome-wide examination of histone modifications and DNA methylation that accompany changes in gene expression in CD4+ T cell subsets. Such analysis has provided insights into the mechanisms underlying T helper cell plasticity (Wei et al., 2009). Trimethylation of histone H3 lysine 4 (H3K4me3) is a permissive mark found at the promoters and the enhancers of active genes, whereas trimethylation of histone H3 lysine 27 (H3K27me3) is present in broad domains that encompass inactive genes. H3K4me3 and H3K27me3 modifications are both present at some genomic regions, and these bivalent modifications have been suggested to poise genes ready for either activation or repression during differentiation.

The epigenetic marks found at the *Ifng*, *Il4*, and *Il17* gene loci correlated precisely with Th1 and Th2 cell lineages, with H3K4me3 at the *Ifng* locus and H3K27me3 at the *Il4* and *Il17* loci in Th1 cells and H3K4me3 at the *Il4* locus and H3K27me3 at the *Ifng* and *Il17* loci in Th2 cells. Intriguingly, the *Tbx21* (encoding T-bet) and *Gata3* genes displayed a bivalent status in Th2 and Th1 cells, respectively, consistent with a report suggesting that interconversion can occur between Th1 and Th2 cells under appropriate inflammatory conditions (Krawczyk et al., 2007). Consistent with the plasticity evident for Treg cells, *Tbx21*, *Gata3*, and *Rorc* were all bivalently modified in Treg cells (Figure 2). Such bivalent modifications may allow specific lineage regulator gene loci to be activated under different polarizing conditions, thus reprogramming Treg cells into other lineages. Similarly, in vitro-differentiated Th17 cells displayed bivalent epigenetic status at the *Tbx21* and *Gata3* loci, and such a result suggests that these cells are preconditioned to be redirected toward Th1 or Th2 cell fates. Such reprogramming appears to be unidirectional, given that repressive epigenetic configuration was observed at both the *Il17a* and *Rorc* loci in Th1 and Th2 cells (Wei et al., 2009).

Thymus-derived nTreg cells and peripherally derived iTreg cells appear to differ in their propensity for reprogramming. This is reflected in the DNA methylation status of the Foxp3 locus and in histone marks at the *Rorc* locus. H3K27me3 was found at the *Il17a* locus, whereas H3K4me3 was found at the *Rorc* locus in iTreg cells, consistent with the notion that RORγt expression was induced by TGF-β, but RORγt-directed IL-17 expression was inhibited by Foxp3 (Wei et al., 2009; Zhou et al., 2008a) (Figure 2). In contrast, in nTreg cells, bivalent H3K4me3 and H3K27me3 were present at the *Rorc* locus, allowing for potential coexpression of Foxp3 and RORγt.

When nTreg cells were cultured in Th17 or Th1 cell-polarizing conditions, a substantial fraction of IL-17+Foxp3+ or IFNγ+ Foxp3+ cells arose without downregulation of Foxp3 expression (Xu et al., 2007; Yang et al., 2008a; Wei et al., 2009). In contrast, TGF-β-induced Treg cells readily lost Foxp3 expression and acquired IL-17 expression when subjected to Th17 cell-polarizing conditions (Yang et al., 2008a). There is accumulating evidence that the difference in stability of Foxp3 expression between nTreg cells and iTreg cells is due to epigenetic differences at the *Foxp3* locus (Huehn et al., 2009). Besides the promoter and an intronic enhancer, a highly conserved CpG-rich element is present upstream of exon −1 in the *Foxp3* locus and is referred to as the Treg cell-specific demethylated region (TSDR) (Floess et al., 2007; Kim and Leonard, 2007; Baron et al., 2007; Nagar et al., 2008). This region is fully demethylated in nTreg cells but remains methylated in TGF-β-induced Treg cells and activated human conventional T cells that transiently express Foxp3. Consistent with this, inhibition of DNA methylation with azacytidine conferred stability of Foxp3 expression induced by TGF-β. Intriguingly, azacytidine, even in the absence of exogenous TGF-β, also promoted de novo induction of Foxp3 expression, most likely through an indirect mechanism, highlighting the importance of DNA methylation for the regulation of Foxp3 transcription (Polansky et al., 2008). Furthermore, in vivo-derived antigen-specific Treg cells have stable Foxp3

expression, consistent with the demethylated status of their TSDR (Polansky et al., 2008). Further studies on regulation of DNA methylation at the TSDR will probably provide important insights into plasticity of Treg cell differentiation.

All together, these data on epigenetic modification of histones and DNA highlight the plasticity of $CD4^+$ T cell differentiation programs, suggesting that the ''lineage-specific'' transcription factors may not necessarily have mutually exclusive expression patterns. Lineage specification is therefore not only determined by the epigenetic status of signature cytokine loci but also relies on the epigenetic states of key transcription factors. With current technological advances, we should expect in the near future a comprehensive epigenetic profile of T helper cell lineages, including the analyses of further histone modifications and of multiple target gene loci associated with the individual lineages. It is important to mention that current epigenetic studies are based on a snapshot analysis of cells within heterogeneous populations. Thus, the interpretation of current data may be compromised by the noise as a result of heterogeneity. The current epigenetic view also fails to reveal the dynamic chromatin changes that occur during effector T cell differentiation. Long-range inter- and intrachromosomal interactions of cytokine gene loci regulate T helper cell differentiation (Rowell et al., 2008). It will be informative to analyze these interactions during redifferentiation of T helper cells as well. Lineage-restricted transcription factors play critical roles in epigenetic regulation of cytokine gene expression, such as initiation of chromatin remodeling and maintenance of chromatin structure. However, the mechanisms underlying epigenetic regulation (including heritable changes in expression of target genes) by these factors are largely unknown. Understanding the mechanisms by which bivalent modifications at the transcription factor loci are achieved will help to delineate T helper cell lineage commitment and plasticity.

Finally, it is important to mention that most of the current studies showing the plasticity of T helper cell differentiation program are performed at the population level. Therefore, purity of the population has to be assured by vigorous cell sorting to avoid potential contaminants. Lineage-specific transcription factor or cytokine reporter mouse models have been invaluable tools to study T helper cell differentiation and will continue to provide a better understanding of T helper cell plasticity, which should be ideally evaluated at the single-cell level. Identification of in vitro culture conditions that mimic in vivo environments will allow us to dissect the signaling pathways and the transcriptional regulatory networks of the T helper cell differentiation program.

Conclusions

T helper cell lineage commitment was originally viewed as a unidirectional process with nonreversible terminal differentiation of Th1 and Th2 cells. Each T helper cell subset expresses its lineage-specific transcription factors and mutually exclusive cytokines. The discovery of two new subsets of T helper cells, Treg cells and Th17 cells, and their capacity to produce cytokines that would be considered hallmarks of opposing lineages suggest that the commitment of T helper cell lineages is more plastic than previously appreciated. Epigenetic modifications of cytokine genes and key transcriptional regulator genes direct the T helper cell differentiation program. The flexibility of Treg and Th17 cell differentiation may provide us with a model system to study the contributions of reversible and dynamic chromatin changes in T cell development. The plasticity and unstable phenotypes of Tregs and Th17 cells will have important biological implications for designing therapeutic regimens to combat infections and control autoimmunity.

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