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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 naïve 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 naïve 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-12Rβ2 (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 non-lymphoid 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
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 IL-10 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+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 or 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−/− mice (Figure 1B).

Abortive Th1 Cell Differentiation of Treg Cells

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 Ifngr1−/− mice displayed a slight (~2-fold) decrease in T-bet protein expression relative to CXCR3+ Treg cells from wild-type animals (Figure S2), further...
Abortive Th1 Cell Differentiation of Treg Cells

The homeostasis of Treg cells is controlled in part by IL-2 produced by CD4^+CD44^+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^−/−Tcdr^−/− and Ifng^−/− donors into irradiated Tcrb^−/−Tcdr^−/− 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^−/−Tcdr^−/− and wild-type BM cells. We found that the frequency of T-bet^+ CXCR3^+ Treg cells in 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 three independent experiments.

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-12Rb2-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-12Rb1, naive CD4^+ cells lack expression of IL-12Rb2 and
Abortive Th1 Cell Differentiation of Treg 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 naïve phenotypes 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-/- Foxp3gfp 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-hydroxytamoxifen (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 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 Foxp3gfp 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 Flar by sorted Foxp3+CXCR3+ or Foxp3- CXCR3+ CD4+ splenocytes isolated from Foxp3gfp mice. Data in all panels are representative of at least two independent experiments.
Abortive Th1 Cell Differentiation of Treg Cells

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.

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 inactivity 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...
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−CD44hi 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 Tcrd−/− Tcrd−/− mice with a 1:1 mixture of splenocytes from wild-type and Ii12rb2−/− 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 Ii12rb2−/− 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-Jéze et al., 2009). Treg cells are also sensitive to changes in the amount of IL-2 produced by CD4+Foxp3−CD44hi 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 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.
Abortive Th1 Cell Differentiation of Treg Cells

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 (Lettieri 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 (Lettieri 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-pre-treated 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...
Tg(TcraTcrb)1100Mjb/J (OT-I) mice were purchased from the Jackson Laboratory. B6.129S7-Ifngr1tm1Agt/J (Ifngr1), B6.129P2-Il12rb2tm1Mom Tcrdtm1Mom (Il12rb2), B6.129S7-Tg(Ifngtm1Ts/J (Ifng), B6.SJL-Ptprca<sup>Aria</sup>/BoyAltac (CD45.1) mice were purchased from Taconic Farms. Foxp3<sup>+</sup> mice were provided by A. Rudensky (Memorial Sloan-Kettering Cancer Center). Tissues from Start<sup>−/−</sup> 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.

**Bone Marrow Chimeras**

BM cells were deplicted of CD4<sup>+</sup> cells with anti-CD4 microbeads (Miltenyi Biotech) and injected retro-orbitally into lethally irradiated (1000 Rad) Tcrb<sup>−/−</sup>/Tcrd<sup>−/−</sup> mice. Chimeras received 6 to 10 x 10<sup>5</sup> cells of a 1:1 mixture of wild-type (CD45.1<sup>+</sup>) and knockout (CD45.2<sup>+</sup>) BM cells mixed with 1 x 10<sup>6</sup> wild-type or Ifng<sup>−/−</sup> 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<sup>+</sup> 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%CO2 prior to staining.

**Phospho-STAT Staining**

Cells were stimulated for 30 min in the presence or absence of 25 ng/mL recombinant mouse (m)IL-12 (eBioscience), IFN-γ (Biolegend) or IL-27 (Peprotech) for 15 min at 37°C, 5%CO2 prior to staining.

**EXPERIMENTAL PROCEDURES**

**Mice**

CS7Bl/6J, B6.129P2-Tcrb<sup>+</sup>Mom Tcrd<sup>+</sup>Mom/J (Tcrb<sup>−/−</sup>/Tcrd<sup>−/−</sup>), B6.129S7-Ifngr1<sup>−/−</sup>/Tg(Ifng<sup>−/−</sup>) J (Ifng<sup>−/−</sup>), B6.129S7-Il12rb2<sup>−/−</sup>/Tcrd<sup>−/−</sup>/Tg(Ifng<sup>−/−</sup>) J (Il12rb2<sup>−/−</sup>), B6.Cg-Tg(TcrbTcrd)425Cbn/J (OT-1), and B6-Tg(TcrbTcrb)1100Mjb/J (OT-1) mice were purchased from the Jackson Laboratory. B6.SJL-Ptprca<sup>Aria</sup>/BoyAltac (CD45.1) mice were purchased from Taconic Farms. Foxp3<sup>+</sup> mice were provided by A. Rudensky (Memorial Sloan-Kettering Cancer Center). Tissues from Start<sup>−/−</sup> 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. 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.
(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 (Y963; 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+ 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 (5.1; 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 at 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.

Adaptive 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 Il12rb2-/- mice after growth to log phase (OD600 = 0.1–0.4) in brain heart infusion media and were washed and cultured in complete RPMI containing 500 U/ml rmIL-2 and cytokine stimulus and cultured in fresh medium containing cytokines above.

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-/-Tcdr-/- 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
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+ 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 Ifi12b2 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 (Mm9999905_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’-TGACAGGATGCACAAAGGAG-3’, anti-sense primer 5’-GCCCTCAGGGAGGCAAT-3’, and probe 5’-FAM-AGTCGCTTGCTGCTAGCAGCAT-TAMRA-3’. In all other assays, expression of Il12rb1, Il12rb2, Il12a, Nkg7, Il21, Il2d, and Ifar were assessed with Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) and normalized to expression of Gapdh.

SUPPLEMENTAL INFORMATION
Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.05.031.

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