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T-bet controls regulatory T cell homeostasis and function during type-1 inflammation

Meghan A. Koch[1,2], **Glady's Tucker-Heard**[3], **Nikole R. Perdue**[1], **Justin R. Killebrew**[1,2], **Kevin B. Urdahl**[2,3], and **Daniel J. Campbell**[1,2]

[1] Benaroya Research Institute, Seattle, WA 98101, USA

[2] Department of Immunology, University of Washington School of Medicine, Seattle, WA 98195

[3] Department of Peditrics, University of Washington School of Medicine, Seattle, WA 98195

Abstract

Several subsets of Foxp3$^+$ regulatory T (T_{reg}) cells work in concert to maintain immune homeostasis. However, the molecular bases underlying the phenotypic and functional diversity of T_{reg} cells remain obscure. We show that in response to interferon-γ, Foxp3$^+$ T_{reg} cells upregulated the T helper 1 (T_H1)-specifying transcription factor T-bet. T-bet promoted expression of the chemokine receptor CXCR3 on T_{reg} cells, and T-bet$^+$ T_{reg} cells accumulated at sites of T_H1-mediated inflammation. Furthermore, T-bet expression was required for the homeostasis and function of T_{reg} cells during type-1 inflammation. Thus, within a subset of CD4$^+$ T cells, the activities of Foxp3 and T-bet are overlaid, resulting in T_{reg} cells with unique homeostatic and migratory properties optimized for suppression of T_H1 responses *in vivo*.

INTRODUCTION

CD4$^+$ T cells adopt one of several functional fates defined by their cytokine production and/or suppressive activity. This functional specialization is due to differential expression of transcription factors that initiate distinct programs of gene expression controlling cytokine production and migration1. For instance, interferon (IFN)-γ (http://www.signaling-gateway.org/molecule/query?afcsid=A001238)-producing CD4$^+$ T helper type-1 (T_H1) cells are required for the elimination or control of many intracellular pathogens2, and the transcription factor T-bet is thought to be both necessary and sufficient for T_H1 cell differentiation3. Accordingly, T-bet directly activates transcription of a set of genes important for T_H1 cell function, including those encoding IFN-γ and the chemokine receptor CXCR3 (http://www.signaling-gateway.org/molecule/query?afcsid=A000635)3, 4. Conversely, the transcription factor Foxp3 (http://www.signaling-gateway.org/molecule/query?afcsid=A002750) is required for the development of CD4$^+$ regulatory T (T_{reg}) cells5.

Correspondence should be addressed to D.J.C. (campbell@benaroyaresearch.org).

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Within cells, Foxp3 coordinates a transcriptional program resulting in the expression of genes important for their regulatory activity, while preventing production of pro-inflammatory cytokines6, 7. The importance of Foxp3$^+$ T_{reg} cells in maintaining immune tolerance is highlighted by the rapid and fatal autoimmunity that develops in Foxp3-deficient mice and humans8-11.

Although beneficial during infection, strong T_H1 responses must be counterbalanced to prevent unwanted tissue destruction and immunopathology. In addition, many autoimmune diseases are thought to result from dysregulated T_H1 responses to self-antigens. The mechanisms used to dampen T_H1 immune responses *in vivo* are complex and involve multiple cell types. For example, 'self-regulation' via interleukin (IL)-10 produced by highly activated T_H1 cells is required for limiting immunopathology during several persistent parasitic infections12, 13. However, Foxp3$^+$ T_{reg} cells are also essential for the proper regulation of T_H1 responses *in vivo*, and can modulate T_H1-mediated delayed-type hypersensitivity responses14, inhibit T_H1 responses during autoimmunity15, and prevent pathogen clearance during persistent intracellular infection16, 17. In addition, loss of T_{reg} cells results in uncontrolled T_H1 responses, further demonstrating the important and non-redundant function of T_{reg} cells in dampening type-1 inflammation18, 19.

T_{reg} cells can be divided into several subsets based on their differential expression of surface homing receptors, and can be readily identified in both lymphoid and non-lymphoid tissues20, 21. Accordingly, blocking their migration to specific anatomical locales results in development of immunopathology in the contexts of autoimmunity, infection and transplantation21-25. T_{reg} cells can also be partitioned into distinct subsets based on their use of a variety of immunosuppressive mechanisms5. For example, IL-10 produced by T_{reg} cells is required for the control of inflammatory responses at mucosal surfaces, but is dispensable for suppression of deleterious immune responses in other tissues26. Although these findings highlight the phenotypic and functional diversity of T_{reg} cells, the contributions made by the various T_{reg} cell subsets to the control of different types of immune responses remain poorly understood. Additionally, little is known about the external cues and intracellular factors responsible for the differentiation and function of distinct T_{reg} cell populations.

Our data demonstrate that the T_H1-specifying transcription factor T-bet controls T_{reg} cell migration, homeostasis and function during type-1 inflammatory responses, and that the IFN-γ-receptor (IFN-γR) plays a key role in the induction of T-bet expression by T_{reg} cells. These results provide new insights into the molecular bases for the phenotypic and functional diversity of T_{reg} cells, and demonstrate that like conventional CD4$^+$ effector T cells, Foxp3$^+$ T_{reg} cells undergo peripheral differentiation in response to the cytokine environment by altering their homeostatic and migratory properties, thereby enabling them to effectively function during strong T_H1 responses.

RESULTS

A subset of CD4$^+$Foxp3$^+$ T cells expresses T-bet

In addition to being expressed by T_H1 cells, the chemokine receptor CXCR3 is also found on a subset of Foxp3$^+$CD4$^+$ cells (Fig. 1a)20. The CXCR3 ligands CXCL9, 10 and 11 are all induced by IFN-γ and enable the efficient recruitment of CXCR3$^+$ cells to sites of type-1 inflammation27-29. Based on this migratory potential, we hypothesized that CXCR3$^+$ T_{reg} cells may be molecularly specialized to effectively inhibit T_H1 responses. CXCR3 expression in T_H1 cells generated *in vitro* is T-bet dependent, and T-bet directly binds to and transactivates the *Cxcr3* promoter in transfected cells4, 30. Therefore, to determine if expression of CXCR3 in T_{reg} cells is also T-bet-dependent, we examined T-bet-deficient (*Tbx21*$^{-/-}$) mice. There was a near complete absence of CXCR3$^+$CD4$^+$Foxp3$^+$ cells in these animals, whereas expression of other homing receptors, including P- selectin ligands, CD103 and CCR6, was similar in wild-type and T-bet-deficient T_{reg} cells (Fig. 1b and Supplementary Fig. 1 online). Accordingly, T-bet-deficient T_{reg} cells failed to migrate toward the CXCR3 ligand CXCL10 *in vitro*, whereas their responses to the CCR7 ligand CCL21 and the CXCR4 ligand CXCL12 were unaltered (Fig.1c and data not shown).

The lack of CXCR3$^+$ T_{reg} cells in T-bet-deficient mice suggests that T-bet directly induces CXCR3 expression in these cells. Alternatively, T_{reg} cells may upregulate CXCR3 in a T-bet-independent manner during T_H1 responses, and the absence of CXCR3$^+$ T_{reg} cells in T-bet-deficient mice may be a consequence of the impaired T_H1 cell development in these animals. To distinguish between these possibilities, we generated mixed bone marrow (BM) chimeras by transplanting a 1:1 ratio of BM cells from wild-type (CD45.1$^+$) and T-bet-deficient (CD45.2$^+$) donors into irradiated *Rag1*$^{-/-}$ recipients. In the resulting mixed chimeric animals, CXCR3$^+$ T_{reg} cells were derived almost entirely from the CD45.1$^+$ wild-type donor, demonstrating that T-bet is required in a cell-intrinsic manner for T_{reg} cell expression of CXCR3 (Fig. 1d). Accordingly, T-bet mRNA was enriched in CXCR3$^+$ T_{reg} cells, and T-bet protein was detected exclusively within the CXCR3$^+$ T_{reg} cell subset by flow cytometry (Fig. 1e and Supplementary Fig. 2 online).

T_{reg} cells upregulate T-bet during type-1 inflammation

The frequency of CXCR3$^+$ T_{reg} cells was significantly lower in the thymus than in the spleen, and the percentage of splenic T_{reg} cells expressing CXCR3 increased with age (Supplementary Fig. 3 online). This suggests that T_{reg} cells upregulate T-bet in response to specific peripheral cues, likely associated with induction of type-1 inflammation. To determine if T-bet$^+$ T_{reg} cells differentiate and/or expand during strong T_H1-promoting conditions *in vivo*, we injected wild-type mice with an agonistic CD40-specific antibody. This treatment induces strong T_H1 responses *in vivo*, and protects Balb/c mice infected with *Leishmania major* by converting their characteristic T_H2 response to a protective T_H1 response31. Indeed, both the frequency and absolute number of T-bet$^+$CXCR3$^+$ T_{reg} cells in spleen and lymph nodes were markedly increased in anti-CD40-treated mice compared with control mice given rat IgG (Fig. 2a and data not shown). The increase in T-bet$^+$ T_{reg} cells in anti-CD40 treated animals was not simply a byproduct of enhanced proliferation, as robust proliferation induced by IL-2 immune complexes (IL-2C) did not increase the proportion of

CXCR3$^+$ T_{reg} cells (Supplementary Fig. 4 online)32. To determine if T-bet$^+$ T_{reg} cells are derived from T-bet$^-$Foxp3$^+$ precursors, we sorted CD4$^+$Foxp3$^+$CXCR3$^-$CD62L$^+$ cells from the spleen and peripheral lymph nodes of reporter mice with a GFP cassette knocked in to the *Foxp3* locus (Foxp3^{gfp} mice) and then transferred these cells into mice lacking endogenous T cells (TCRβδ-KO mice) (Fig. 2b). Unlike rat IgG treatment, anti-CD40 treatment resulted in upregulation of T-bet and CXCR3 expression in the majority of transferred T_{reg} cells (Fig. 2c). Notably, anti-CD40 treatment did not induce Foxp3 expression in transferred CD4$^+$Foxp3$^-$CXCR3$^-$CD62L$^+$ T cells. Thus, T_H1-inducing conditions promote *de novo* induction of T-bet expression within Foxp3$^+$T-bet$^-$ T_{reg} cells, and in this experimental system T-bet$^+$ T_{reg} cells were not peripherally induced from naïve CD4$^+$Foxp3$^-$ cells.

T-bet is first expressed in developing T_H1 cells following T cell receptor ligation coupled with signaling through the IFN-γ receptor (IFN-γR) via its associated signaling adaptor STAT133. Additionally, stable T-bet expression and full commitment to the T_H1 lineage depends on IL-12 signaling through its cognate receptor34. To determine if T-bet induction in T_{reg} cells occurs through a similar mechanism, we analyzed CD4$^+$Foxp3$^+$ cells isolated from mice lacking IFN-γR1, STAT1 and IL-12p40. Interestingly, there was a substantial reduction in the frequency of CXCR3$^+$T-bet$^+$ T_{reg} cells in mice lacking either STAT1 or IFN-γR1 (Fig 3a). In contrast, relative to age-matched controls, there was no decrease in the fraction of CXCR3$^+$ T_{reg} cells in IL-12p40-deficient mice (data not shown). In addition, mice lacking either IL-4 or STAT6--two molecules critical for T_H2 cell differentiation--also contained normal frequencies of CXCR3$^+$ T_{reg} cells (Supplementary Fig. 5 online). Together, these findings indicate that T-bet expression in T_{reg} cells is induced during T_H1 responses by an IFN-γ-dependent, IL-12-independent signaling pathway. To determine if IFN-γR expression in T_{reg} cells is required for optimal expression of T-bet and CXCR3, we constructed mixed BM chimeras using wild-type and *Ifngr1*$^{-/-}$ donors, and determined the contribution of each donor to various lymphocyte populations. Whereas wild-type and *Ifngr1*$^{-/-}$ BM contributed equally to the generation of B cells and CXCR3$^-$ T_{reg} cells, CXCR3$^+$ T_{reg} cells were derived predominantly from the wild-type donor (Fig 3b,c), demonstrating that IFN-γ responsiveness is required for optimal expression of T-bet and CXCR3 by T_{reg} cells. Together, these data provide a molecular link between IFN-γ produced during strong type-1 inflammatory responses and expression of T-bet by T_{reg} cells, and support the hypothesis that T-bet is selectively induced in T_{reg} cells during T_H1-mediated inflammation.

Functional characterization of T-bet$^+$ T_{reg} cells

Within CD4$^+$ T cells, T-bet and Foxp3 direct distinct transcriptional programs that can result in opposing functional outcomes. T-bet binds to and transactivates the *Ifng* locus, and is required for IFN-γ production by CD4$^+$ T cells35. However, Foxp3 can suppress IFN-γ expression, and T_{reg} cells do not generally produce pro-inflammatory cytokines. Therefore, we examined IFN-γ production by splenocytes isolated from Foxp3^{gfp} mice following *in vitro* stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Fig 4a). As expected, IFN-γ production among Foxp3$^-$ cells was largely restricted to the T-bet$^+$ population. However, very few Foxp3$^+$T-bet$^+$ cells produced IFN-γ. Additionally, CXCR3$^+$

T_{reg} cells sorted from anti-CD40-treated Foxp3^{gfp} mice efficiently suppressed the proliferation of CD4^{+}CD25^{-} T cells *in vitro*, demonstrating their suppressive capacity (Fig. 4b).

CXCR3^{+} T_{reg} cells expressed high amounts of GITR, CTLA-4 and CD103, and low amounts of CD25, consistent with the phenotype of 'effector/memory-like' T_{reg} cells (Fig. 4c)20. Accordingly, CXCR3^{+} T_{reg} cells contained abundant mRNA for the T_{reg} cell-associated effector molecules IL-10, TGF-β and granzyme B (Supplementary Fig. 6 online). Furthermore, sorted Foxp3^{+}CXCR3^{+} cells maintained expression of both CXCR3 and T-bet for at least two weeks following adoptive transfer into lympho-replete hosts, indicating that T-bet expression is a stable characteristic of this T_{reg} cell subset (Supplementary Fig. 7 online and data not shown).

T-bet regulates T_{reg} cell homeostasis

T-bet controls the proliferation and selection of developing T_H1 cells *in vivo*1, 34. As such, we hypothesized that T-bet may be important for T_{reg} cell proliferation and/or survival in the T_H1-promoting conditions induced by anti-CD40 treatment. To test this, we co-injected purified CD45.2^{+} T-bet-deficient and CD45.1^{+} wild-type T_{reg} cells into TCRβδ-KO mice, treated the recipient animals with anti-CD40, IL-2C, or rat IgG and examined the frequency and absolute number of each population in the spleen of recipient animals one week later (Fig 5a). Consistent with a role for T-bet in regulating T_{reg} cell homeostasis, T-bet-deficient T_{reg} cells were outcompeted by wild-type cells following anti-CD40 treatment (Fig. 5b). Indeed, the absolute number of T-bet-deficient T_{reg} cells recovered from anti-CD40-treated mice was lower than in rat IgG-treated controls, suggesting that T-bet promotes the survival and/or proliferation of T_{reg} cells in type-1 inflammatory conditions (Fig. 5b). Furthermore, both wild-type and T-bet-deficient T_{reg} cells underwent robust expansion in animals given IL-2C, demonstrating that the failure of T-bet-deficient T_{reg} cells to expand in anti-CD40-treated mice was not due to a general inability to survive and proliferate *in vivo.*

To directly compare the proliferation of wild-type and T-bet-deficient T_{reg} cells, we administered the nucleotide analogue 5-bromo-2′-deoxyuridine (BrdU) in the drinking water of recipient mice during the final 48 hours of anti-CD40, IL-2C or rat IgG treatment (Fig. 5c). Due to the lymphopenic environment present in the TCRβδ-KO animals, the majority of both wild-type and T-bet-deficient T_{reg} cells incorporated BrdU in rat IgG-treated mice. Treatment with IL-2C further enhanced proliferation of both populations, consistent with their similar accumulation under these conditions. In contrast, the proliferative response of T-bet-deficient T_{reg} cells following anti-CD40 treatment was significantly attenuated. The T-bet target genes CXCR3 and IL-12Rβ2 have been implicated in T_H1 cell differentiation and selection34, 36, 37. However, proliferation of both CXCR3- and IL-12Rβ2-deficient T_{reg} cells was equivalent to wild-type following anti-CD40 treatment (Supplementary Fig. 8 online).

Because T-bet is induced in T_{reg} cells and controls their proliferation following anti-CD40 treatment, we reasoned that T-bet may also be important for T_{reg} cell fitness during persistent infections dominated by T_H1 immune responses. Following aerosol infection with *Mtb*, both T_H1 effector cells and T_{reg} cells proliferate in the draining mediastinal lymph

node (dLN) and traffic in parallel to granulomas in the lungs, a nidus of IFN-γ-mediated inflammation17, 38. The "balanced" responses of T_H1 cells and T_{reg} cells established within pulmonary granulomas leads to the control, but not the eradication, of tuberculous bacilli. In *Mtb*-infected mice, T-bet$^+$ T_{reg} cells were highly enriched in three principle sites of microbial replication: the lungs, the dLN and the spleen (Fig. 6a). In contrast, few T-bet$^+$ T_{reg} cells were found in the uninvolved mesenteric lymph nodes (mLN), nor did they accumulate in the lungs of mice with chronic T_H2-mediated pulmonary inflammation caused by overexpression of the pro-allergic cytokine thymic stromal lymphopoietin (*SPC-Tslp* mice, Supplementary Fig. 9 online)39.

To determine if T-bet expression by T_{reg} cells is important for their competitive fitness during persistent infection, we constructed mixed BM chimeras using wild-type and T-bet-deficient donors, and calculated the ratio of wild-type:T-bet-deficient T_{reg} cells present in the lungs, dLN and spleen following *Mtb* infection (Fig. 6b). As control populations, we examined the ratio of wild-type:T-bet-deficient CD4$^+$CD44^{hi}Foxp3$^-$ effector T cells (T_{eff}), and CD4$^-$CD8$^-$ (DN) cells, which are predominantly B cells. There was a 3-5 fold enrichment of wild-type compared to T-bet-deficient T_{reg} cells in the tissues of infected animals, indicating that T-bet-deficient T_{reg} cells were outcompeted by wild-type cells during *Mtb* infection (Fig 6b). T-bet-deficient CD4$^+$Foxp3$^-$CD44^{hi} effector T cells were also outcompeted by wild-type effector T cells, consistent with the obligate role of T-bet in directing T_H1 cell differentiation and accumulation34. In contrast, wild-type and T-bet-deficient DN cells were present in a 1:1 ratio in each tissue, demonstrating equal engraftment of hematopoietic precursors. These data corroborate our results in anti-CD40-treated mice and demonstrate that T-bet regulates the homeostasis of T_{reg} cells during the strong T_H1 responses elicited by *Mtb* infection.

Functional impairment of T-bet-deficient T_{reg} cells

T-bet deficient T_{reg} cells can block T cell proliferation *in vitro*40, and function *in vivo* in experimental models of asthma and colitis41-43. However, their ability to specifically regulate T_H1 responses has not been directly examined. Therefore, we compared the ability of wild-type and T-bet-deficient T_{reg} cells to control T_H1 responses following transfer into Foxp3-deficient *scurfy* (*sf*) mice. Due to a spontaneous mutation in *Foxp3*, these animals lack functional T_{reg} cells and succumb to severe multi-organ autoimmunity associated with an accumulation of CD4$^+$T-bet$^+$ T_H1 cells that produce IFN-γ (Fig. 7a)44. Transfer of purified T_{reg} cells into neonatal *sf* mice prevents disease; thus, this is a sensitive experimental system to examine the homeostasis and function of T_{reg} cells *in vivo*21. Consistent with the strong T_H1 responses observed in *sf* mice, we found that most wild-type T_{reg} cells recovered from the spleens of recipient *sf* animals expressed T-bet (Fig. 7b). However, compared to mice given wild-type cells, the frequency of CD4$^+$Foxp3$^+$ cells recovered from recipients of T-bet-deficient T_{reg} cells was significantly reduced (Fig. 7c). Additionally, there was an increase in the fraction of CD4$^+$CD44^{hi} effector T cells expressing T-bet and producing IFN-γ in recipients of T-bet-deficient T_{reg} cells (Fig. 7d,e). In contrast, the frequency of IL-4 and IL-17 producing CD4$^+$ T cells did not differ between *sf* recipients of wild-type or T-bet-deficient T_{reg} cells (Supplemental Fig. 10 online). Moreover, whereas all animals receiving wild-type T_{reg} cells remained healthy throughout

the 60 day experiment, 7 out of 10 *sf* mice given T-bet-deficient T_{reg} cells failed to thrive, appeared runted, and displayed other symptoms of inflammatory disease such as lymphadenopathy and splenomegaly (Fig. 7f, Supplemental Fig. 11, and Supplemental Table 1 online). Together, these data demonstrate that T-bet is essential for T_{reg} cell homeostasis and control of T_H1 responses in *sf* mice.

Real-time PCR analysis of sorted $Foxp3^+$ cells showed only modest (~2-fold) reductions in expression of the immunosuppressive cytokines TGF-β and IL-10 in T-bet-deficient compared to wild-type T_{reg} cells (data not shown). Thus, impaired expression of these genes is unlikely to account for the inability of T-bet-deficient T_{reg} cells to control T_H1 responses in *sf* mice. Instead, our data indicate that T-bet functions largely to endow T_{reg} cells with the homeostatic and migratory properties required for the suppression of strong T_H1 responses *in vivo*.

Discussion

We identified the T_H1-associated transcription factor T-bet as a key regulator of the migration, proliferation and survival of T_{reg} cells during T_H1-mediated immune responses *in vivo*. These data have several important implications for understanding T_{reg} cell-mediated immunoregulation and the functional differentiation of $CD4^+$ T cells. First, they demonstrate that like conventional naïve $CD4^+$ T cells, T_{reg} cells undergo molecular differentiation in response to the cytokine environment, resulting in their phenotypic and functional specialization. In addition, they show T-bet is important not only for the differentiation of T_H1 cells, but also for the control and regulation of T_H1 responses; thus the role of T-bet in coordinating type-1 inflammation *in vivo* is more complicated than previously appreciated. Finally, by demonstrating that Foxp3 and T-bet operate within the same cell to produce a unique functional outcome, our data challenge current models which posit that the functional specialization of $CD4^+$ T cells is due to differential and exclusive expression of a limited set of 'master' transcription factors.

T-bet appears to control multiple aspects of T_{reg} cell biology during type 1-inflammatory responses. By inducing expression of the chemokine receptor CXCR3, T-bet can help promote T_{reg} cell migration to sites of T_H1-mediated inflammatory responses. CXCR3 is expressed by T_H1 cells, by nearly all activated $CD8^+$ T cells, and by the majority of natural killer (NK) and NKT cells. During T_H1 responses, CXCR3 expression facilitates efficient recruitment of these effector populations in response to the IFN-γ-inducible CXCR3 ligands. Indeed, we found that $CXCR3^+$T-bet^+ T_{reg} cells accumulate at sites of T_H1-mediated inflammation during persistent *Mtb* infection. Although the importance of CXCR3 in inflammatory cell migration depends on the model used and the tissue examined, two recent reports have demonstrated a critical role for CXCR3-mediated trafficking of T_{reg} cells to the central nervous system and liver, highlighting the importance of T-bet-induced CXCR3 expression in the localization and function of T_{reg} cells45, 46.

The molecular mechanisms by which T-bet controls the homeostasis of T_{reg} cells during T_H1 inflammation are not clear. Early in T_H1 cell differentiation, STAT1-induced T-bet confers responsiveness to the cytokine IL-1234. Acting via STAT4, IL-12 then induces

growth and survival of developing T_H1 cells. However, we found normal numbers of T-bet$^+$CXCR3$^+$ T_{reg} cells in IL-12p40-deficient mice, and T_{reg} cells lacking IL-12Rβ2 showed no homeostatic defect in anti-CD40 treated mice. T-bet also regulates the development and homeostasis of both NK and NKT cells, largely through induction of CD122, which allows these cells to respond to the cytokine IL-1547. CD122 is also a component of the high-affinity IL-2 receptor, and is required for the differentiation and homeostasis of T_{reg} cells48. However, T-bet-deficient T_{reg} cells proliferated and expanded normally following IL-2C treatment, and thus it is unlikely that impaired IL-2 responsiveness underlies their altered homeostasis. Instead, T-bet likely controls the homeostasis of T_H1 cells, NK and NKT cells, and T_{reg} cells by distinct mechanisms. T-bet may act in T_{reg} cells by directly controlling expression of cell cycle or anti-apoptotic genes4, or by conferring sensitivity to undefined growth and survival factors that regulate T_{reg} cell homeostasis primarily during type-1 inflammation.

Recently, the transcription factor interferon-regulatory factor-4 (IRF-4), which functions in T_H2 cell differentiation, was shown to be required for T_{reg} cell-mediated control of T_H2 responses *in vivo*18. Together with our results, these findings indicate that as a general strategy, T_{reg} cells may utilize selective aspects of effector T cell differentiation programs to tune their migratory, homeostatic and functional properties without acquiring pro-inflammatory effector functions. However, whereas IRF-4 appears to be expressed uniformly by nearly all peripheral T_{reg} cells, T-bet is only expressed by a subset of T_{reg} cells defined by surface expression of CXCR3. Additionally, our data suggest a molecular pathway by which T_{reg} cells upregulate T-bet in response to STAT1-mediated IFN-γR signaling. Indeed, recent genome-wide histone methylation analyses indicate that *Tbx21* exists in a poised epigenetic state in T_{reg} cells, and can readily be upregulated under T_H1-polarizing conditions *in vitro*49. Collectively, these results demonstrate that T_{reg} cells can sense and respond to the local cytokine environment by undergoing molecular specialization that enables them to function in specific inflammatory settings.

T-bet coordinates T_H1 cell development and function by directly inducing expression of genes such as *Ifng*, *Il12rb2*, *Spp1*, *Runx3*, *Hlx* and *Cxcr3*, and by silencing *Il4* to block T_H2 differentiation1. Interestingly, we noted that CXCR3$^+$ T_{reg} cells express ∼10-20-fold lower amounts of T-bet when compared with fully differentiated CD4$^+$Foxp3$^-$CXCR3$^+$ T_H1 cells. The ability of T-bet to bind to particular target loci and promote gene expression may be concentration-dependent4. In addition, Foxp3 can directly repress *Ifng* expression, and chromatin immunoprecipitation studies detected Foxp3 bound to the *Spp1* locus, which encodes the type-1 cytokine osteopontin6, 50. Therefore, we propose that coupled with the direct repressive functions of Foxp3, the low concentration of T-bet in CXCR3$^+$ T_{reg} cells prevents the production of pro-inflammatory T_H1 cytokines, while permitting expression of T-bet target genes that influence localization and homeostasis of T_{reg} cells during T_H1-mediated inflammatory responses. Consistent with this model, T_{reg} cells that lost Foxp3 expression upregulated T-bet and acquired the ability to produce IFN-γ, suggesting an active role for Foxp3 in preventing full T_H1 cell differentiation in T_{reg} cells19.

Our results provide a new framework for understanding how T_{reg} cells sense and respond to strong T_H1 responses, and how this leads to their phenotypic differentiation and

specialization. Further defining the molecular mechanisms by which T-bet and Foxp3, when present in combination, control the homeostasis, migration and function of T_{reg} cells is essential for determining how T_{reg} cells maintain normal immune homeostasis during T_H1 responses *in vivo*, and for understanding how the activities of two so-called 'master regulators' of $CD4^+$ T cell differentiation can be overlaid in the same cell to produce a unique functional outcome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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ONLINE METHODS

Mice

C57BL/6J, Balb/c, B6.129P2-CXCR3^{tm1Dgen}/J (*Cxcr3*$^{-/-}$), B6.129P2-*Tcrb*^{tm1Mom} *Tcrd*^{tm1Mom}/J (TCRβδ-KO), B6.129S7-*Ifngr1*^{tm1Agt}/J (*Ifngr1*$^{-/-}$) and B6.Cg-Foxp3sf/J (*sf*) mice were purchased from The Jackson Laboratory. B6.SJL-*Ptprc*^{a}/BoyAiTac (CD45.1) mice were purchased from Taconic Farms. *Tbx21*$^{-/-}$ and Foxp3^{gfp} mice (on C57BL/6 background) were generously provided by A. Weinmann and A. Rudensky, respectively (University of Washington, Seattle, WA). *SPC-Tslp*, *Il4*$^{-/-}$ and *Stat6*$^{-/-}$ mice (on Balb/c background) were provided by S. Ziegler (Benaroya Research Institute, Seattle, WA). Splenocytes from *Stat1*$^{-/-}$ mice were provided by M. Krishna-Kaja. All animals were housed and bred at the Benaroya Research Institute (Seattle, WA) and all experiments were performed in accordance with the guidelines of the Benaroya Research Institute Animal Care and Use Committee.

BM chimeras and neonatal transfers

BM cells from the femurs of WT, *Tbx21*$^{-/-}$ and *Ifngr1*$^{-/-}$ mice were obtained and depleted of $CD4^+$ cells using anti-CD4 microbeads (Miltenyi Biotech). 8×10^6 cells of a 1:1 mixture of WT (CD45.1) and *Tbx21*$^{-/-}$ (CD45.2) or *Ifngr1*$^{-/-}$ (CD45.2) BM were injected retro-orbitally into RAG1$^{-/-}$ mice following lethal irradiation of 1000 Rad. For neonatal adoptive transfers, $CD4^+CD25^+$ cells were isolated from the spleens and LNs of WT or *Tbx21*$^{-/-}$ mice by magnetic separation using a CD4 T cell isolation kit (Invitrogen), followed by CD25 positive isolation kit (Miltenyi Biotech). 1×10^6 $CD4^+CD25^+$ cells (>90% purity) were resuspended in 25ul PBS, and injected i.p. into 1-2 day old *sf* neonates. Mice were monitored for external signs of disease and killed after 25-60 days for analysis.

Flow cytometry and cell sorting

Cell isolation was performed as described21. For flow cytometry, cells were surface stained with the following directly conjugated antibodies specific for murine proteins: anti-CD4 (GK1.5), anti-CD62L (MEL-14), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD103 (2E7), anti-CD25 (PC61.5), anti-CD8 (53-6.7), anti CTLA-4 (UC10-4B9), anti-GITR (YGL-386), RatIgG2a (eBR2a) were from eBioscience, and anti-CXCR3 (220803), and anti-CCR6 (140706) were from R&D systems. To assess expression of functional P-selectin ligands, cells were first incubated with P- selectin-human IgM fusion proteins, followed by biotinylated goat-anti-human IgM (Jackson Immunoresearch) and streptavidin-PE (eBioscience). Data were acquired on FACsCalibur or LSRII flow cytometers (BD Biosciences) and analyzed using FlowJo software (Treestar). For cell sorting experiments, $CD4^+$ cells were enriched using Dynal CD4 T cell negative isolation kit (Invitrogen), stained for desired cell surface markers, and isolated using a FACS Vantage (BD Biosciences).

Intracellular staining

For intracellular staining of IFN-γIL-4, IL-17, Foxp3 and/or T-bet, lymphocytes were surface stained, then permeabilized with the eBioscience FixPerm buffer. Cells were then washed and stained with anti-IFN-γ (XMG1, eBioscience), anti-IL-4 (11B11, eBioscience), anti-IL-17 (TC11-18H10.1, Biolegend), anti-Foxp3 (FKJ-16s; eBioscience) and/or purified anti-T-bet (4B10; Santa Cruz Biotech) or mIgG1 isotype (P3, eBioscience) in staining media containing HBSS, 1%BSA, 10mM Hepes and 0.5% saponin for 30min. To detect T-bet, secondary staining was done with anti-mIgG1-APC or -PE (A85-1; BD Pharmingen). For BrdU incorporation, BrdU was continuously administered in drinking water at a concentration of 0.8mg/mL for 2 days before sacrifice. For intracellular Foxp3 and BrdU staining, a modified BrdU staining protocol was used. Lymphocytes were isolated and surface stained, followed by fixation in eBioscience FixPerm for 30 min. Cells were then stained for BrdU following the BrdU Flow Kit manufacturer's instructions (BD Pharmingen).

In vitro suppression assay

Lymphocytes were isolated from the spleens of anti-CD40 treated $Foxp3^{gfp}$ mice, enriched for $CD4^+$ cells using a Dynal CD4 T cell-negative isolation kit (Invitrogen), and $Foxp3^+CXCR3^+$ cells were FACs sorted. $CD4^+CD25^-$ effector lymphocytes were isolated from spleens and LNs of congenically marked B6.SJL mice using Dynal CD4 T cell-negative isolation kit. $CD25^-$ cells were isolated by staining with anti-CD25-PE followed by magnetic separation using anti-PE microbeads (Miltenyi Biotech). Final suspensions of $CD4^+CXCR3^+GFP^+$ cells and $CD4^+CD25^-$ cells were >95% pure. $CD4^+CD25^-$ cells were incubated with 0.8μM carboxyfluorescein cuccinimidyl ester (CFSE) in PBS for 9 min in a 37°C water bath, washed with 100% bovine calf serum and resuspended in complete medium. In each culture well, CFSE-labeled $CD4^+CD25^-$ T cells were incubated with T cell depleted, irradiated (2500 Rad) splenocytes with or without addition of sorted T_{reg} cells. All stimulated cultures received 5μg/mL anti-CD3 (2C11) and 2μg/mL anti-CD28 (37.51).

Proliferation of $CD45.1^+CD4^+CD25^-$ T cells during co-incubation with varying ratios of T_{reg} cells was measured by assessing relative CFSE dilution after 110 hours of culture.

Chemotaxis assay

All recombinant murine chemokines were purchased from Peprotech. Red blood cell-depleted splenocytes isolated from WT, *Tbx21*$^{-/-}$ and *Cxcr3*$^{-/-}$ mice were incubated at 37°C, 5% CO_2 in complete media for 1 h at 2×10^6/mL. Cells were then washed and resuspended at 10×10^6/mL and 1×10^6 cells were added to the top chamber of a 5-μM pore transwell (Costar). Chemokines were diluted in complete medium and added to the bottom of culture chambers to a final concentration of 100nM. Complete medium alone was added to the bottom of control chambers. Chemotaxis toward each chemokine or media control was measured in triplicate. After 90min of incubation at 37°C, 5%$C0_2$, 100μl was harvested from the bottom of each well and added to a fixed number of 15μm latex beads (Polysciences, Inc) to calculate the overall migration toward each chemokine or media. Replicate wells were then combined and stained for CD4 and Foxp3. The % specific migration was calculated by normalizing the frequency of migrated $CD4^+Foxp3^+$ T_{reg} cells to the input population.

In vitro T cell stimulation and cytokine analysis

6×10^6 red blood cell-depleted wild-type splenocytes were stimulated with 50ng/mL PMA and 1μg/mL ionomycin in the presence of 10μg/mL monensin in 1ml of complete media for 4 h at 37°C, 5%CO_2. Following stimulation, cells were harvested, surface stained, permeabilized with eBioscience FixPerm and stained with antibodies against cytokines and transcription factors.

Mycobacterium tuberculosis infection

Mice were infected with sonicated *Mtb* strain H37Rv using an aerosol infection chamber (Glas Col). A set of wild-type mice were sacrificed 1 day post infection to determine the infectious dose. In each experiment, 50-100 colony forming units (CFUs) were deposited in the lungs of each mouse.

Anti-CD40 and IL-2C administration

Mice were injected intraperitoneally (i.p) with 25μg anti-CD40 (Clone IC10, eBioscience) or 25μg Rat IgG (Sigma) in PBS on days 0, 2, and 4. For IL-2C treatment, 50μg anti-IL-2 (JES6) was incubated with 1.5μg recombinant mouse IL-2 (carrier free, eBioscience) in PBS overnight at 4°C. Mice were injected with IL-2C i.p. on days 0 and 2. All mice were sacrificed on day 6. For competition experiments, cells were adoptively transferred via retro-orbital injection on day -1.

Quantitative PCR

RNA extraction was performed using Qiagen RNeasy columns (Qiagen) and cDNA was generated using Omniscript RT Kit (Qiagen) according to the manufacturer's instructions.

Presynthesized Taqman Gene Expression Assays (Applied Biosystems) were used to amplify *Tbx21* (Mm00450960_m1), *Il10* (Mm99999052_m1), *Gzmb* (Mm00442834_m1), and *Tgfb1* (Mm01178820_m1) mRNA transcripts. *Actb* was used as an internal control with the sense primer TGACAGGATGCAGAAGGAGAT, anti-sense primer GCGCTCAGGAGGAGCAAT, and probe FAM-ACTGCTCTGGCTCCTAGCACCATTAMRA. Target gene values are expressed relative to *Actb*.

Statistical analysis

Statistical significance was determined by unpaired student's two-way repeated measures ANOVA, or two-tailed Student's t test as indicated in figure legends.

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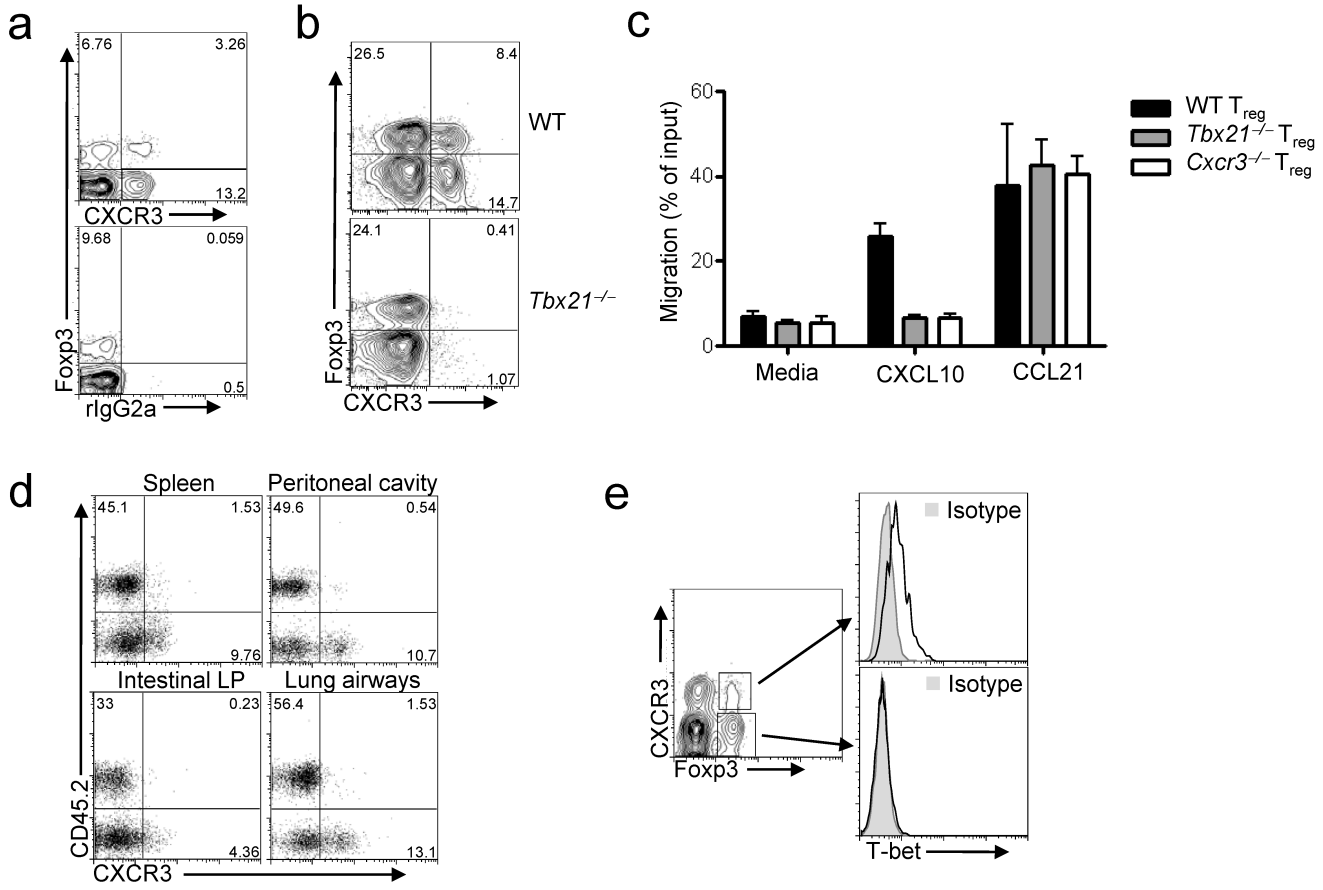

Figure 1. CXCR3 expression on T_{reg} cells is T-bet-dependent

(a,b) Representative flow cytometric analysis of CXCR3 and Foxp3 expression by splenocytes isolated from wild-type (WT) mice, (**a**) or age-matched WT and *Tbx21*$^{-/-}$ mice (**b**). Plots are gated on CD4^{+} splenocytes. rIgG2a, isotype control. Numbers display the frequency of cells expressing the indicated markers. Data are representative of greater than six mice analyzed in this fashion. (**c)** Migration of CD4^{+}Foxp3^{+} splenocytes isolated from the indicated mice in response to media alone, 100nM CXCL10 or 100nM CCL21 in a transwell chemotaxis assay. Data are mean and s.d. of triplicate measurements. (**d)** CD45.2 and CXCR3 expression on CD4^{+}Foxp3^{+} cells recovered from the indicated tissues of recipients of a mixture of CD45.1^{+} WT and CD45.2^{+} *Tbx21*$^{-/-}$ BM. Numbers depict the percent of cells positive for the indicated markers. Data are representative of three independent experiments with four mice analyzed per experiment. (**e)** T-bet expression (open histograms) in CD4^{+}Foxp3^{+}CXCR3^{+} or CD4^{+}Foxp3^{+}CXCR3^{-} splenocytes. Histograms (right) correspond to indicated gates (left). Data are representative of greater than ten mice analyzed in this fashion.

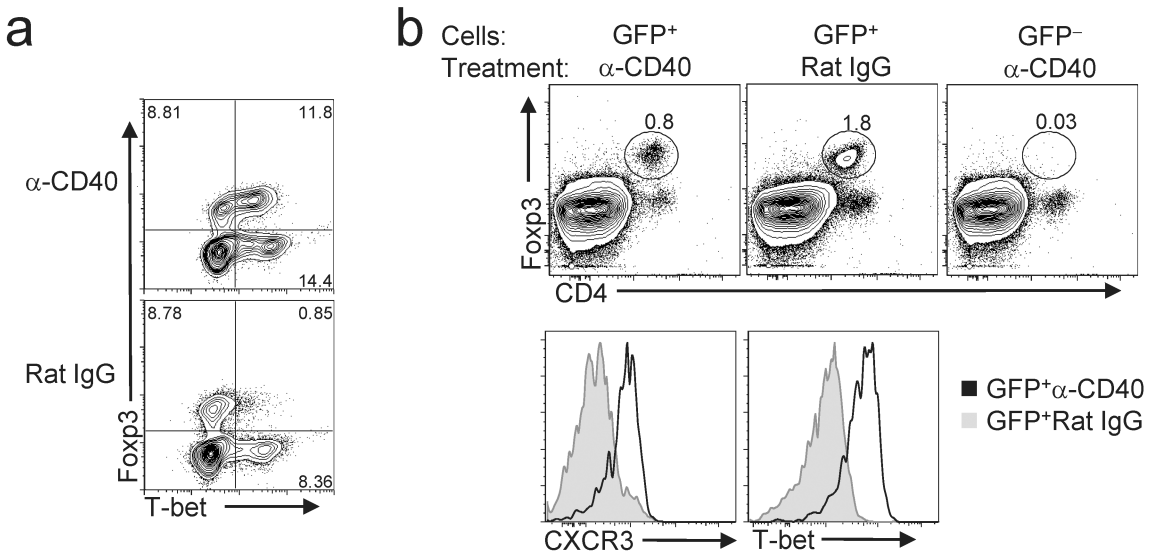

Figure 2. T-bet^{+} T$_{reg}$ cells upregulate T-bet *in vivo* following anti-CD40 treatment

(a) Analysis of T-bet and Foxp3 expression in splenocytes from mice treated with anti-CD40 (top) or rat IgG (bottom). Plots are gated on CD4^{+} cells. Numbers in plots represent the percentage of cells positive for the indicated markers. Data are representative of three independent experiments. **(b)** (Top) Splenocytes from TCRβδ-KO recipients of the indicated cells and subjected to the indicated treatment were analyzed by flow cytometry. Dot plots are gated on lymphocytes. Histograms display CXCR3 and T-bet expression in CD4^{+}Foxp3^{+} cells isolated from anti-CD40- (open histograms) or rat IgG- (shaded histograms) treated mice as indicated. Numbers in dot plots indicate the percentage of Foxp3^{+}CD4^{+} cells of total lymphocytes. Data are representative of two independent experiments with three mice per group.

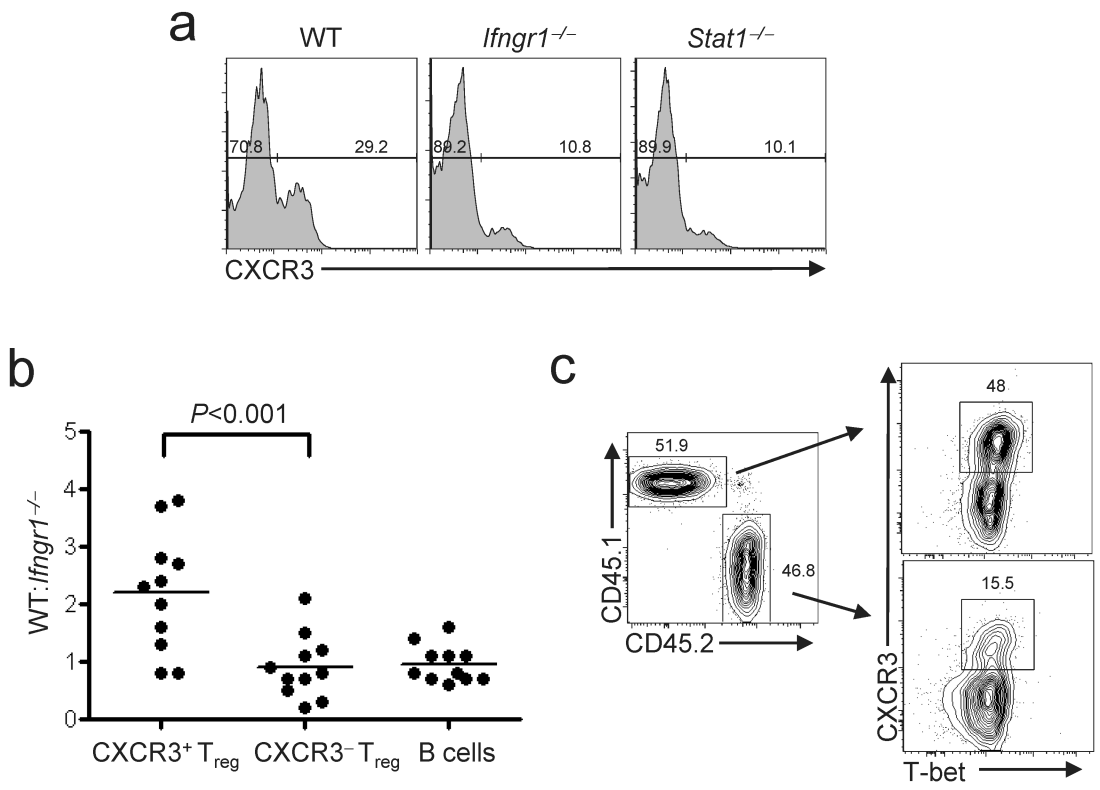

Figure 3. IFN-γR and STAT1 are promote expression of T-bet and CXCR3 by T_{reg} cells

(a) CXCR3 expression on splenocytes from WT, *Ifngr1*$^{-/-}$, or *Stat1*$^{-/-}$ mice as indicated (n≥ three per genotype). Histograms are gated on CD4^{+}Foxp3^{+} cells. Numbers indicate the percent of CXCR3^{+} cells among total CD4^{+}Foxp3^{+}. **(b)** Graph depicts the ratio of WT:*Ifngr1*$^{-/-}$-derived lymphocytes among CXCR3^{+}CD4^{+}Foxp3^{+}, CXCR3^{-}CD4^{+}Foxp3^{+} or CD4^{-}B220^{+} peripheral blood lymphocytes of 11 mixed BM chimeras. Each point represents an individual mouse. Statistical significance was determined using a two-way repeated measures ANOVA. A Bonferroni post-test was used to obtain the *P*-value for the indicated pairwise comparison. **(c)** CXCR3 and T-bet expression on splenocytes isolated from a WT:*Ifngr1*$^{-/-}$ BM chimera. Plots are gated on CD4^{+}Foxp3^{+} cells from WT-derived (CD45.1^{+}) or *Ifngr1*$^{-/-}$ -derived (CD45.2^{+}) BM as indicated. Numbers in dot plots indicate the percent of cells positive for CXCR3. Data are representative of four mice analyzed in this fashion.

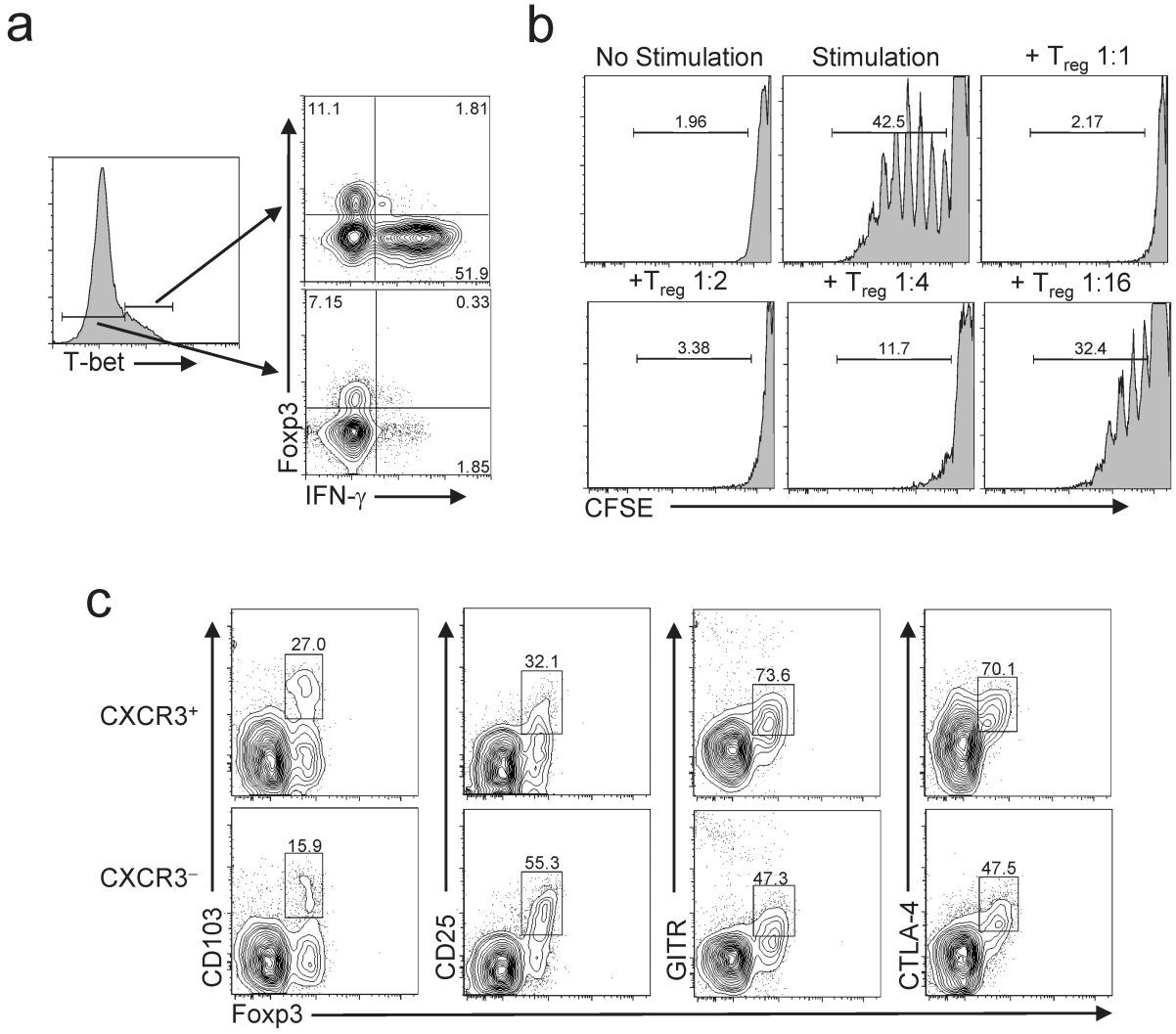

Figure 4. Functional characterization of T-bet$^+$ Foxp3$^+$ T$_{reg}$ cells

(a) IFN-γ and Foxp3 expression by T-bet$^+$CD4$^+$ (top right) and T-bet$^-$CD4$^+$ (bottom right) lymphocytes isolated from WT mice following stimulation with PMA and ionomycin. Left histogram is gated on total CD4$^+$ cells, and indicates gates used to define the populations depicted in the dot plots. Numbers in plot indicate the percent of cells positive for the indicated markers. Data are representative of greater than five mice analyzed in this fashion. **(b)** Proliferation of CFSE-labeled CD4$^+$CD25$^-$ T cells incubated with irradiated splenocytes, anti-CD3 and anti-CD28, with or without varying concentrations of CXCR3$^+$ T$_{reg}$ cells. Numbers above histograms indicate T$_{reg}$:T$_{eff}$ cell ratio in each culture. No stimulation, control without irradiated splenocytes and stimulatory antibodies. Numbers indicate the percent of T$_{eff}$ cells that are CFSE$^-$. **(c)** Expression of the indicated markers on gated CD4$^+$CXCR3$^+$ (top) or CD4$^+$CXCR3$^-$ (bottom) splenocytes from WT mice. Numbers represent the frequency of cells positive for each marker as a fraction of total CD4$^+$Foxp3$^+$ cells. Data are representative of three mice analyzed in this fashion.

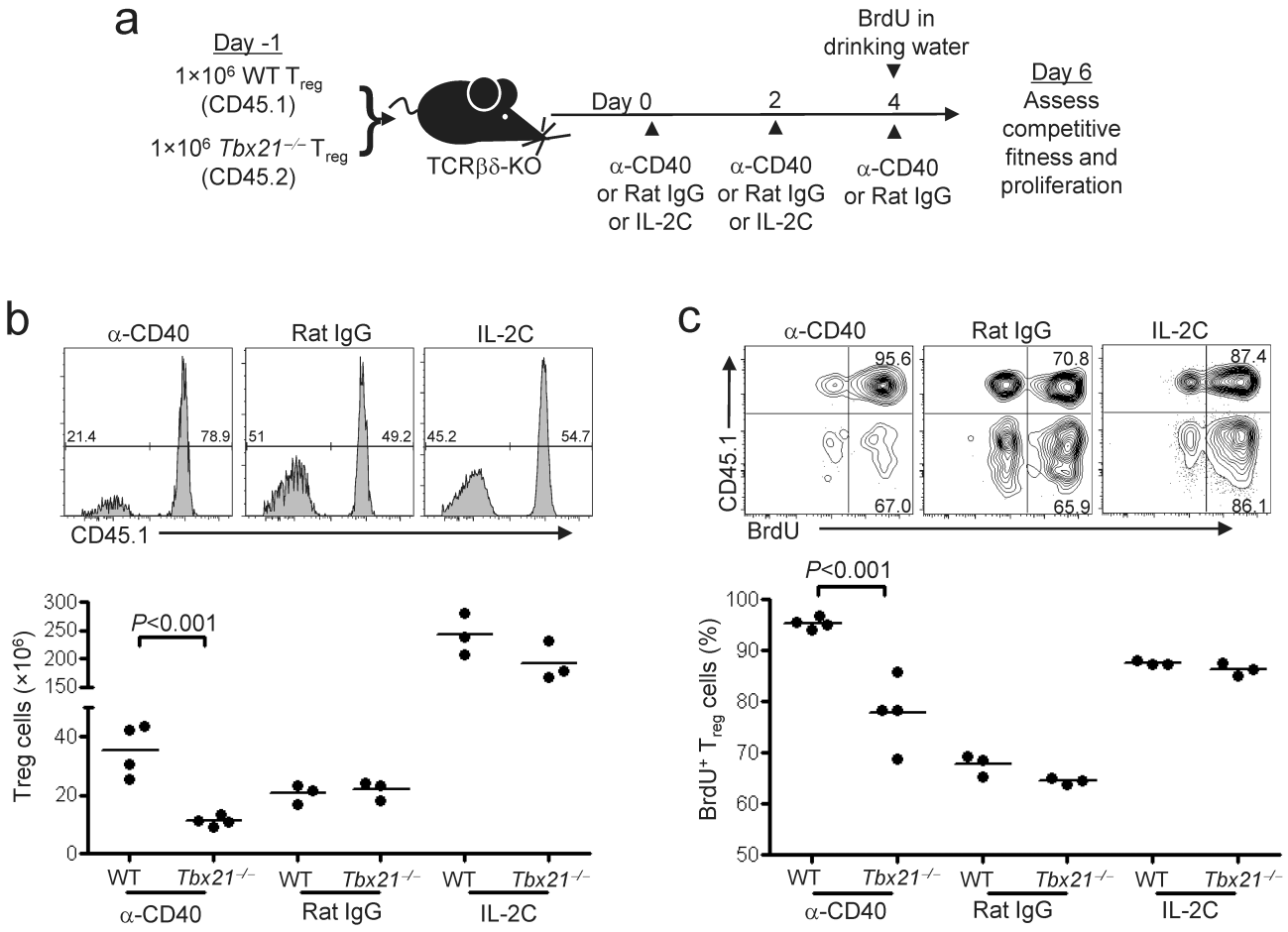

Figure 5. Decreased proliferation of T-bet-deficient T_{reg} cells following anti-CD40 treatment

(a) Experimental design showing cell transfer and treatment schedule. Briefly, a mixture of CD45.1$^+$ WT and CD45.2$^+$ *Tbx21*$^{-/-}$ T_{reg} cells were injected into TCRβδ-KO mice, followed by treatment with the indicated antibodies. BrdU was added to the drinking water when indicated. **(b)** CD45.1 expression on splenocytes of recipient mice was analyzed by flow cytometry. Histograms are gated on Foxp3$^+$CD4$^+$TCRβ$^+$B220$^-$ cells, and numbers indicate the percent of cells positive and negative for CD45.1. Graphs show absolute numbers of WT- and *Tbx21*$^{-/-}$-derived T_{reg} cells recovered from the spleens of recipient mice. Each point represents an individual treated mouse. **(c)** BrdU incorporation by splenocytes of recipient mice was analyzed by flow cytometry. Dot plots are gated on Foxp3$^+$CD4$^+$TCRβ$^+$B220$^-$ splenocytes. Numbers in plots indicate percentage of BrdU$^+$ cells among total WT- (CD45.1$^+$) or *Tbx21*$^{-/-}$-derived (CD45.1$^-$) T_{reg} cells. Graphs depict the frequency of BrdU$^+$ cells among WT and *Tbx21*$^{-/-}$-derived T_{reg} cell populations. For **b** and **c**, statistical significance was determined using a two-way repeated measures ANOVA. Bonferroni post-tests were used to obtain the *P*-values for the indicated pairwise comparisons. Data are representative of three independent experiments with three or greater mice per group

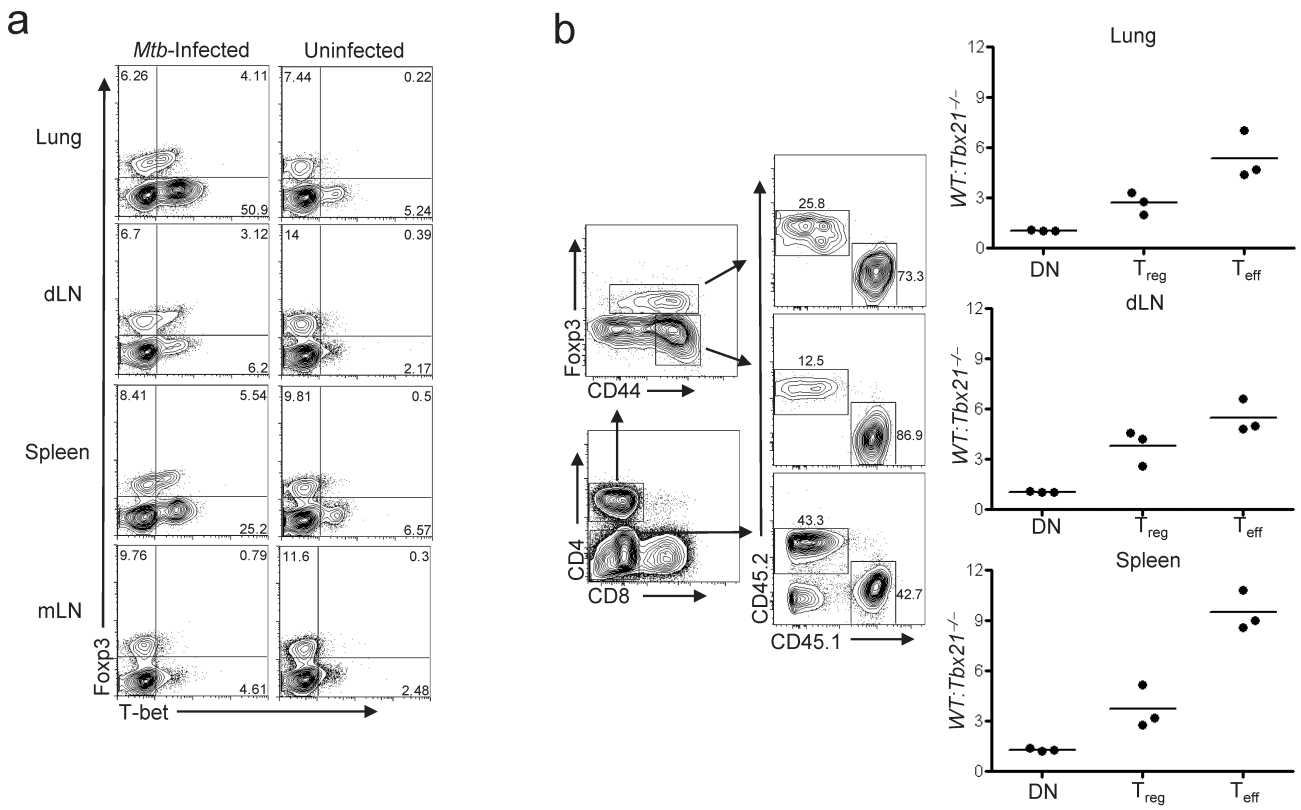

Figure 6. Impaired homeostasis of T-bet-deficient T_{reg} cells during persistent *Mtb* infection

(a) T-bet and Foxp3 expression on cells isolated from the indicated tissues of an *Mtb*-infected mouse (left) or an uninfected age-matched control (right). Plots are gated on $CD4^+$ T cells. Numbers display the percent of cells in each of the indicated quadrants. Data are representative of five independent experiments. **(b)** (Left) Recipients of a mixture of WT ($CD45.1^+$) and *Tbx21*$^{-/-}$ ($CD45.2^+$) BM were infected with *Mtb* for 105 days. Cells isolated from the indicated organs were analyzed by flow cytometry. Contour plots depict gating strategy, with numbers indicating the percent of cells positive for the indicated markers. Graphs depict the ratio of WT and *Tbx21*$^{-/-}$-derived cells among gated $CD4^-CD8^-$ DN, $CD4^+Foxp3^+$ T_{reg} and $CD4^+Foxp3^-CD44^{hi}$ T_{eff} populations. Each point represents a value from an individual infected BM chimera. Data are representative of two independent experiments (n=3 per experiment).

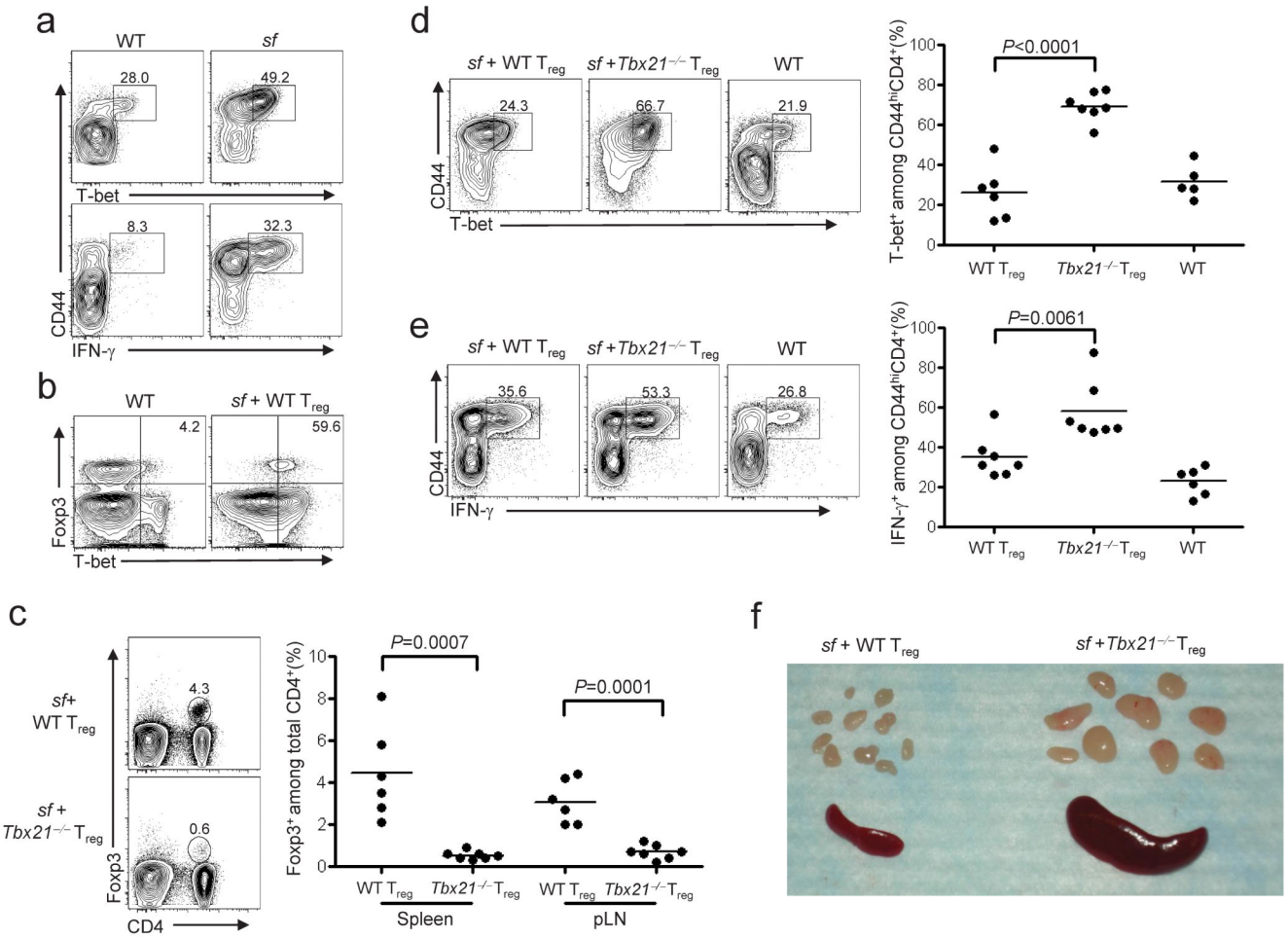

Figure 7. T-bet expression in T_{reg} cells is critical for control of T_H1-mediated inflammatory responses

(a) CD44 and T-bet expression (top) or IFN-γ production (bottom) by splenocytes isolated from age-matched WT or scurfy (*sf*) mice, as measured by flow cytometry. Plots are gated on $CD4^+Foxp3^-$ cells. Numbers in plots indicate the percent of T-bet$^+$ (top) or IFN-γ$^+$ (bottom) cells among total $CD44^{hi}$ $CD4^+Foxp3^-$cells. Data are representative of three independent experiments. **(b)** T-bet and Foxp3 expression by splenocytes isolated from age-matched WT mice or *sf* mice given WT T_{reg} cells. Plots are gated on $CD4^+CD8^-$ lymphocytes. Numbers in plots indicate the percentage of $CD4^+Foxp3^+$ cells expressing T-bet. Data are representative of six independent experiments. **(c)** Cells isolated from the spleen and peripheral lymph nodes (pLN) of *sf* neonate recipients of WT or *Tbx21*$^{-/-}$ T_{reg} cells were analyzed by flow cytometry. Numbers in plots indicate fraction of Foxp3$^+$ T cells among total CD4$^+$ reg splenocytes. **(d, e)** Flow cytometric and quantitative analysis of splenocytes isolated from *sf* mice given WT or *Tbx21*$^{-/-}$ T_{reg} cells, or from age-matched WT mice. Splenocytes in **e** were stimulated with PMA and ionomycin prior to analysis. Plots are gated on $CD4^+Foxp3^-$ cells. Numbers in plots display percentage of T-bet$^+$ (**d**) and IFN-γ$^+$ (**e**) cells among total $CD4^+Foxp3^-CD44^{hi}$ cells. **(f)** Photograph of representative spleen and peripheral LNs isolated from 6 week old *sf* mice given WT (left) or *Tbx21*$^{-/-}$ (right) T_{reg}

cells as neonates. Representative of six independent experiments. For **c**, **d** and **e**, each point represents an individual mouse, and significance was measured using two-tailed, unpaired student's *t* tests.