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T-bet+ Treg Cells Undergo Abortive Th1 Cell Differentiation due to Impaired Expression of IL-12 Receptor β2

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SUMMARY

Foxp3+ regulatory T (Treg) cells limit inflammatory responses and maintain immune homeostasis. Although comprised of several phenotypically and functionally distinct subsets, the differentiation of specialized Treg cell populations within the periphery is poorly characterized. We demonstrate that the development of T-bet+ Treg cells that potently inhibit T helper 1 (Th1) cell responses was dependent on the transcription factor STAT1 and occurred directly in response to interferon-γ produced by effector T cells. Additionally, delayed induction of the IL-12Rβ2 receptor component after STAT1 activation helped ensure that Treg cells do not readily complete STAT4-dependent Th1 cell development and lose their ability to suppress effector T cell proliferation. Thus, we define a pathway of abortive Th1 cell development that results in the specialization of peripheral Treg cells and demonstrate that impaired expression of a single cytokine receptor helps maintain Treg cell-suppressive function in the context of inflammatory Th1 cell responses.

INTRODUCTION

The appropriate differentiation of naive T helper cells into functionally specialized effector lineages is critical for generating productive immune responses against different types of pathogens. T helper 1 (Th1) cells are required for immunity to intracellular pathogens and develop from naive precursors via a multistep mechanism involving sequential activation of the transcription factors STAT1, T-bet, and STAT4. Initially, T cell receptor (TCR) stimulation in conjunction with STAT1 activation by cytokines such as IFN-γ induce low-level expression of the transcription factor T-bet, which in turn drives expression of the inducible IL-12 receptor component IL-12Rβ (Afkarian et al., 2002; Mullen et al., 2001). This allows the cells to undergo IL-12-dependent activation of STAT4, whereupon STAT4 and T-bet act independently and synergistically to drive expression of the signature inflammatory cytokine IFN-γ, as well as other

genes required for the proper function of Th1 cells (Thieu et al., 2008; Wei et al., 2010).

In addition to effector Th subsets, Foxp3+ regulatory T (Treg) cells comprise a distinct CD4+ T cell lineage important for dampening inflammation and preventing autoimmunity (Sakaguchi et al., 2008). Treg cell differentiation occurs primarily in the thymus, where developing T cells upregulate Foxp3 upon recognition of self-antigen and stimulation with IL-2 or IL-15. Thus, the TCR repertoire of Treg cells is believed to be heavily biased toward autoreactivity (Lio and Hsieh, 2011). Like conventional CD4+ Th cells, Treg cells can be subdivided into distinct subsets on the basis of differential expression of homing receptors and activation markers (Huehn et al., 2004; Min et al., 2007), and multiple functional mechanisms of suppression have been ascribed to Treg cells (Vignali et al., 2008; Shevach, 2009; Tang and Bluestone, 2008). Indeed, the ability of Treg cells to control different types of Th cell responses depends on their expression of specific Th cell-associated transcription factors (Chaudhry et al., 2009; Koch et al., 2009; Zheng et al., 2009). For example, although T-bet is considered the "master regulator" of Th1 cell differentiation, Treg cells selectively upregulate T-bet during type 1 inflammatory responses in vivo, and T-bet-deficient Treg cells display diminished expression of the Th1 cell-associated chemokine receptor CXCR3 and fail to rescue Foxp3-deficient mice from Th1 cell-mediated inflammatory disease (Koch et al., 2009). Thus, the phenotypic and functional heterogeneity of Treg cells helps ensure that they can modulate different types of immune responses in both lymphoid and nonlymphoid tissue sites (Campbell and Koch, 2011).

The diversity of peripheral Treg cells suggests that they alter their migratory, functional, and homeostatic properties in response to contextual cues from the immune environment. However, the mechanisms guiding the development of specialized Treg cell subsets, and the ways in which they mirror and diverge from the comparatively well-characterized pathways of effector T cell differentiation, have not been extensively explored. In addition, the autoreactivity of Treg cells presents a significant danger if they were to acquire proinflammatory effector functions. Thus, strict mechanisms that limit the functional reprogramming of Treg cells during strong inflammatory responses are thought to exist. For instance, despite upregulating T-bet during Th1 cell inflammatory responses, T-bet+ Treg cells do not typically produce IFN-γ (Koch et al., 2009). However, IFN-γ-producing Treg cells have been observed during the
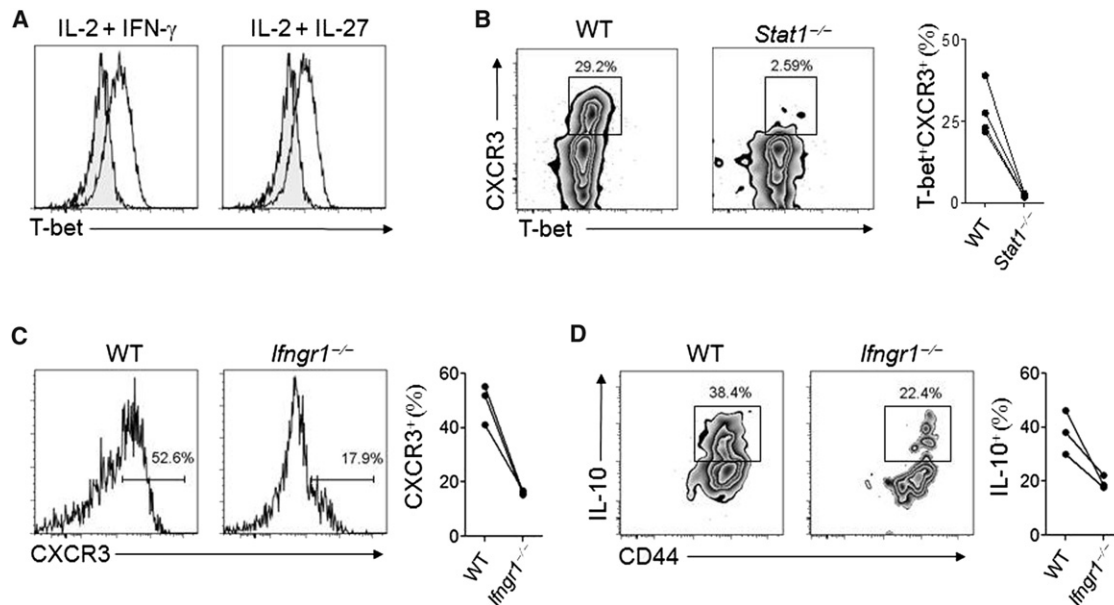
Figure 1. STAT1- and IFN-γR-Dependent Expression of T-bet by Treg Cells

(A) Flow cytometric analysis of T-bet expression by CD4+Foxp3+ Treg cells stimulated with anti-CD3/anti-CD28 in media containing IL-2 alone (shaded histograms) or IL-2 + IFN-γ or IL-27 (open histograms) for 5 days as indicated.

(B) Flow cytometric analysis of CXCR3 and T-bet expression by gated wild-type- or Stat1−/−-derived CD4+Foxp3+ splenocytes in mixed bone marrow chimeras. Data are representative of two independent experiments with at least three mice analyzed per experiment. Graph depicts the frequency of CXCR3+T-bet+ cells among total WT and Stat1−/−-derived CD4+Foxp3+ splenic Treg cells in each BM chimera examined. Linked points represent the values from individual chimeric mice.

(C) Flow cytometric analysis of CXCR3 expression by gated wild-type- or Ifngr1−/−-derived CD4+Foxp3+ Treg cells in the brains of mixed bone marrow chimeras 14 days after induction of EAE.

(D) Flow cytometric analysis of CD44 and IL-10 expression by CD4+Foxp3+ Treg cells from either wild-type or Ifngr1−/− donors in the brains of mixed bone marrow chimeras 19 days after induction of EAE. Graphs in (C) and (D) depict the frequency of CXCR3+ or IL-10+ cells among the wild-type or Ifngr1−/− Treg cells in each chimera examined. Linked points represent the values from individual chimeric mice.

dysregulated immune responses associated with autoimmune disease or infection-induced immunopathologies (Oldenhove et al., 2009; Zhao et al., 2011). This has led to the notion that Treg cells retain a considerable degree of functional plasticity (Zhou et al., 2009), although this notion has been questioned in subsequent studies (Rubtsov et al., 2010; Miyao et al., 2012).

In this study, we examined the cellular and molecular pathway leading to the upregulation of T-bet by Treg cells and explored the mechanisms limiting their ability to acquire proinflammatory effector functions in Th1 cell-polarizing inflammatory environments. We demonstrate that activation of STAT1 by effector T cell-derived IFN-γ induced T-bet expression in Treg cells. However, delayed induction of the IL-12 receptor component IL-12 Rβ2 prevented Treg cells from completing STAT4-dependent Th1 cell differentiation during acute type 1 inflammatory responses. Thus, abortive Th1 cell differentiation driven by activation of STAT1, but not STAT4, results in the generation of functionally specialized T-bet+ Treg cells that effectively modulate Th1 cell responses without acquiring proinflammatory effector functions.

RESULTS

STAT1-Dependent Expression of T-bet by Treg Cells

We recently identified and characterized a population of T-bet+ CXCR3+ Treg cells that express elevated amounts of *Il10* and

that are important for regulating strong Th1 cell responses (Koch et al., 2009). Moreover, the frequency of this Treg cell population is increased during Th1 cell responses, but substantially reduced in mice lacking STAT1. This suggests that like effector T cells, Treg cells upregulate T-bet during Th1 cell responses after STAT1 activation. Consistent with this, nearly all Foxp3+ Treg cells rapidly phosphorylated STAT1 after treatment with the STAT1-activating cytokines IFN-γ or IL-27 (Figure S1 available online). Additionally, in vitro stimulation in the presence of either IFN-γ or IL-27 potently induced T-bet expression in sorted CD4+Foxp3+CD62L+CXCR3− naive phenotype Treg cells (Figure 1A). To determine whether Treg cells are exposed to STAT1-activating cytokines in vivo, we constructed mixed bone marrow (BM) chimeras using congenically marked wild-type and Stat1−/− donors and compared the phenotype of Foxp3+ Treg cells derived from each donor 8 weeks later. Indeed, whereas ~25%–30% of the splenic Treg cells from the wild-type donor were T-bet+CXCR3+, this population of Treg cells was nearly absent from the Stat1−/− cells (Figure 1B).

Treg cell expression of T-bet and CXCR3 is reduced in mice lacking the STAT1-activating IFN-γ receptor (IFN-γR [Koch et al., 2009]), indicating that IFN-γ is the principle STAT1-activating cytokine driving the development of T-bet+CXCR3+ Treg cells. Moreover, CXCR3+ Treg cells from Ifngr−/− mice displayed a slight (<2-fold) decrease in T-bet protein expression relative to CXCR3+ Treg cells from wild-type animals (Figure S2), further
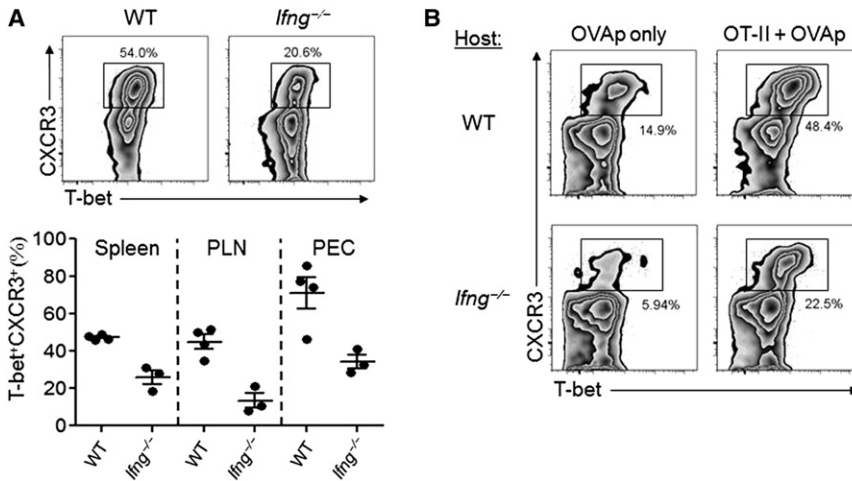
Figure 2. T Cell-Derived IFN-γ Induces T-bet Expression by Treg Cells

(A) Representative flow cytometric analysis of CXCR3 and T-bet expression by CD4⁺Foxp3⁺ cells isolated from the spleens of mixed bone marrow chimeras containing T cells derived from WT (left) or $Ifng^{-/-}$ (right) donors. The graph shows the frequency of CXCR3⁺ T-bet⁺ cells among total CD4⁺ Foxp3⁺ (Treg) cells isolated from the spleens, peripheral lymph nodes (PLN), and peritoneal exudate cells (PEC) of the indicated chimeras. Each point represents an individual mouse. Data are representative of two independent experiments. Error bars denote SEM.

(B) Representative flow cytometric analysis of CXCR3 and T-bet expression by gated CD4⁺ Foxp3⁺ cells isolated from the spleens of wild-type (top panels) and $Ifng^{-/-}$ (bottom panels) mice immunized twice with OVAp after transfer of Th1 polarized OVA-specific CD4⁺ T cells (right panels) or without transfer (left panels). Numbers depict the proportion of cells positive for CXCR3 and T-bet. Data are representative of three independent experiments.

highlighting the importance of IFN-γR signaling for optimal T-bet induction in Treg cells. However, tissue inflammation results in the upregulation of numerous inflammatory and regulatory cytokines, several of which (i.e., IL-27) can activate STAT1 and may induce T-bet and CXCR3 expression in Treg cells in the absence of IFN-γ. To determine whether these cytokines can compensate for loss of IFN-γ signaling during strong inflammatory responses in vivo, we induced experimental autoimmune encephalomyelitis (EAE), a demyelinating inflammatory disease mediated by autoreactive Th1 and Th17 cells (Dardalhon et al., 2008), in mixed BM chimeras containing a 1:1 ratio of congenically marked wild-type and $Ifngr1^{-/-}$ cells. Loss of IFN-γR-mediated signaling did not impair the accumulation of CD4⁺ T cells in the central nervous system (CNS; data not shown). However, expression of T-bet and CXCR3 were substantially reduced in $Ifngr1^{-/-}$ Treg cells in both the CNS and spleen (Figure 1C and data not shown). Additionally, consistent with the large amount of $Il10$ expressed by CXCR3⁺ Treg cells, $Ifngr1^{-/-}$ Treg cells in the CNS showed decreased IL-10 production at day 19 (Figure 1D), a late time point associated with Treg cell-mediated amelioration of disease (Korn et al., 2007; McGeachy et al., 2005). Thus, even during strong inflammatory responses associated with EAE, IFN-γ promotes the functional specialization of Treg cells by controlling the expression of molecules such as CXCR3 and IL-10 that are critical for Treg cell-mediated disease suppression (McGeachy et al., 2005; Müller et al., 2007).

T Cell Derived-IFN-γ Drives T-bet⁺ Treg Cell Differentiation

The homeostasis of Treg cells is controlled in part by IL-2 produced by CD4⁺CD44^hi^Foxp3⁻ effector T cells (Setoguchi et al., 2005), establishing a link between effector T cell activation and Treg cell abundance. To determine whether effector T cells also control Treg cell specialization via production of IFN-γ, we injected a 9:1 ratio of BM cells from $Tcrb^{-/-}Tcrd^{-/-}$ and $Ifng^{-/-}$ donors into irradiated $Tcrb^{-/-}Tcrd^{-/-}$ recipients in order to construct animals in which all nonhematopoietic cells, as well

as 90% of non-T cells, were IFN-γ sufficient, whereas all T cells were derived from $Ifng^{-/-}$ donor BM. Control chimeras were generated using a 9:1 mixture of $Tcrb^{-/-}Tcrd^{-/-}$ and wild-type BM cells. We found the that frequency of T-bet⁺ CXCR3⁺ Treg cells in the spleens, peripheral lymph nodes, and peritoneal cavities of mice lacking T cell-derived IFN-γ was substantially lower than in the wild-type control chimeras (Figure 2A).

To determine whether IFN-γ produced by effector T cells is sufficient to induce CXCR3 and T-bet expression by Treg cells, we transferred OVA-specific CD4⁺ Th1 cells from OT-II TCR transgenic mice into wild-type and $Ifng^{-/-}$ recipients. These mice were then given OVA₃₂₃₋₃₃₉ peptide (OVAp) in PBS on days 1 and 4 after transfer, and we examined the endogenous Treg cells in these animals 3 days later. Indeed, in both wild-type and $Ifng^{-/-}$ mice given OT-II Th1 cells and OVAp, we observed a 3- to 4-fold increase in the fraction of T-bet⁺CXCR3⁺ Treg cells as compared to mice receiving OVAp alone (Figure 2B). Similarly, activation of effector CD8⁺ T cells from OT-I TCR transgenic mice in vivo also increased Treg cell expression of T-bet and CXCR3 (Figure S3). Thus, through the production of IFN-γ, both CD4⁺ and CD8⁺ effector T cells can directly control the specialization of Treg cells.

IL-12R Signaling Is Dispensable for the Development of T-bet⁺ Treg Cells

In addition to the STAT1 activating cytokines, IL-12 has an essential role in Th1 cell differentiation (Trinchieri, 2003; Hsieh et al., 1993; Manetti et al., 1993), and activation of STAT4 via the IL-12 receptor is necessary for expression of genes required for Th1 cell function (Thieu et al., 2008; Wei et al., 2010). However, Treg cell expression of T-bet and CXCR3 was unaltered in IL-12Rβ2-deficient mice (Figures 3A and S2), indicating that STAT4 activation via IL-12R is dispensable for the differentiation of T-bet⁺ Treg cells.

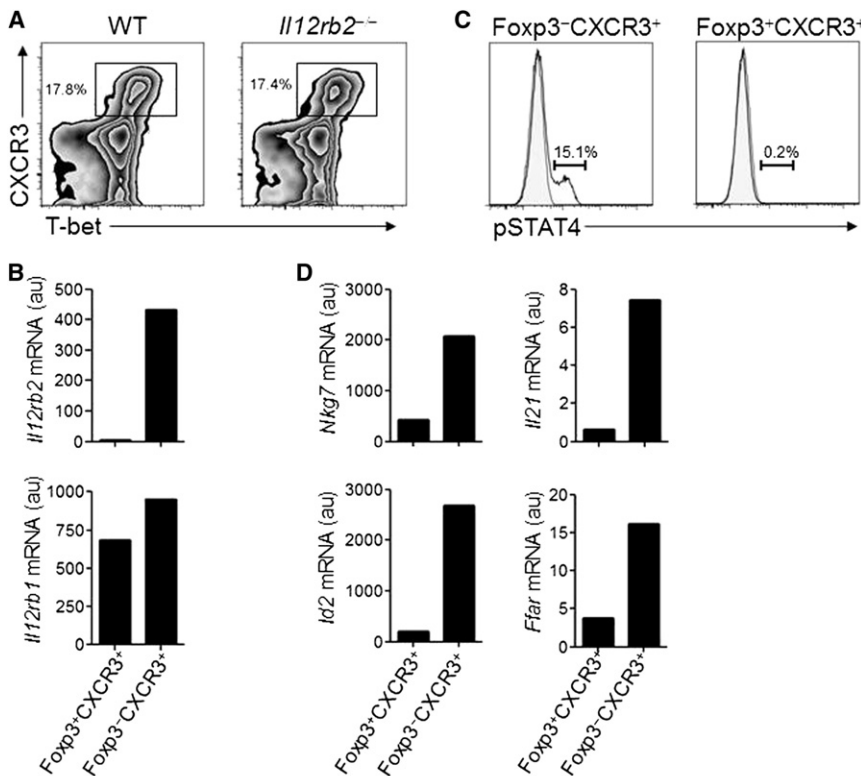Although most T cells express the IL-12 receptor subunit IL-12Rβ1, naive CD4⁺ T cells lack expression of IL-12Rβ2 and

Figure 3. Treg Cells Do Not Express *Il12rb2* or Respond to IL-12 Ex Vivo

(A) Flow cytometric analysis of CXCR3 and T-bet expression by gated CD4$^+$Foxp3$^+$ splenocytes isolated from age-matched wild-type and *Il12rb2*$^{-/-}$ mice.

(B) Representative qPCR analysis of *Il12rb2* and *Il12rb1* mRNA expression by sorted Foxp3$^+$CXCR3$^+$ or Foxp3$^-$CXCR3$^+$ CD4$^+$ splenocytes isolated from Foxp3^{gfp} mice.

(C) Representative flow cytometric analysis of STAT4 phosphorylation by sorted Foxp3$^+$CXCR3$^+$ or Foxp3$^-$CXCR3$^+$ CD4$^+$ splenocytes treated for 30 min with (open histograms) or without (shaded histograms) 25 mg/ml rmIL-12.

(D) Representative qPCR analysis of expression the STAT4 regulated genes *Nkg7*, *Id2*, *Il21*, and *Ffar* by sorted Foxp3$^+$CXCR3$^+$ or Foxp3$^-$CXCR3$^+$ CD4$^+$ splenocytes isolated from Foxp3^{gfp} mice. Data in all panels are representative of at least two independent experiments.

are not IL-12 responsive (Szabo et al., 1997). Induction of IL-12Rβ2 downstream of STAT1 and T-bet is a key checkpoint in Th1 cell development (Afkarian et al., 2002; Mullen et al., 2001). Indeed, whereas both Foxp3$^+$CXCR3$^+$ Treg cells and Foxp3$^-$CXCR3$^+$ Th1 cells expressed *Il12rb1*, expression of *Il12rb2* was ~100-fold lower in CXCR3$^+$ Treg cells (Figure 3B). Accordingly, these cells failed to phosphorylate STAT4 in response to IL-12 in vitro (Figure 3C). To determine whether Foxp3$^+$CXCR3$^+$ Treg cells showed evidence of STAT4 activation, we compared expression of several STAT4-controlled genes in sorted Foxp3$^+$CXCR3$^+$ Treg cells and Foxp3$^-$CXCR3$^+$ Th1 cells (Wei et al., 2010). Consistent with their lack of IL-12 responsiveness, expression of each of these genes was substantially lower in the CXCR3$^+$ Treg cells compared with the Foxp3$^-$ Th1 cells (Figure 3D). Thus, despite having undergone STAT1-dependent T-bet upregulation, CXCR3$^+$ Treg cells failed to acquire IL-12 responsiveness through upregulation of IL-12Rβ2 and consequently lacked the STAT4-dependent genetic signature characteristic of fully differentiated Th1 cells.

Differential Regulation of IL-12Rβ2 Expression in Foxp3$^+$ and Foxp3$^-$ Cells

The striking lack of IL-12 responsiveness in T-bet$^+$CXCR3$^+$ Treg cells suggests that regulation of IL-12Rβ2 expression is different in Foxp3$^+$ and Foxp3$^-$ cells. Alternatively, chronic TCR stimulation can inhibit IL-12Rβ2 induction (Schulz et al., 2009), and this may limit its expression in autoreactive Treg cells. To differentiate between these possibilities, we compared acquisition of IL-12 responsiveness in Foxp3$^+$ and Foxp3$^-$ CD4$^+$ T cells stimulated in vitro under identical conditions. Sorted naive phenotype CD4$^+$CD62L$^+$CXCR3$^-$Foxp3$^-$ and CD4$^+$CD62L$^+$CXCR3$^-$Foxp3$^+$ T cells were activated in the presence of IL-2 and IFN-γ for 3 days, at which point the cells were removed from the TCR stimulus and cultured in cytokines for three additional days. We measured STAT4 phosphorylation after IL-12 treatment in the Foxp3$^+$ and Foxp3$^-$ cells daily starting at day 2 of culture. IL-12 responsiveness in the Foxp3$^-$ cells was evident by day 2 of activation, peaked by day 4, and was maintained throughout the remainder of the culture (Figure 4A, top). In comparison, acquisition of IL-12 responsiveness was substantially delayed in the Foxp3$^+$ Treg cells, which did not become IL-12 responsive until day 4 of culture (Figure 4A, bottom). However, by day 6 the Foxp3$^+$ and Foxp3$^-$ cells displayed roughly equivalent STAT4 phosphorylation after IL-12 treatment. Importantly, Treg cells became IL-12 responsive on day 4 of culture even when removed from the TCR stimulus at earlier time points (day 1 or 2) postactivation (data not shown), indicating that TCR stimulation is not the primary factor inhibiting their expression of *Il12rb2*.

The delay in acquisition of IL-12 responsiveness suggests that T-bet-dependent induction of *Il12rb2* expression is impaired in Treg cells. To directly test this, we sorted Foxp3$^+$ and Foxp3$^-$ CD4$^+$ T cells from T-bet-deficient *Tbx21*$^{-/-}$Foxp3^{gfp} mice, stimulated them in vitro with plate-bound anti-CD3 and anti-CD28, and infected them with a retroviral construct encoding a T-bet-estrogen receptor (T-bet-ER) fusion protein whose activity is modulated by the estrogen analog 4-hydroxytamoxifin (4-HT). The infected cells were cultured for 3 days with varying concentrations of 4-HT, at which point we assessed their expression of *Cxcr3* and *Il12rb2*. As expected, activation of T-bet led to robust induction of *Cxcr3* mRNA expression in both Foxp3$^+$ and Foxp3$^-$ cells (Figure S4). However, induction of *Il12rb2* was observed only in Foxp3$^-$ cells (Figure 4B), demonstrating that the regulation of this gene is markedly different in Foxp3$^+$ and Foxp3$^-$ CD4$^+$ T cells.
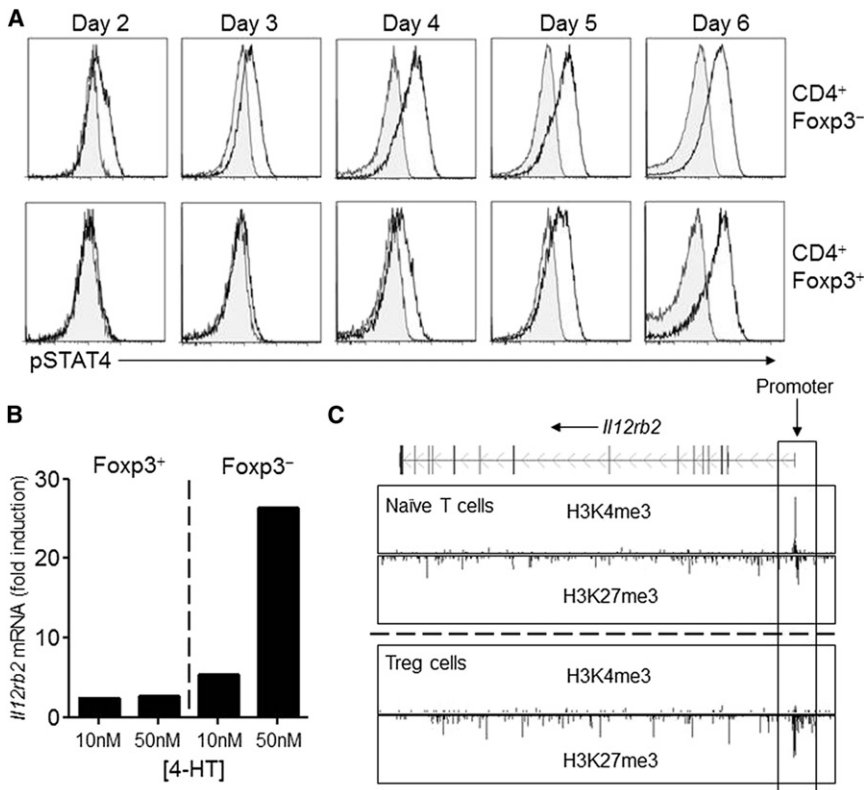
Figure 4. Differential Regulation of *Il12rb2* in Foxp3+ and Foxp3− T Cells

(A) Flow cytometric analysis of STAT4 phosphorylation by CD4+Foxp3− (top) or CD4+Foxp3+ (bottom) cells isolated at the indicated time points after activation in the presence of IFN-γ and treated for 30 min with (open histograms) or without (shaded histograms) 25 mg/ml rmIL-12. Data are representative of three independent experiments.

(B) qPCR analysis of *Il12rb2* mRNA expression by CD4+Foxp3+ and CD4+Foxp3− cells isolated from *Tbx21−/−*Foxp3*gfp* mice, infected with the T-bet-ER encoding retroviruses, and cultured for 3 days with the indicated doses of 4-HT. Data are presented as fold induction of mRNA expression over infected cells not given 4-HT and are representative of two independent experiments.

(C) Distribution of H3K4me3 and H3K27me3 histone modifications along the *Il12rb2* locus in the indicated CD4+ T cell subsets. ChIP-Seq data (Wei et al., 2009) was mapped to the Feb. 2006 (NCBI36/mm8) mouse genome assembly with the UCSC genome browser. Annotation of the *Il12rb2* locus appears above, and the peaks represent the density of sequence tags assigned to each chromosomal region. The region surrounding the proximal promoter and exon 1 of *Il12rb2* is boxed.

To explore the molecular basis for the differential regulation of *Il12rb2* in Foxp3+ and Foxp3− cells, we compared the epigenetic status of the *Il12rb2* promoter in naive CD4+ T cells and Treg cells through analysis of genome-wide histone modification data (Wei et al., 2009). In naive CD4+ T cells, the *Il12rb2* promoter is marked by both permissive H3K4me3 and repressive H3K27me3 histone modifications (Figure 4C, top). Consistent with the rapid induction of *Il12rb2* in developing Th1 cells, this bivalent state is believed to repress gene transcription while keeping the promoter "poised" for activation-induced expression (Bernstein et al., 2006). By contrast, only H3K27me3 marks are found at the *Il12rb2* promoter in Treg cells (Figure 4C, bottom). Thus, expression in Treg cells probably requires substantial epigenetic remodeling of the *Il12rb2* promoter, involving both the removal of repressive histone modifications and the addition of permissive modifications.

IL-12 Can Functionally Reprogram Treg Cells

Although IL-12 has been reported to induce IFN-γ-production by Foxp3+ Treg cells in vitro, it does so inefficiently, with only ∼5% of CD4+Foxp3+ cells producing IFN-γ after activation for 3 days in the presence of IL-12 (Wei et al., 2009). Moreover, a recent study demonstrating that Foxp3+ cells contain a small population of effector T cells suggests that at least some of the observed IFN-γ production could result from these contaminating cells (Miyao et al., 2012). Indeed, we found that IL-12 alone failed to induce IFN-γ expression by sorted CD4+CD62L+CXCR3−Foxp3+ naive phenotype Treg cells, which are less likely to contain contaminating activated effector cells (Figure S5).

The stringent regulation of IL-12Rβ2 expression in autoreactive Treg cells may serve as a tolerance mechanism that prevents them from undergoing IL-12-dependent functional differentiation and acquiring potentially dangerous proinflammatory properties such as the ability to produce IFN-γ. Alternatively, the inability of Treg cells to produce IFN-γ may result from direct inhibition of *Ifng* expression by Foxp3 or other Treg cell-specific repressive mechanisms. To determine how IL-12 receptor signaling functionally impacts Treg cells, we activated sorted CD4+Foxp3+CD62L+CXCR3− naive phenotype Treg cells in the presence of IFN-γ for 6 days to render the cells IL-12 responsive. We then cultured these cells with or without IL-12 for 3 additional days, examined their ability to produce IFN-γ, and assessed their suppressive function in vitro. Despite the reported ability of Foxp3 to directly inhibit IFN-γ expression (Ono et al., 2007), IL-12 potently induced IFN-γ production in both Foxp3+ and Foxp3− cells that had been "primed" with IFN-γ (Figure 5A). In addition, IL-12 treatment substantially reduced the ability of Treg cells to inhibit effector T cell proliferation (Figure 5B). Thus, IL-12 can alter the balance between pro- and anti-inflammatory functions in Treg cells, emphasizing the importance of regulating IL-12 responsiveness in order to maintain Treg cell-mediated immune suppression during strong type 1 inflammatory responses.

Treg Cells Undergo Abortive Th1 Cell Development during Acute Bacterial Infection

Although robust, production of IL-12 during infection with intracellular pathogens is typically very transient. Because Treg cells are poised to respond to IFN-γ, but display delayed induction of
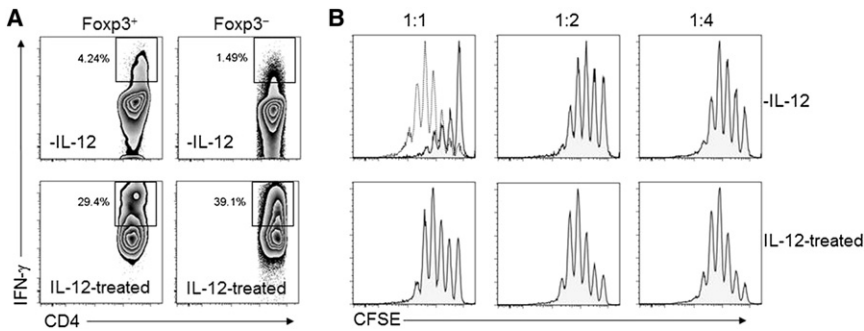
Figure 5. IL-12 Can Functionally Reprogram Treg Cells

(A) Flow cytometric analysis of CD4 and IFN-γ expression by gated CD4$^+$Foxp3$^+$ (left) or Foxp3$^-$ (right) cells after stimulation with PMA and ionomycin. Prior to analysis, sorted Foxp3$^+$ and Foxp3$^-$ naive phenotype cells were activated and cultured for 6 days in the presence of IL-2 and IFN-γ, after which they were washed and incubated for 3 days in media containing IL-2 with or without IL-12, as indicated.

(B) Flow cytometric analysis of CFSE dilution by labeled CD4$^+$CD25$^-$ effector T cells activated in the presence of the indicated cellular ratios of CD4$^+$Foxp3$^+$ Treg cells sorted from Foxp3^{gfp} mice activated in the presence of IFN-γ and/or IL-12 as described in (A). Proliferation of effector T cells in the absence of Treg cells is displayed in the dashed histogram overlay in the upper-left panel. Data are representative of three independent experiments.

IL-12Rβ2, we predicted that during acute type 1 inflammatory responses they would undergo partial Th1 cell differentiation driven by activation of STAT1 but not STAT4. To test this, we monitored cytokine responses in different splenic lymphocyte populations throughout the course of acute infection with the intracellular bacterial pathogen *L. monocytogenes*, which provokes a strong, IL-12-dependent Th1 response.

Consistent with the transient appearance of IL-12p70 in the serum of *L. monocytogenes*-infected mice (Way et al., 2007; Seki et al., 2002), the *Il12a* and *Il12b* genes were strongly upregulated in the spleen within 1 day of infection, after which their expression was rapidly extinguished (Figure 6A and data not shown). Moreover, direct ex vivo analysis of CD4$^+$Foxp3$^-$CD44^{hi} T cells (without any in vitro restimulation) showed that ∼25% of these effector T cells in the spleen were phospho(p)-STAT4$^+$ on day 1 after infection and that a substantial fraction of the pSTAT4$^+$ cells were also producing IFN-γ (Figures 6B and 6C). However, mirroring the sharp decline in IL-12 cytokine expression, the frequency of pSTAT4$^+$ IFN-γ-producing effector T cells fell dramatically on days 2 and 3 after infection. By contrast, less than 5% of splenic Treg cells were pSTAT4$^+$ directly ex vivo at any time point examined during infection (Figures 6B, middle, and 6C). Indeed, IL-12 responsiveness, as measured by the ability to phosphorylate STAT4 after in vitro IL-12 treatment, emerged in only a small fraction of Treg cells between days 3–5 postinfection, after production of IL-12 had ceased (Figure 6B, right).

Coincident with the robust IFN-γ production by effector T cells, we observed a dramatic increase in STAT1 phosphorylation in Treg cells on days 1 and 2 postinfection (Figure 7A). Accordingly, expression of T-bet and CXCR3 was dramatically elevated in Treg cells from *L. monocytogenes* infected mice. However, consistent with their inability to respond to IL-12 early in infection, very few Treg cells acquired the ability to produce IFN-γ (Figure 7B). To determine whether IL-12 receptor signaling is required for the small degree of IFN-γ production by Treg cells during *L. monocytogenes* infection, we reconstituted *Tcrb*$^{-/-}$ *Tcrd*$^{-/-}$ mice with a 1:1 mixture of splenocytes from wild-type and *Il12rb2*$^{-/-}$ donors and infected them with *L. monocytogenes* 10 days later. Indeed, whereas a small fraction of wild-type Treg cells acquired the ability to produce IFN-γ after infection, this was blunted in the *Il12rb2*$^{-/-}$ Treg cells (Figure 7C). Taken together, these data confirm our in vitro findings regarding the ability of Treg cells to respond to IFN-γ and

IL-12 in an in vivo setting of acute inflammation and indicate that the relative inability of Treg cells to respond to IL-12 limits their functional reprogramming into IFN-γ-producing cells.

DISCUSSION

In this study, we examined how Treg cells respond to inflammatory cytokines produced during strong Th1 cell responses in vitro and in vivo. We define a mechanism whereby impaired upregulation of IL-12Rβ2 causes Treg cells to undergo abortive Th1 cell differentiation driven by activation of STAT1 but not STAT4. This results in the phenotypic and functional specialization of Treg cells via induction of T-bet and prevents them from IL-12-dependent functional reprogramming leading to their acquisition of potentially harmful proinflammatory effector functions.

Increasing evidence indicates that Treg cells both sense and respond to changes in the immune environment and that this is necessary for proper immunoregulation. For instance, IL-10-dependent activation of STAT3 is important for the ability of Treg cells to effectively restrain Th17 cell-mediated inflammatory responses (Chaudhry et al., 2011). Additionally, Treg cell abundance is tightly linked to the size of the dendritic cell compartment, given that depletion or expansion of dendritic cells in vivo serves to reduce or augment Treg cell numbers, respectively (Darrasse-Jèze et al., 2009). Treg cells are also sensitive to changes in the amount of IL-2 produced by CD4$^+$Foxp3$^-$CD44^{hi} T cells, and this is thought to be a mechanism by which Treg cell abundance can be rapidly altered as the number of effector T cells fluctuates (Setoguchi et al., 2005). Here, we show that Treg cells upregulated expression of the key transcription factor T-bet downstream of STAT1 activation in direct response to IFN-γ produced by CD4$^+$ and CD8$^+$ effector T cells. Thus, in addition to controlling Treg cell abundance, cytokines produced by effector T cells can also drive the phenotypic and functional specialization of Treg cells. However, although we demonstrated that T cells are a key source of the IFN-γ that acted on Treg cells in vivo, other IFN-γ-producing populations, such as NK and NKT cells, may also influence the phenotypic and functional specialization of Treg cells when they are potently activated. Additionally, other STAT1-activating cytokines such as IL-27 or type 1 interferons may also help drive T-bet expression by Treg cells when produced in abundance during parasitic or viral infections.
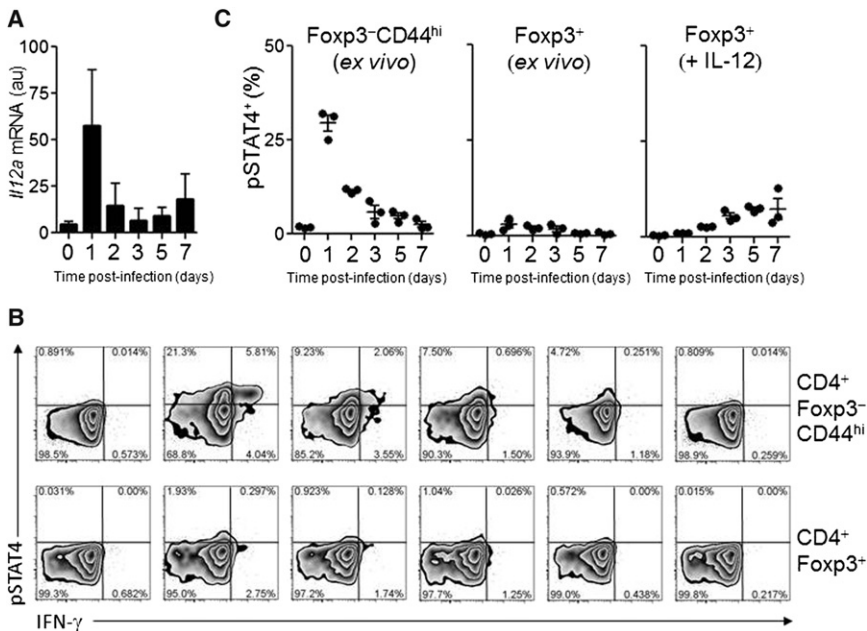
Figure 6. Treg Cells Do Not Phosphorylate STAT4 during Acute *L. monocytogenes* Infection

(A) qPCR analysis of *Il12a* expression in total spleen of mice at the indicated times during acute *L. monocytogenes* infection. The mean and SEM of measurements from three different animals at each time point are presented.

(B) Representative flow cytometric analysis of pSTAT4 and IFN-γ expression by gated effector (top) and regulatory (bottom) splenic CD4+ T cells at the indicated times during *L. monocytogenes* infection. Analyses were performed directly ex vivo (without any in vitro restimulation) by isolating cells directly into Fix and Perm buffer.

(C) Graphs depicting the percentage of pSTAT4+ cells among gated CD4+Foxp3−CD44hi effector T cells (left) or CD4+Foxp3+ Treg cells (middle and right) from the spleens of individual mice analyzed either directly ex vivo (left and middle) or after IL-12 stimulation in vitro (right) at the indicated times during acute *L. monocytogenes* infection. Error bars in all panels denote SEM.

Although STAT1 activation initiates Th1 cell development, IL-12-dependent STAT4 activation is required for full Th1 cell differentiation. Indeed, STAT4 and T-bet have distinct, yet overlapping functions in establishing the Th1 cell-specific gene expression program. For instance, STAT4 induces the high level of T-bet expression necessary for the terminal differentiation of Th1 effector cells, and activated STAT4 synergizes with T-bet to drive expression of IFN-γ (Thieu et al., 2008; Mullen et al., 2001). Similarly, IL-12 controls the level of T-bet expression in CD8+ effector T cells and dictates whether CD8+ T cells become "short-lived effector cells" or long-lived "memory-precursor effector cells" (Joshi et al., 2007). Consistent with their lack of ex vivo IL-12 responsiveness, T-bet+CXCR3+ Treg cells express 5- to 10-fold less T-bet than fully differentiated Th1 cells and do not produce IFN-γ (Koch et al., 2009). Moreover, we show that they lack expression of other STAT4 regulated genes, and thus we conclude that these Treg cells have undergone a form of incomplete Th1 cell differentiation driven by activation of STAT1 but not STAT4.

Despite being a key checkpoint in Th1 cell development, the molecular control of *Il12Rb2* expression is still poorly understood. Although its expression after STAT1 activation is T-bet dependent, it is unclear whether T-bet acts directly on the *Il12rb2* locus. Notably, a number of IFN-γ activation site (GAS) elements are found upstream of the *Il12rb2* coding sequence, indicating that expression of this gene may be directly regulated by STAT1 (Letimier et al., 2007). Moreover, IL-12-dependent STAT4 activation can feed-forward and enhance *Il12rb2* expression (Becskei and Grusby, 2007). In addition, TCR stimulation alone results in recruitment of the Brg1-containing BAF chromatin remodeling complex to the *Il12rb2* locus, which promotes histone hyperacetylation and potentiates STAT-induced *Il12rb2* expression (Letimier et al., 2007). However, the presence of abundant H3K27me3 histone modifications at the *Il12rb2* promoter probably acts to temporally delay IL-12Rβ2 induction in Treg cells until H3K27 demethylases such as Jumonji 3 can

be recruited and remove this chromatin mark (De Santa et al., 2007). Although Foxp3 is a potent transcriptional repressor, it is not found at or near the *Il12rb2* locus (Zheng et al., 2007; Marson et al., 2007), and deletion of Foxp3 in established Treg cells does not result in *Il12rb2* upregulation (Williams and Rudensky, 2007). In addition, IL-12 can potently induce IFN-γ production from TGF-β-induced Treg cells in vitro (Feng et al., 2011), which are believed to retain a greater degree of phenotypic and functional plasticity than the in vivo-derived natural Treg cells we examined. Thus, repression of *Il12rb2* expression appears to be part of the Foxp3-independent program of Treg cell differentiation, and further studies are needed to establish the precise molecular mechanisms that regulate *Il12rb2* expression in effector and regulatory T cells.

During acute inflammation, cells of the innate immune system transiently produce IL-12 after pathogen recognition, and we showed that delayed induction of IL-12Rβ2 prevented Treg cells from sensing and responding to IL-12 during acute *L. monocytogenes* infection. However, acquisition of IL-12 responsiveness after prolonged STAT1 activation predicts that Treg cells would be more susceptible to functional reprogramming during sustained or dysregulated inflammatory responses. Accordingly, we observed that IL-12 potentiated production of IFN-γ and decreased the suppressive function of IFN-γ pretreated Treg cells. Moreover, unlike the transient IL-12 production we observed during *L. monocytogenes* infection, sustained IL-12 production during the lethal intestinal inflammation caused by oral infection with *Toxoplasma* is associated with functional reprogramming of Treg cells into IFN-γ-producing Th1-like cells (Oldenhove et al., 2009), and IFN-γ-producing Treg cells are also found in the CNS during chronic corona-virus-induced encephalomyelitis (Zhao et al., 2011). Finally, consistent with our results indicating that sustained STAT1 activation renders Treg cells susceptible to IL-12-mediated functional reprogramming, IFN-γ production is observed in Treg cells displaying elevated STAT1 activation due to loss of the microRNA miR-146a
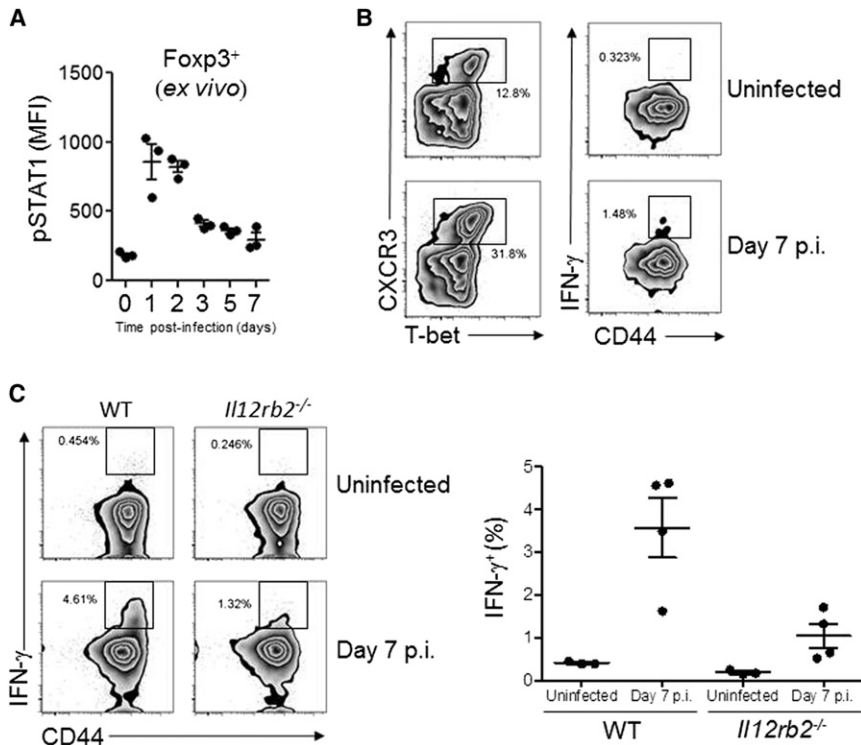
Figure 7. Treg Cells Undergo Abortive Th1 Differentiation during Acute *L. monocytogenes* Infection

(A) A graph depicting the mean fluorescence intensity of pSTAT1 in gated CD4+Foxp3+ Treg cells from the spleens of individual mice analyzed directly ex vivo at the indicated times during acute *L. monocytogenes* infection.

(B) Representative flow cytometric analysis of T-bet and CXCR3 (left) or CD44 and IFN-γ (right) expression by gated CD4+Foxp3+ splenic Treg cells from either uninfected mice or at day 7 after *L. monocytogenes* infection.

(C) Representative flow cytometric analysis (left) of CD44 and IFN-γ expression by gated wild-type and *Il12rb2*−/− Treg cells from *TCRb*−/− *TCRd*−/− mice reconstituted with a 1:1 mixture of wild-type and *Il12rb2*−/− splenocytes 10 days prior to infection with *L. monocytogenes*. The graph (right) depicts the frequency of IFN-γ+ cells among total WT and *Il12rb2*−/−-derived CD4+Foxp3+ splenic Treg cells in each animal examined. Error bars in all panels denote SEM.

(Lu et al., 2010). Thus, although Foxp3 has been reported to inhibit expression of IFN-γ by Treg cells (Ono et al., 2007), IL-12 appears to be sufficient to overcome this blockade, and impaired IL-12Rβ2 induction helps limit the functional reprogramming of Treg cells during Th1 cell inflammation. This also provides a molecular mechanism that helps reconcile conflicting data regarding the susceptibility of Treg cells to functional reprogramming in resolving versus chronic inflammatory conditions (Oldenhove et al., 2009; Rubtsov et al., 2010; Zhao et al., 2011).

By demonstrating that cytokines produced by activated effector T cells can influence the specialization of Treg cells, our data help elucidate how immune homeostasis is maintained after the initiation of robust T cell responses. We also identify the regulated expression of IL-12Rβ2 as a key checkpoint that functions to prevent Treg cells from obtaining proinflammatory effector functions and that may contribute to the "functional plasticity" of Treg cells observed during uncontrolled inflammation. Further examining how Treg cells respond to specific signals in the immune environment will provide new insights into how Treg cell activity is controlled during different types of immune responses, and is vital for developing and optimizing cellular and molecular therapies aimed at manipulating Treg cell activity in the contexts of autoimmunity, cancer, transplantation, and chronic infection.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6J, B6.129P2-*Tcrb*tm1Mom *Tcrd*tm1Mom/J (*Tcrb*−/−*Tcrd*−/−), B6.129S7-*Ifngr1*tm1Agt/J (*Ifngr1*−/−), B6.129S7-*Ifng*tm1Ts/J (*Ifng*−/−), B6.129S1-*Il12rb2*tm1Jm/J (*Il12rb2*−/−), B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-II), and B6-Tg(TcraTcrb)1100Mjb/J (OT-I) mice were purchased from the Jackson

Laboratory. B6.SJL-*Ptprc*[a]/BoyAiTac (CD45.1) mice were purchased from Taconic Farms. Foxp3*gfp* mice were provided by A. Rudensky (Memorial Sloan-Kettering Cancer Center). Tissues from *Stat1*−/− mice were provided by M. Krishna-Kaja (University of Washington). All animals were housed and bred at the Benaroya Research Institute Seattle, WA and experiments were performed in accordance within the guidelines of the Animal Care and Use Committees of the Benaroya Research Institute.

Cell Isolations

Lymphocytes were isolated from the spleens and lymph nodes (LNs) by tissue disruption with glass slides followed by filtration through a 100 μM filter. CNS lymphocytes were recovered from perfused animals by grinding brain and spinal cord tissue through a 100 μm filter with the plunger of a 5 mL syringe. Cells were pelleted by centrifugation at 3200 rpm for 15 min and resuspended in 6 mL of 37% Percoll (GE Healthcare). Cells were centrifuged again at 3200 rpm for 15 min (brake off) and lymphocytes were recovered after evacuation of the fatty supernatant.

Bone Marrow Chimeras

BM cells were depleted of CD4+ cells with anti-CD4 microbeads (Miltenyi Biotech) and injected retro-orbitally into lethally irradiated (1000 Rad) *Tcrb*−/− *Tcrd*−/− mice. Chimeras received 6 to 10 × 10^6 cells of a 1:1 mixture of wild-type (CD45.1+) and knockout (CD45.2+) BM, or 9 × 10^6 TCRβδ-KO BM cells mixed with 1 × 10^6 wild-type or *Ifng*−/− BM cells as indicated.

Flow Cytometry and Cell Sorting

Data were acquired on LSRII flow cytometers (BD Biosciences) and analyzed with FlowJo software (Treestar). For cell sorting experiments, CD4+ cells were enriched with Dynal CD4 T cell negative isolation kit (Invitrogen), stained for desired cell surface markers, and isolated with a FACS Vantage or FACS Aria (BD Biosciences). For intracellular staining of IFN-γ, Foxp3, and/or T-bet, lymphocytes were surface stained, then permeabilized with the eBioscience FixPerm buffer. For intracellular cytokine staining after restimulation, cells were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μg/mL ionomycin in the presence of 10 μg/mL monensin for 4 hr at 37°C, 5%CO$_2$ prior to staining.

Phospho-STAT Staining

Cells were stimulated for 30 min in the presence or absence of 25 ng/mL recombinant mouse (rm)IL-12 (eBioscience), IFN-γ (Biolegend) or IL-27

(eBioscience). Cells were then and fixed for 20 min in BD Fix and Perm buffer at room temperature (BD Biosciences), washed with BD Perm Wash buffer, and fixed in 90% ice-cold methanol for 30min on ice. Cells were washed two times with BD Perm/Wash and stained with antibodies against cell surface and intracellular markers, including pSTAT4 (Y693; BD Biosciences) and pSTAT1 (Y701; BD Biosciences), in BD Perm Wash for 45 min at room temperature. For direct ex vivo pSTAT and cytokine staining, ~1/5 of each spleen was ground between glass slides in BD Fix and Perm buffer, left for 20 min at room temperature, washed, fixed in 90% methanol, and stained as described above.

In Vitro T Cell Culture

$CD4^+CD62L^+CXCR3^-$ Foxp3$^+$ and Foxp3$^-$ cells were FACS sorted from Foxp3^{gfp} mice. A total of 1.5 to 2×10^6 cells were stimulated with plate-bound anti-CD3 (2C11; UCSF hybridoma core) and anti-CD28 (37.51; UCSF hybridoma core) in medium containing 500 units/mL rmIL-2. rmIFN-γ, rmIL-27, and rmIL-12p70 were added to some cultures at 25 ng/mL as indicated. All cytokines used were from eBioscience or Biolegend. Cells were cultured for 72 hr at 37°C, at which point they were analyzed or removed from the CD3 and CD28 stimulus and cultured in fresh medium containing cytokines for 48–72 more hours at 37°C. In some assays, after 144 hr of culture, cells were washed and cultured for an additional 3 days with 500 units/mL IL-2 in the presence or absence of 25 ng/mL IL-12.

Adoptive Transfer and OVA Peptide Administration

A total of 5×10^6 polarized OT-II$^+$CD4$^+$ or OT-I$^+$CD8$^+$ cells were adoptively transferred into wild-type or Ifng$^{-/-}$ mice via retro-orbital injection on day 0 and recipients were injected intraperitoneally with 200 μg OVA peptide$_{323-339}$ (for OT-II adoptive transfers) or OVA peptide$_{257-264}$ (for OT-I adoptive transfers) in PBS on days 1 and 4. All mice were sacrificed for analysis on day 7.

In Vitro Suppression Assays

A total of 7.5×10^5 CFSE-labeled CD4$^+$CD25$^-$ T cells were incubated with 7.5×10^5 irradiated (2,500 Rad) Tcrb$^{-/-}$Tcrd$^{-/-}$ splenocytes (CD45.2$^+$), with or without addition of Treg cells at the indicated ratios, and stimulated with 0.15 μg/ml soluble anti-CD3 (2C11) for 72 hr.

L. Monocytogenes Infection

The recombinant LM-OVA strain was provided by M. Bevan (University of Washington). A total of 10^4 cfu LM-OVA were injected intravenously into mice after growth to log phase (OD$_{600}$ = 0.1–0.4) in brain heart infusion media (BD Biosciences) at 37°C.

Induction of EAE

Mice were immunized subcutaneously (s.c.) in the flank with 200 μg myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide in CFA. A total of 200 ng of pertussis toxin in PBS was injected intravenously on day 0 and day 2 after immunization.

Retroviral Transduction Assays

FACS-purified CD4$^+$Foxp3$^+$ and CD4$^+$Foxp3$^-$ cells from Tbx21$^{-/-}$ Foxp3^{gfp} mice were plated in a 24-well flat-bottom plate precoated with 1 μg/ml anti-CD3 (2C11) and 1 μg/ml anti-CD28 (37.51) in medium with 500 units/ml rmIL-2. After 72 hr, cells were resuspended in retroviral supernatant containing 8 μg/ml polybrene (Sigma-Aldrich) and were centrifuged at 2500 rpm for 2 hr at 37°C, then cultured at 37°C in complete RPMI containing 500 U/ml rmIL-2 and 4-hydroxytamoxifen (Sigma-Aldrich). Infected cells were isolated 72 hr later with anti-human CD2 microbeads (Miltenyi Biotech) and expression of Cxcr3 and Il12rb2 was assessed by quantitative PCR.

Quantitative PCR

RNA extraction was performed with QIAGEN RNeasy columns (QIAGEN) and cDNA was generated with an Omniscript RT Kit (QIAGEN) according to the manufacturer's instructions. Presynthesized Taqman Gene Expression Assays (Applied Biosystems) were used for amplifying Il12Rb2 (Mm00434200_m1) or Cxcr3 (Mm99999054_s1) mRNA transcripts in analysis of T-bet-ER transduced cells. Target gene values are expressed relative to Actb, which was analyzed with the sense primer 5′-TGACAGGATGCAGAAGG AGAT-3′, anti-sense primer 5′-GCGCTCAGGAGGAGCAAT-3′, and probe 5′-FAM-ACTGCTCTGGCTCCTAGCACCAT-TAMRA-3′. In all other assays, expression of Il12rb1, Il12rb2, Il12a, Nkg7, Il21, Id2, and Ffar were assessed with Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) and normalized to expression of Gapdh.

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