HHS Public Access

Author manuscript

Nat Immunol. Author manuscript; available in PMC 2015 August 01.

Published in final edited form as:

Nat Immunol. 2015 February ; 16(2): 197–206. doi:10.1038/ni.3053.

Dynamic expression of T-bet and GATA3 by regulatory T cells maintains immune tolerance

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Abstract

Regulatory T (T_{reg}) cells can express the transcription factors T-bet and GATA3 but the function of this expression and whether such cells represent stable subsets is still unknown. By using multiple reporter tools, we show that the expression of T-bet and GATA3 in T_{reg} cells is dynamically influenced by the cytokine environment. T_{reg} cell-specific deletion of either *Tbx21* or *Gata3* genes singly did not result in loss of T_{reg} cell functions; however, mice with combined deficiency of both genes in T_{reg} cells developed severe autoimmune-like diseases. Loss of T_{reg} cell function was correlated with RORγt transcription factor upregulation and reduced Foxp3 expression. Thus, in the steady state, activated T_{reg} cells transiently upregulate either T-bet or GATA3 to maintain T cell homeostasis.

Upon antigen stimulation through their T cell receptor (TCR), naive $CD4^+$ T cells differentiate into distinct effector lineages including type 1 T helper (T_H1), type 2 T helper (T_H2) and interleukin-17 (IL-17)-producing T helper (T_H17) cells; this process is influenced by the strength of TCR signaling as well as the cytokine environment[1]. The differentiation of each T_H lineage is determined by the induction of specific key transcription factors: T-bet is important for the differentiation of T_H1 cells[2]; GATA3 is indispensable for the generation of T_H2 cells[3]; and RORγt plays a critical role in determining the fate of T_H17 cells[4]. Not only do these transcription factors promote the differentiation toward one lineage, they also repress acquisition of other fates. For example, T-bet suppresses the expression and functions of GATA3[5], thus preventing the activation of an endogenous T_H2 differentiation pathway during T_H1 differentiation[6, 7]. T-bet also suppresses RORγt expression by interacting and modulating the function of Runx1, which is an important transcription factor for inducing RORγt expression during T_H17 differentiation[8, 9].

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COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

Author contributions: F. Y. performed all the experiments; S. S. and J. E. helped in some experiments and made suggestions to the manuscript; L. F. made the transgenic mouse strains; F. Y. and J. Z. designed the experiments, analyzed the data and wrote the paper.

Regulatory T (T_{reg}) cells, consisting of thymus-derived T_{reg} (tT_{reg}) cells and peripherally derived T_{reg} (pT_{reg}) cells, are crucial for the maintenance of immune tolerance and homeostasis[10, 11, 12, 13]. The transcription factor Foxp3 plays a central role in T_{reg} generation and function. The cytokine TGF-β is required for the induction of RORγt and Foxp3 and is thus involved in the differentiation of both T_H17 and T_{reg} cells[14, 15]. Consequently, RORγt and Foxp3 are co-expressed at early stages of T_H17 and T_{reg} differentiation and may antagonize each other[16]. Indeed, in some cases, loss of T_{reg} suppressive functions during inflammation is associated with upregulation of RORγt and IL-17 production in T_{reg} cells[17].

T-bet expression is found in a subset of T_{reg} cells[18]. Although T-bet expression in these T_{reg} cells has been shown to be important for the maintenance of T_{reg} homeostasis during type 1 immune responses, the physiological significance of T-bet expression in T_{reg} cells in the steady state is unknown. Furthermore, there is no report on characterizing mice with T_{reg} cell-specific deletion of *Tbx21* (encoding T-bet) even though it is known that some T_{reg} cells express GATA3 in the steady state[19, 20, 21]. GATA3 can be induced when T_{reg} cells become activated. It has been reported that T_{reg}-specific deletion of GATA3 in mice results in spontaneous autoimmunity starting from 16 weeks of age[21]; however, other reports indicate that GATA3 is only critical for T_{reg} functions during inflammation and mice with T_{reg}-specific GATA3 deletion do not develop any disease until 6 months of age[19, 20].

Although T-bet- and GATA3-expressing T_{reg} cells have been well documented, it is not clear whether the T-bet- (T_H1-) and GATA3-expressing (T_H2-like) T_{reg} cells represent stable T_{reg} subsets. Furthermore, whether and how T-bet and GATA3 regulate the function of T_{reg} cells, especially in the steady state, is not known. Here we report that T-bet and GATA3-expressing T_{reg} cells could be detected in the steady state; however, their expression in T_{reg} cells was highly dynamic. Thus, T-bet-expressing T_{reg} cells do not represent a stable T_{reg} subset. Single deletion of either *Tbx21* or *Gata3* gene specifically in T_{reg} cells by *Foxp3*-Cre did not alter T_{reg} functions. However, combined deletion of both *Tbx21* and *Gata3* in T_{reg} cells allowed the development of aggressive autoimmune-like diseases in mice at very young age.

RESULTS

Generation of T-bet:GATA3:Foxp3 tri-color reporter mice

To facilitate investigation on the relationship between T-bet and GATA3-expressing T_{reg} cells, a tri-color reporter mouse strain, in which the expression of T-bet, GATA3 and Foxp3 are depicted by different fluorescent proteins, was first constructed. Foxp3-mRFP knock-in mice[22] and GATA3-GFP knock-in mice[23], in which mRFP and GFP faithfully marks the expression of Foxp3 or GATA3, respectively, have been reported. A third fluorescent marker is required for reporting T-bet expression, but a previously generated T-bet-ZsGreen reporter mouse strain[6] is not useful for this purpose since green fluorescence is also used to report GATA3 expression.

Utilizing a similar strategy to that previously described[6], we prepared a BAC transgenic T-bet reporter mouse strain, in which AmCyan indicates T-bet expression. AmCyan-

expressing cells but not AmCyan negative cells directly sorted from the spleens of the intact reporter mice stained positive for T-bet protein (Supplementary Fig. 1a). To further evaluate the faithfulness of this new T-bet-AmCyan reporter, naive $CD4^+$ T cells ($CD4^+CD25^-CD45RB^{hi}AmCyan^-$) were isolated by cell sorting from the transgenic mice and cultured under T_H1 or T_H2 polarizing conditions for 4 days. Virtually all cells from the T_H1 cell-polarizing culture (>90%) expressed AmCyan, whereas, under T_H2 conditions, most if not all (>97%) the cells remained Amcyan negative (Supplementary Fig. 1b). These results indicate that the T-bet-AmCyan reporter faithfully reflects the expression of endogenous T-bet, just as a previous T-bet-ZsGreen reporter does. The T-bet-AmCyan reporter line was then bred with GATA3-GFP and Foxp3-RFP knock-in mice to generate a T-bet-AmCyan:GATA3-GFP:Foxp3-mRFP tri-color reporter mouse strain.

T-bet and GATA3 expression can be induced in T_{reg} cells

We first re-examined the expression pattern of T-bet and GATA3 in T_{reg} cells in the spleen, peripheral lymph node (PLN), and mesenteric lymph node (MLN) of the tri-color reporter mice in the steady state. About 20-30% of T_{reg} cells expressed GATA3 in the steady state (Fig. 1a,b). In agreement with previous reports[6, 18], only a small proportion of *ex vivo*-isolated T_{reg} cells expressed T-bet (Fig. 1a,b). The T-bet-expressing T_{reg} cells were enriched in the spleen; ~10% of total T_{reg} cells in the spleen expressed T-bet but only 3-5% of T_{reg} cells in lymph nodes did so. Thus, the existence of T-bet- and GATA3-expressing T_{reg} cells in the steady state was confirmed in the tri-color reporter mouse model. Some T_{reg} cells expressed both T-bet and GATA3; the percentage of $GATA3^+$ cells among the $T\text{-}bet^+$ population was identical to the percentage of $GATA3^+$ cells among the $T\text{-}bet^-$ population (Fig. 1a). The vast majority of the T-bet-AmCyan positive T_{reg} cells expressed CXCR3 whether they were GATA3-GFP positive or not, consistent with the idea that CXCR3 is a gene target of T-bet (Supplementary Fig. 2a**)**. T_{reg} cell subsets were then separated based on their T-bet and GATA3 expression by cell sorting (Supplementary Fig. 2b**)**. Sorted $T\text{-bet-AmCyan}^+GATA3\text{-}GFP^-$ but not the $T\text{-bet-AmCyan}^-GATA3\text{-}GFP^-$ or $T\text{-bet-AmCyan}^-GATA3\text{-}GFP^+$ T_{reg} cells expressed T-bet (Supplementary Fig. 2c**)**. These results indicate that T-bet-AmCyan faithfully reports T-bet expression in T_{reg} cells.

Since more than 60% of the T_{reg} cells did not express either T-bet or GATA3, we next examined whether T-bet and GATA3 expression could be induced in such cells. Highly purified T-bet and GATA3 non-expressing T_{reg} cells by cell sorting (Supplementary Fig. 2b**)** were cultured with plate-bound anti-CD3 and anti-CD28 in the presence of IL-2 with addition of either IL-4 or IFN-γ. Foxp3 expression was stably maintained in these cultures **(**Fig. 1c**)**. About 16% of T_{reg} cells upregulated $GATA3\text{-}GFP^+$ when stimulated with IL-4, and more than 50% T_{reg} cells became $T\text{-bet-AmCyan}^+$ when cultured with IFN-γ (Fig. 1c).

To test whether T-bet and GATA3 expression could be induced in non-expressing T_{reg} cells *in vivo*, T-bet and GATA3 non-expressing T_{reg} cells were transferred into *Rag1*-deficient (*Rag1* $^{-/-}$) mice either alone or together with CD45.1 congenic naive ($CD4^+CD25^-CD45RB^{hi}$) T cells. Consistent with the report that T_{reg} cells are unstable when transferred alone into lymphopenic environments[24], more than 50% of the transferred cells became Foxp3 negative T cells (data not shown). T-bet and GATA3 were induced in a

portion of remaining Foxp3$^+$ cells; some cells expressing both T-bet and GATA3 (Fig. 1d). When co-transferred with naive T cells, Foxp3$^+$ cells retained Foxp3 expression, and T-bet and GATA3 were induced in these T_{reg} cells whether isolated from spleen, MLN or small intestine lamina propria (siLP) (Fig. 1e). T-bet was more efficiently induced in the siLP. The proportions of T-bet- and GATA3-expressing T_{reg} cells observed after transfer were similar to that in the steady state (Fig. 1a**)**. These results indicate that T-bet and GATA3 expression can be induced in non-expressing T_{reg} cells both *in vitro* and *in vivo*.

It has been previously reported that IFN-γ and STAT1 but not IL-12 and STAT4 are involved in T-bet induction in T_{reg} cells[18, 25, 26]. To confirm the signaling pathways required for T-bet expression in T_{reg} cells in our reporter system, we examined T_{reg} cells isolated from spleen and lymph nodes of the T-bet-ZsGreen reporter mice that were wild-type, *Stat1*$^{-/-}$, *Stat4*$^{-/-}$ or *IFN-gr1*$^{-/-}$. In agreement with the previous studies, our results showed that deficiency in STAT1 but not STAT4, dramatically reduced T-bet expression in T_{reg} cells (Fig. 1f and Supplementary Fig. 3). There was a partial reduction of T-bet-expressing T_{reg} cells in *IFN-gr1*$^{-/-}$ mice. Thus, these results indicate that T-bet expression in T_{reg} cells in the steady state depends on STAT1 activating cytokines including IFN-γ.

Dynamic expression of T-bet and GATA3 in T_{reg} cells

To assess whether the T-bet- and the GATA3-expressing T_{reg} cells represent stable subsets, we purified T-bet$^-$GATA3$^+$ and T-bet$^+$GATA3$^-$ T_{reg} cells by cell sorting (Supplementary Fig. 2b**)**. T-bet$^-$GATA3$^+$ T_{reg} cells were cultured with plate-bound anti-CD3, anti-CD28 and IL-2, in the presence or absence of TGF-β, with addition of either IL-4 or IFN-γ (Fig. 2a). Foxp3 expression was stably maintained under all conditions. In the absence of IL-4, IFN-γ and TGF-β, some T_{reg} cells lost GATA3 expression while others gained T-bet expression. TGF-β further reduced GATA3 expression but prevented T-bet upregulation. GATA3 expression was largely maintained when IL-4 was present, but T-bet expression was not induced. However, even in the presence of TGF-β, IFN-γ induced T-bet expression in ~40% T_{reg} cells that had been T-bet$^-$GATA3$^+$, while GATA3 expression was strikingly diminished. Similarly, when T-bet$^+$GATA3$^-$ T_{reg} cells were cultured with plate-bound anti-CD3 and anti-CD28 with IL-2 and TGF-β, T-bet expression in the majority of the cells was lost although Foxp3 expression was retained (Fig. 2b and data not shown). IFN-γ was able to maintain T-bet expression whereas IL-4 induced GATA3 expression in T cells that originated from T-bet$^+$GATA3$^-$ reg T_{reg} cells.

To examine whether T-bet- and GATA3-expressing T_{reg} cells are stable *in vivo*, we sorted T-bet$^-$GATA3$^+$ T cells were transferred into *Rag1*$^{-/-}$ reg mice. As expected, more than 50% of them became Foxp3 negative T cells (data not shown). Nevertheless, ~50% of the cells that maintained Foxp3 expression turned into T-bet$^+$GATA3$^+$, T-bet$^+$GATA3$^-$ or T-bet$^-$GATA3$^-$ T_{reg} cells (Fig. 2c). To further test the stability of the T-bet- and GATA3-expressing T_{reg} cells in an environment where Foxp3 expression can be maintained, T-bet$^-$GATA3$^+$ or T-bet$^+$GATA3$^-$ T were transferred into *Rag1*$^{-/-}$ reg mice together with congenic CD45.1 naive CD4$^+$ T cells (Fig. 2d). Most of the transferred T_{reg} cells retained Foxp3 expression as expected, however, the pattern of T-bet and GATA3 expression in T_{reg} cells became very similar in the recipient mice that received either T-bet$^-$GATA3$^+$ or T-

bet^{+}GATA3^{-} T_{reg} cells. We also transferred sorted T-bet^{-}GATA3^{+} cells into wild type congenic animals. The results support the idea that the expression of T-bet and GATA3 in T_{reg} cells are dynamic (data not shown). Overall, our results indicate that the expression of T-bet and GATA3 in T_{reg} cells is dynamic both *in vitro* and *in vivo*, and that T-bet- and GATA3-expressing T_{reg} cells do not represent stable T_{reg} subsets.

Fate-mapping T-bet-expressing T_{reg} cells

To confirm the dynamic feature of T-bet expression in T_{reg} cells in intact animals, we prepared a T-bet fate-mapping mouse strain. Offspring of T-bet-ZsGreen-T2A-CreER^{T2} and Rosa26-loxP-STOP-loxP-tdTomato mice (T-bet-fate-mapping mice) were treated with tamoxifen. In such mice, ZsGreen indicates T-bet expression while tdTomato reflects a cohort of cells that expressed T-bet at the time of tamoxifen treatment (Fig. 3a). Before tamoxifen injection, all the T cells including CD8 T cells were tdTomato negative as expected (Fig. 3a **and data not shown**). One week after a single tamoxifen injection into T-bet-fate-mapping mice, the majority of the CD8 memory-like (CD8^{+}CD44^{hi}) cells, which constantly express T-bet, were tdTomato positive (Fig. 3a). Only a small fraction of the ZsGreen-expressing CD8^{+}CD44^{hi} cells were not marked by tdTomato indicating a high efficiency of Cre-mediated reporter activation. Virtually all these tdTomato positive CD8 T cells were also expressing ZsGreen suggesting that T-bet expression in CD8 T cells is stable. However, within the CD4^{+}CD25^{+} T_{reg} compartment, the ZsGreen^{-} tdTomato^{+} population, which represents T-bet^{-} T_{reg} cells that had previously expressed T-bet, were present at a substantial proportion. In fact, these cells outnumbered the ZsGreen^{+}tdTomato^{+} population, which represents T-bet-expressing T_{reg} cells, in the peripheral and mesenteric lymph nodes (Fig. 3b). The fact that more than 60% of lymph node T-bet-expressing T_{reg} cells became T-bet non-expressing T_{reg} cells within 1 week confirms that T-bet expression in T_{reg} cells is highly dynamic. By contrast, the vast majority of the NK cells, CD8 memory-like (CD8^{+}CD44^{hi}) cells, or CD4^{+}CD25^{-} cells that were tdTomato positive continued to express ZsGreen suggesting that T-bet expression in NK, CD8 and conventional CD4 T cells is relatively stable (Fig. 3c). Although most of the ZsGreen^{+}tdTomato^{+} T_{reg} cells expressed CXCR3 as expected, a substantial proportion of ZsGreen^{-}tdTomato^{+} T_{reg} cells were also CXCR3^{+}, suggesting that the T-bet-expressed cells had up-regulated CXCR3 expression (Fig. 3d).

To test whether ZsGreen^{+} T_{reg} cells can give rise to ZsGreen^{-}tdTomato^{+} T_{reg} cells we sorted CD4^{+}CD25^{+}ZsGreen^{+} cells from the T-bet fate-mapping mice and adoptively transferred them together with effector T cells into *Rag1*$^{-/-}$ recipient mice treated with tamoxifen. Two weeks after transfer, ~ half of tdTomato^{+} T_{reg} cells had lost ZsGreen expression (Fig. 3e). By using the T-bet fate-mapping reporter, we confirmed that T-bet^{-} T_{reg} cells were originated from T-bet^{+} T_{reg} cells. Thus, our data indicate that T-bet expression in T_{reg} cells is highly dynamic in the steady state in intact animals.

T-bet or GATA3 single KO does not affect T_{reg} cell function

To study the functions of T-bet and GATA3 in T_{reg} cells, we generated mice with T_{reg}-specific deletion of either *Tbx21* or *Gata3* by crossing *Tbx21*$^{fl/fl}$ mice[27] or *Gata3*$^{fl/fl}$ mice[28] to *Foxp3*-Cre mice expressing yellow fluorescent protein (YFP)-Cre recombinase fusion

protein under the control of *Foxp3* locus[29]. *Tbx21*$^{fl/fl}$*Foxp3*-Cre and *Gata3*$^{fl/fl}$*Foxp3*-Cre mice had normal number of CD4$^+$ and CD8$^+$ T cells in spleen and lymph nodes (Fig. 4a and data not shown). The percentage of CD4$^+$Foxp3$^+$ T_{reg} cells from *Tbx21*$^{fl/fl}$*Foxp3*-Cre and *Gata3*$^{fl/fl}$*Foxp3*-Cre mice were similar to *Foxp3*-Cre mice (Fig. 4a). Furthermore, the vast majority of the CD4$^+$ and CD8$^+$ T cells in all the mice displayed naive phenotype (data not shown).

Extending a previous report[20] showing that *Gata3*$^{fl/fl}$*Foxp3*-Cre mice were healthy at a young age, we found that our *Gata3*$^{fl/fl}$*Foxp3*-Cre mice kept for more than six months did not develop any obvious abnormality before they were euthanized (data not shown). Similarly, no apparent abnormality was noted in adult *Tbx21*$^{fl/fl}$-*Foxp3*-Cre mice up to six months (data not shown). Therefore, the absence of either T-bet or GATA3 individually had no major impact on the function of T_{reg} cells in the steady state.

To assess whether the absence of either T-bet or GATA3 could have functional consequences on the ability of T_{reg} cells to control tissue inflammation, we utilized the well-described inflammatory bowel disease (IBD) model induced by T cell transfer into *Rag1*$^{-/-}$ recipients[30]. CD45.1 congenic naive CD4$^+$ T cells were transferred into *Rag1*$^{-/-}$ mice either alone or together with T_{reg} cells from *Foxp3*-Cre, *Tbx21*$^{fl/fl}$*Foxp3*-Cre or *Gata3*$^{fl/fl}$*Foxp3*-Cre mice. As expected, transferring naive CD4$^+$ T cells alone led to severe inflammation associated with significant weight loss and colon thickening (Fig. 4b). Co-transferring T_{reg} cells from *Foxp3*-Cre mice with naive CD4$^+$ T cells prevented disease. Similarly, T_{reg} cells from either *Tbx21*$^{fl/fl}$*Foxp3*-Cre or *Gata3*$^{fl/fl}$*Foxp3*-Cre mice were as effective as wild-type T_{reg} cells in preventing weight loss. The total cell number of congenic CD45.1$^+$CD4$^+$ T cells found in the small intestine lamina propria (siLP) was strikingly reduced in all the groups in which T_{reg} cells were co-transferred (Fig. 4c). These results indicate that T_{reg} cells deficient in either T-bet or GATA3 have substantial suppressive functions in this IBD model.

To assess the cross-regulation between T-bet and GATA3 in T_{reg} cells, T_{reg} cells from *Foxp3*-Cre, *Tbx21*$^{fl/fl}$*Foxp3*-Cre and *Gata3*$^{fl/fl}$*Foxp3*-Cre mice were cultured with plate-bound anti-CD3 and anti-CD28 in the presence of IL-2 with addition of either IL-4 or IFN-γ. *Tbx21*$^{fl/fl}$*Foxp3*-Cre T_{reg} cells expressed higher amounts of GATA3 protein in the presence of IFN-γ, whereas *Gata3*$^{fl/fl}$-*Foxp3*-Cre T_{reg} cells expressed higher levels of T-bet in the presence to IL-4 compared to WT T_{reg} cells cultured under the same conditions (Fig. 4d). A modest staining of GATA3 was noted in the group of IL-4-stimulated *Gata3*-deficient cells presumably because anti-GATA3 also recognizes the truncated GATA3 protein lacking functional zinc fingers encoded by exon 4. These results suggest that cross-regulation between T-bet and GATA3 may occur in T_{reg} cells within certain cytokine environments.

T-bet and GATA3 double deficiency results in autoimmunity

Since the expression of T-bet and GATA3 in T_{reg} cells are both dynamic and since T-bet and GATA3 may regulate each other under certain conditions, we asked whether T-bet and GATA3 have a redundant role in modulating T_{reg} cell functions. To test this, we generated *Tbx21*$^{fl/fl}$*Gata3*$^{fl/fl}$*Foxp3*-Cre double knockout (DKO) mice harboring T_{reg}-specific deletion of both T-bet and GATA3.

DKO mice were born at the expected Mendelian ratio and appeared to be as healthy as their littermates (either *Tbx21*$^{fl/fl}$*Gata3*$^{fl/+}$*Foxp3*-Cre or *Tbx21*$^{fl/fl}$*Gata3*$^{+/+}$*Foxp3*-Cre) during the first month of life. However, by 6-8 weeks of age, DKO mice displayed splenomegaly and lymphadenopathy (Fig. 5a), which was reflected by the increased total cell number (Fig. 5b). T-bet and GATA3 double ablation in T_{reg} cells led to marked increase in activated $CD4^+Foxp3^-CD62L^{lo}CD44^{hi}$ effector T cells (Fig. 5c). Histopathological evaluation of the DKO mice showed spleen and lymph node hyperplasia and massive cell infiltration in the liver, lung, kidney, small intestine and colon (Fig. 5d and data not shown), whereas control littermates did not show any noticeable pathology.

Consistent with their activated phenotype, increased capacity to produce each of the effector cytokines, including IFN-γ, IL-4 and IL-17, was observed (Fig. 5e). To test the consequences of IL-4 up-regulation, we measured levels of serum immunoglobulin isotypes in 8-week-old control, DKO and each single knockout mice. Consistent with an uncontrolled IL-4 and IFN-γ production in these mice, IgE serum concentrations were massively elevated in the DKO mice and serum IgG1 and IgG2a level was also significantly increased (Fig. 5f). Thus, both Th1- and Th2-related antibody responses are dysregulated when both T-bet and GATA3 are deficient in T_{reg} cells.

T_{reg} cells lacking both T-bet and GATA3 are unstable

We next explored whether T-bet and GATA3 double deficiency in T_{reg} cells impairs their generation, maintenance, and/or suppressive functions. DKO mice exhibited no significant alteration in the development of $Foxp3^+CD4^+$ T_{reg} cells in the thymus compared to WT mice (Supplementary Fig. 4a and 4b). Although the frequency of splenic $Foxp3^+$ T_{reg} cells in the DKO was slightly reduced compared to the control mice, the frequency of $Foxp3^+$ T_{reg} cells was significantly increased in the lymph nodes of the DKO mice (Supplementary Fig. 4c). DKO T_{reg} cells proliferate at a similar rate as WT T_{reg} cells judged by Ki-67 staining and DKO T_{reg} cells expressed normal levels of Bcl-2 (Supplementary Fig. 5). Therefore, DKO T_{reg} cells do not have proliferative or survival defects and the dramatic phenotype observed in the DKO mice is not because of reduced T_{reg} cells.

We next tested whether T-bet and GATA3 double deficiency in T_{reg} cells results in changes in the expression of T_{reg} signature genes. The expression of CD25 and CTLA-4 were normal while the expression GITR and CD103 were reduced in the DKO Treg cells compared to WT T_{reg} cells (Supplementary Fig. 6). There was a substantial reduction of Nrp-1 expression in the DKO T_{reg} cells. Although Nrp-1 has been reported to potentiate T_{reg} cell functions, it is dispensable for maintaining immune homeostasis[31]. Thus, defective expression in Nrp-1 in DKO T_{reg} cells cannot explain the autoimmune phenotype seen in these mice. The intensity of Foxp3 expression was substantially reduced in T_{reg} cells from the DKO mice compared to T_{reg} cells from the control mice (Fig. 5g). Consistent with a previous report, Foxp3 expression was only slightly reduced in *Gata3*$^{fl/fl}$*Foxp3*-Cre mice in comparison of *Foxp3*-Cre and *Tbx21*$^{fl/fl}$*Foxp3*-Cre mice. Since reduced Foxp3 expression often translates to T_{reg} cell instability, we performed transfer experiments with total splenocytes from the DKO mice. *Rag1*$^{-/-}$ mice, which received splenocytes from 8-week old DKO mice, developed IBD as manifested by weight loss (Fig. 5h). As expected, the

percentage of $CD4^+Foxp3^+$ T_{reg} cells were markedly decreased upon transfer confirming the instability of the T_{reg} cells lacking the expression of both T-bet and GATA3; higher proportion of these T_{reg} cells expressed RORγt compared to control T_{reg} cells (Fig. 5i). These results suggest that T_{reg} cell-specific deficiency of both T-bet and GATA3 affects their function and stability.

Double-deficient T_{reg} cells fail to suppress IBD

To assess suppressive function of T_{reg} cells in vivo, CD45.1 congenic naive T cells were transferred into *Rag1*$^{-/-}$ mice either alone or together with T_{reg} cells from 8-week-old DKO or control *Foxp3*-Cre mice. As expected, transfer of naive T cells alone led to severe inflammation associated with significant weight loss and colon pathology including massive inflammatory cell infiltration that was prevented when control *Foxp3*-Cre T_{reg} cells were co-transferred (Fig. 6a,b). However, T_{reg} cells from the DKO mice failed to suppress the disease. Similarly, the number of congenic $CD45.1^+CD4^+$ T cells in the siLP was significantly reduced by co-transferring control *Foxp3*-Cre T_{reg} cells but not DKO T_{reg} cells (Fig. 6c). Many DKO T_{reg} cells had lost Foxp3 protein expression whereas control T_{reg} cells were largely stable as expected (Fig. 6d). In the DKO cells that still expressed Foxp3, a significantly elevated proportion also expressed RORγt protein (Fig. 6e). Accordingly, DKO T_{reg} cells significantly upregulated the T_H17 effector cytokine IL-17A (Fig. 6f). Thus, T_{reg} cells deficient in both T-bet and GATA3 are defective in homeostasis and suppressive functions, associated with their abnormal T_H17 phenotype.

RORγt suppression by T-bet and GATA3 is cell intrinsic

To further assess whether the role of T-bet and GATA3 in T_{reg} cells is cell intrinsic, we prepared mixed bone marrow chimeras using the *Foxp3*-Cre, DKO, *Tbx21*$^{fl/fl}$*Foxp3*-Cre or *Gata3*$^{fl/fl}$*Foxp3*-Cre donors together with the CD45.1 congenic donors. The contribution of each donor to $CD4^+Foxp3^+$ T_{reg} cells was then examined. The frequencies of $Foxp3^+$ T_{reg} cells among the $CD45.1^+$ cells were the same in all groups as expected (data not shown). The frequencies of $Foxp3^+$ T_{reg} cells originating from *Foxp3*-Cre, *Tbx21*$^{fl/fl}$*Foxp3*-Cre or *Gata3*$^{fl/fl}$*Foxp3*-Cre bone marrow were similar, but the frequency of $Foxp3^+$ T_{reg} cells developed from DKO bone marrow progenitors was significantly reduced (Fig. 7a). In addition, RORγt protein expression was dramatically increased in $Foxp3^+$ T_{reg} cells derived from the DKO donor compared to T_{reg} cells from other donors and the MFI of Foxp3 from the DKO group was significantly lower (Fig. 7b). In fact, RORγt expression was mostly found within the $Foxp3^{lo}$ population in the DKO group and such cells expressed high amounts of CD44 indicating an activated T_{reg} population (Fig. 7c). The $CD44^{hi}$ T_{reg} cell population was significantly reduced in the DKO group compared to other groups indicating that without T-bet and GATA3, activated T_{reg} cells are either inefficiently generated or poorly maintained. In addition, the vast majority of the $CD44^{hi}$ T_{reg} cells in the DKO group expressed RORγt. Consequently, a substantial proportion of the $Foxp3^+$ T_{reg} cells from DKO donor were capable of producing IL-17A (Fig. 7d). Thus, these results indicate that the defects of DKO T_{reg} cells are cell intrinsic.

DISCUSSION

T_{reg} cells are critical for maintaining immune tolerance. When such cells are defective or absent, various types of autoreactive $CD4^+$ T effectors, including T_H1, T_H2 and T_H17 cells, become activated. The diversity of the T_{reg} cell population has evoked general interest by researchers in the field[32]. Whether defined subsets of T_{reg} cells are responsible for controlling distinct types of T cells either through a common or specific mechanism has been unclear.

It has been suggested that T_{reg} cells might utilize some components of the transcriptional machinery operating in distinct effector cells to effectively control a particular type of immune response, for example by showing that T_{reg} cells could use IRF4 to selectively control T_H2 immune responses[33]. However, IRF4 is not only important for T_H2 cell differentiation, but is also involved in T_H17 cell differentiation. Furthermore, IRF4 is expressed in all activated T_{reg} cells and has broader effects on T_{reg} cell activation[34]. Therefore, it remains possible that IRF4 is critical for optimal T_{reg} cell activation and that suppression appears selective because the control of the differentiation of T_H2 cells requires more robust T_{reg} cell function than does the control of T_H1 or T_H17 cells. The striking observation that T_{reg}-specific deletion of *Stat3* leads to uncontrolled T_H17 responses at 6 weeks of age suggests that T_{reg} cells need to receive comparable signals to those required to induce a particular type of effector cells in order to effectively suppress those effector cells[35]. It remains attractive to test whether STAT1 and STAT6 are indispensable for the T_{reg} cells to suppress T_H1 and T_H2 responses, respectively.

T-bet, the master regulator of T_H1 cells, is expressed by a subset of T_{reg} cells[18]. More importantly, it has been reported that T-bet deficient T_{reg} cells failed to thrive under T_H1 inflammatory environment and that these cells failed to suppress T_H1 response in a transfer model. However, the importance of T-bet expression by T_{reg} cells has not been directly tested in mice with T_{reg}-specific T-bet ablation. Furthermore, whether T-bet expression by T_{reg} cells is required for their ability to suppress T_H1-mediated autoimmunity in the steady state is unknown. Here we report that mice with T_{reg}-specific T-bet deletion did not develop any obvious autoimmunity, indicating that T-bet is dispensable for T_{reg} cell-mediated suppression of auto-reactive T_H1 cells in the steady state.

Similarly, GATA3 is expressed by a subset of T_{reg} cells. However, GATA3 expression in T_{reg} cells does not seem to be required for control T_H2-mediated autoimmunity in the steady state. Although it was reported that T_{reg}-specific ablation of GATA3 resulted in T_H2-related diseases starting at 16 weeks of age[21], two other mouse lines with T_{reg} cell-specific *Gata3* deletion failed to develop obvious abnormalities until the mice were more than half a year old[19, 20].

Our experiments were performed mainly in the steady state or under mild inflammation. Since T_{reg} cells function differently in the steady state versus under inflammatory conditions[36] or during T_H1 biased infections[37], the phenotype and functions of antigen-specific T_H1-like T_{reg} cells and the role of T-bet in T_{reg} cells during T_H1-promoting

infections require further investigation. Similarly, it will be interesting to investigate the function of GATA3 in T_{reg} cells during robust T_H2 responses *in vivo*.

Although ablation of T-bet or GATA3 alone in T_{reg} cells has no major impact on T_{reg} cell functionality in the steady state, mice in which both genes are deleted in T_{reg} cells develop a severe autoimmune-like phenotype beginning at 6-8 weeks of age. These results suggest T-bet and GATA3 are redundant in maintaining T_{reg} cell functions. Reduced functionality of these T_{reg} cells is associated with upregulation of RORγt and downregulation of Foxp3 expression in a cell intrinsic manner. Foxp3 and RORγt are normally co-expressed in T cells treated with large amounts of TGF-β and in a subset of cells in the siLP[16]. Foxp3 antagonizes RORγt function partly by protein-protein interaction. However, it is also possible that RORγt antagonizes Foxp3 function in T_{reg} cells. Therefore, RORγt expression in T_{reg} cells may require careful control. One way that T_{reg} cells could fully suppress RORγt expression is to express T-bet and/or GATA3, both of which are capable of suppressing RORγt expression in effector T cells.

Redundancy of T-bet and GATA3 in T_{reg} cells can be explained by their dynamic expression pattern. Our current study indicates that T-bet-expressing cells can convert into GATA3-expressing cells, and *vice versa*, under the influence of cytokines, both *in vitro* and *in vivo*. Such plasticity or flexibility of T_{reg} cells by changing their pattern of transcription factor expression within the $Foxp3^+$ compartment is different from the previously reported T_{reg} cell plasticity with loss of Foxp3 expression and gain of effector functions under inflammatory conditions[37, 38, 39].

T_{reg} cells need to be activated to acquire full suppressive activity[40, 41]. T-bet expression is associated with activated T_{reg} cells since T-bet-expressing T_{reg} cells expressed higher T_{reg} cell markers, including CD103, GITR and CTLA4, than T-bet-non-expressing T_{reg} cells[18]. We also found that T-bet is mainly expressed by $CD44^{hi}$ T_{reg} cells. Moreover, T-bet and GATA3 double deficient T_{reg} cells contain less $CD44^{hi}$ T_{reg} population. Because of the dynamic expression pattern, some T-bet- and GATA3-non-expressing cells might have expressed these molecules at an earlier time and thus consist of activated T_{reg} cells. We propose that when T_{reg} cells are activated in a local environment, they upregulate T-bet or GATA3 to prevent RORγt expression and to maintain Foxp3 expression.

Overall, we demonstrate that unlike T_H1 and T_H2 effector cells, 'T_H1-like' and 'T_H2-like' T_{reg} cells in the steady state are not stable subsets and that T-bet expression in T_{reg} cells is not required for these cells to suppress T_H1 effectors. Furthermore, our data demonstrate that T-bet and GATA3 have a redundant role in controlling RORγt expression in activated T_{reg} cells indicating that T_{reg} cells can utilize multiple cross-regulatory mechanisms found in T effector cells to overcome the close relationship between T_{reg} and T_H17 cells and thus maintain T cell homeostasis.

ONLINE METHODS

Mice

The C57BL/6 T-bet-AmCyan reporter mouse strain was generated in the same way as we previously prepared the C57BL/6 T-bet-ZsGreen reporter mouse strain except for using a different fluorescent marker. The coding region of the AmCyan was amplified from pAmCyan1-N1 vector (Clontech) by PCR for recombineering using the following primers: 5′- GAC CCT CGG GTC TCT TCG ACG GCT GCT GGA AGG CGC CCA GCC CGC CTC GGA TGG CCC TGT CCA ACA AGT TCA TC -3′ and 5′- CAC TGC ATT CTA GTT GTG GTT TGA TGG GCA TCG TGG AGC CGG GCT GCG GAG ACA TGC TGA CCG GCA CCG AGC C -3′. The modified BAC clone, after sequence verification of the manipulated region, was used to generate transgenic mice by pronuclear microinjection of fertilized C57BL/6 eggs. Total of 6 founders were identified to be positive for AmCyan as assessed by Southern blot and PCR. The offspring of the founder (B6-Tbet-AmCyan-B10) that carried only one copy of the transgene were selectively maintained. Foxp3-mRFP knock-in mice (C57BL/6-*Foxp3*^{tm1Flv}/J)[22] were purchased from the Jackson Laboratory (#008374). GATA3-GFP reporter mice (*Gata3*$^{g/+}$) on the C57BL/6 background were provided by Dr. James Douglas Engel[23]. T-bet-AmCyan reporter mice were bred with *Foxp3*^{tm1Flv}/J to generate T-bet-AmCyan:Foxp3-RFP mice; these mice were further bred with *Gata3*$^{g/+}$ mice to generate T-bet-AmCyan:GATA3-GFP:Foxp3-mRFP tri-color reporter mice. The Tri-color reporter mice were genotyped using the following primers: for T-bet-AmCyan: 5′- GTA GGT GAA GGT TCT CTC GTA G -3′, 5′- GAC AAG AGA CTT ACA CTT AGG AGT G -3′; for *Gata3*$^{g/+}$, 5′- CAG GTG ATC GGA AGA GCA AC -3′, 5′- GTT TGC AGT TAA GGG TAT AG -3′; for *Foxp3*^{tm1Flv}/J, 5′- CAA AAC CAA GAA AAG GTG GGC -3′, 5′- CAG TGC TGT TGC TGT GTA AGG GTC -3′, and 5′- GGA ATG CTC GTC AAG AAG ACA GG -3′.

Tbx21$^{fl/fl}$ mice[27] (kindly provided by Dr. Steve Reiner, University of Columbia) and *Gata3*$^{fl/fl}$ mice[28] were on the C57BL/6 background. *Tbx21*$^{fl/fl}$ mice or *Gata3*$^{fl/fl}$ mice were crossed with Foxp3-IRES-YFP-Cre mice[29] (kindly provided by Dr. Alexander Rudensky, Memorial Sloan Kettering Cancer Center, New York) to generate *Tbx21*$^{fl/fl}$- *Foxp3*-Cre or *Gata3*$^{fl/fl}$-*Foxp3*-Cre mice. *Tbx21*$^{fl/fl}$-*Gata3*$^{fl/fl}$-*Foxp3*-Cre (DKO) mice were generated by crossing *Tbx21*$^{fl/fl}$-*Foxp3*-Cre with *Gata3*$^{fl/fl}$-*Foxp3*-Cre mice. All animals were genotyped using the following primers: for *Foxp3*-Cre, primer pair-1: 5′- CCA GAT GTT GTG GGT GAG TG -3′, 5′- TGG ACC GTA GAT GAA TTT GAG TT -3′ and primer pair-2: 5′- AGG ATG TGA GGG ACT ACC TCC TGT A -3′, 5′- TCC TTC ACT CTG ATT CTG GCA ATT T -3′; for *Tbx21*$^{fl/fl}$, 5′- TAT GAT TAC ACT GCA GCT GTC TTC AG -3′, 5′- CAG GAA TGG GAA CAT TCG CCT GTG -3′, and 5′- CTC TGC CTC CCA TCT CTT AGG AGC -3′; for *Gata3*$^{fl/fl}$, 5′-TCA GGG CAC TAA GGG TTG TTA ACT T-3′, 5′-GAA TTC CAT CCA TGA GAC ACA CAA-3′.

C57BL/6 T-bet-ZsGreen reporter (TBGR, Taconic Line 8419), *IFN-gr1*$^{-/-}$-T-bet-ZsGreen (Taconic Line 8457) and *Stat4*$^{-/-}$-T-bet-ZsGreen mice (Taconic Line 8452) were previously described [6] and deposited into the NIAID-Taconic repository. CD45.1 congenic mice (Line 7) and *Rag1*$^{-/-}$ mice (Line 146) were also from the NIAID-Taconic repository. *Stat1*$^{-/-}$-T-

bet-ZsGreen mice were generated by crossing *Stat1*$^{-/-}$ mice[42] (kindly provided by Dr. Dragana Jankovic of the NIAID, originally from Dr. Joan Durbin) with TBGR mice.

The generation of T-bet-ZsGreen-T2A-CreER^{T2} mouse line will be described in detail in another study. Briefly, ZsGreen-T2A-CreER^{T2}, a DNA cassette containing sequences encoding ZsGreen, a "self-cleaving" T2A peptide[43], and a fusion Cre recombinase with mutated human estrogen receptor ligand-binding domain (CreER^{T2})[44] obtained from Addgene, was inserted into the T-bet translational start site in the BAC clone RP23-237M14. In the resulting BAC transgenic mice, T-bet-expressing cells will express both ZsGreen and CreER^{T2} as separated proteins. The T-bet-ZsGreen-T2ACreER^{T2} mice were then bred with the reporter mice carrying a loxP site–flanked STOP cassette and a DNA sequence encoding red fluorescent protein variant, tdTomato, under the control of ubiquitously expressed ROSA26 locus (JAX mice line #007914)[45]. Offspring of the T-bet-ZsGreen-T2A-CreER^{T2} and Rosa26-loxP-STOP-loxP-tdTomato mice were designated as T-bet-fate-mapping mice. Cre-mediated recombination was induced by tamoxifen treatment. Tamoxifen (Sigma) dissolved in corn oil (Sigma) was injected intraperitoneally (i.p.) into T-bet-fate-mapping mice at day 0 (3mg tamoxifen in 150ul corn oil per mouse per injection). Mice were analyzed at day 7 after the initial injection.

All the mice were bred and/or maintained in the NIAID specific pathogen free animal facility and the experiments were done when mice were 6 to 16 weeks of age under protocols approved by the NIAID Animal Care and Use Committee.

Cell preparation and cell culture

Single cell suspension was prepared directly from different lymphoid organs of mice including lymph nodes, spleen, thymus and bone marrow. Cells from small intestinal lamina propria were prepared as previously described[46]. Lymph node cells and splenocytes from T-bet-AmCyan:GATA3-GFP:Foxp3-RFP tri-color reporter mice were stained with APC-anti-CD4, and then sorted for CD4$^+$RFP$^+$AmCyan$^-$GFP$^-$, CD4$^+$RFP$^+$AmCyan$^+$GFP$^-$ and/or CD4$^+$RFP$^+$AmCyan$^-$GFP$^+$ populations using FACSAria (BD Biosciences). Lymph node cells from *Foxp3*-Cre, *Tbx21*$^{fl/fl}$-*Foxp3*-Cre, *Gata3*$^{fl/fl}$-*Foxp3*-Cre and DKO mice were stained with APC-anti-CD4 and PE-anti-CD25, and then sorted for CD4$^+$CD25$^+$ YFP$^+$ population. Naive CD4$^+$ T cells from CD45.1 congenic mice (Taconic Line 7) were purified by cell sorting for the CD4$^+$CD25$^-$CD45Rb^{hi} population after staining lymph node cells with FITC-anti-CD4, PE-anti-CD25 and APC-anti-CD45Rb.

RPMI 1640 media (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone), 50μM β-mercaptoethanol (Sigma), 1% sodium pyruvate, 1% nonessential amino acids, 1% HEPES, 100U/ml penicillin and 100μg/ml streptomycin, and 2mM L-glutamine (all from Invitrogen) was used as culture medium. The cells were incubated at 37°C and under 5% CO2 for the indicated time. Sorted T_{reg} cells were stimulated with plate-bound anti-CD3 (2C11) and anti-CD28 (37.51) in IL-2 (100U/ml) containing RPMI 1640 media alone or with different combinations of cytokines: TGF-β1 (5ng/ml), IL-4 (1ng/ml) or IFN-γ (1ng/ml) were added as indicated. All cytokines used in cell culture were purchased from PeproTech.

In vitro T cell stimulation and flow cytometry analysis

Staining of cell surface molecules was carried out using PBS with 2% FBS. For intracellular staining of cytokines, cells were first stimulated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 nM ionomycin for 4 hours, or with plate-bound anti-CD3/anti-CD28 for 5 hours, in the presence of 2 mM monensin, and then stained with a cocktail of fixable viability dye (eBioscience) and antibodies to various cell surface markers. They were then fixed with 4% paraformaldehyde for 10 min at room temperature and permeabilized in PBS containing 0.5% Triton X-100 and 0.1% BSA before staining for cytokines. Staining for transcription factors was performed with Foxp3 Staining Buffer Set (eBioscience) according to the manufacturer's instructions. Flow cytometry data were collected with LSR II (BD Biosciences) and results were analyzed by using FlowJo software (Tree Star). Antibodies specific for mouse CD4 (RM4-5), CD8 (53-6.7), CD25 (PC61.5), CD44 (IM7), CD45.1 (A20), CD45.2 (104), CD45Rb (C363.16A), CD357 (GITR, DTA-1), CD152 (CTLA-4, UC10-4B9), Ki-67 (SolA15), IL-4 (11B11), IFN-γ (XMG1.2), IL-17A (TC11-18H10), Foxp3 (FJK-16s), RORγt (AFKJS-9), T-bet (eBio4B10), and Fixable Viability Dye eFluor® 506 were purchased from eBioscience; antibodies specific for CD103 (M290), Bcl-2 (3F11), IFN-γ (XMG1.2), and GATA3 (L50-823) were purchased from BD Biosciences; antibody specific for mouse neuropilin-1 (761705) was purchased from R&D systems; antibody specific for mouse $F_C\gamma$II/III (2.4G2) was prepared by Harlan.

Serum immunoglobulin ELISA

Serum IgE concentrations were measured using Mouse IgE ELISA Ready-SET-Go kit (eBioscience). Serum IgG1 and IgG2b were measured using Mouse Ig Isotyping ELISA Ready-Set-Go kit (eBioscience). Experiments were performed following the manufacturer's instructions.

T cell transfer model of colitis

$CD4^+CD25^+$ YFP^+ T cells from *Foxp3*-Cre, *Tbx21*$^{fl/fl}$ reg -*Foxp3*-Cre, *Gata3*$^{fl/fl}$-*Foxp3*-Cre and DKO mice (CD45.2) and naive $CD4^+CD25^-CD45RB^{hi}$ T cells from CD45.1 congenic mice (Line 7) were prepared by cell sorting and then washed in sterile PBS. Each *Rag1*$^{-/-}$ recipient mouse (Line 146) was injected intravenously with either 2×10^5 naive $CD4^+CD25^-CD45RB^{hi}$ T cells alone, or together with 5×10^4 $CD4^+CD25^+YFP^+$ T_{reg} cells. T_{reg} cells purified from tri-color mice were transferred alone into *Rag1*$^{-/-}$ mice for 2 weeks, or co-transferred with CD45.1 congenic naive $CD4^+CD25^-CD45RB^{hi}$ T cells into *Rag1*$^{-/-}$ mice for 8 weeks before analysis. For splenocytes transfer experiments, 10×10^6 splenocytes from either *Foxp3*-Cre or DKO mice were injected into each *Rag1*$^{-/-}$ recipient (Line 146). Body weight of mice was monitored weekly in all experiments.

Bone marrow chimeras

Bone marrow cells from femurs of *Foxp3*-Cre, *Tbx21*$^{fl/fl}$-*Foxp3*-Cre, *Gata3*$^{fl/fl}$-*Foxp3*-Cre and DKO mice (CD45.2) were mixed with those from CD45.1 congenic mice (Line 7) at 1:1 ratio and then injected retro-orbitally (10 million cells per mouse) into *Rag1*$^{-/-}$ mice (Line 146) after lethal irradiation (450 Rads twice with 2-3 hours apart).

Statistics

Groups were compared with Prism 6 software (GraphPad) using a 2-tailed unpaired Student's t test or an ordinary one-way ANOVA. Data are presented as mean ± SEM. $P < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENT

We thank Drs. Ethan Shevach, Yasmine Belkaid and William Paul for their critical reading of our manuscript and helpful discussions. We also thank Dr. Liying Guo for the help on ELISA experiments, Drs. Nezih Cereb and Soo Young Yang at Histogenetics (Ossining, NY) and Dr. Steven Reiner of Columbia University (NY, NY) for constructing and providing the floxed *Tbx21* mice, and the NIAID flow cytometry core facility for cell sorting. The work is supported by the Division of Intramural Research, NIAID, National Institutes of Health, USA.

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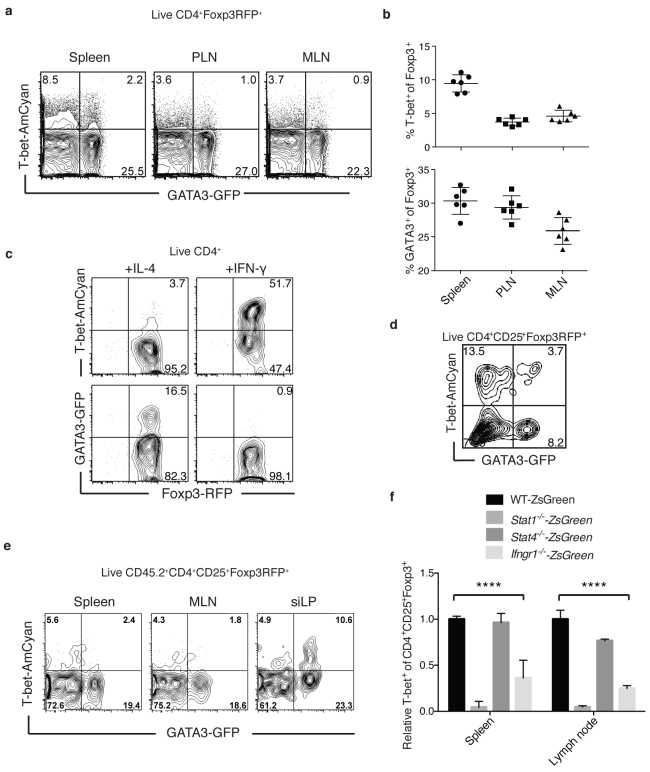

Figure 1.

T-bet and GATA3 expression in T_{reg} cells can be induced by cytokines. (**a**) Flow cytometric analysis of T-bet and GATA3 expressing T_{reg} cells *ex vivo* isolated from spleen, peripheral (PLN) and mesenteric lymph node (MLN) of the T-bet-AmCyan:GATA3-GFP:Foxp3-RFP tri-color reporter mice. Plots were gated on the $CD4^+CD25^+Foxp3RFP^+$ population. (**b**) Graphical summary of the data in (**a**) showing percentage of $T\text{-}bet^+GATA3^-$ (top panel) and $T\text{-}bet^-GATA3^+$ (bottom panel) T_{reg} cells in the spleen, PLN and MLN. Each dot represents an individual mouse. (**c**) Sorted $Foxp3RFP^+T\text{-}betAmCyan^-GATA3GFP^-$ T_{reg} cells (harvested from 3 mice) were cultured with plate-bound anti-CD3+anti-CD28 and IL-2 in the presence of either IL-4 or IFN-γ for 4 days. Flow cytometric analysis of T-bet and GATA3 expressing T_{reg} cells was carried out. Data are representative of three independent experiments with a single culture for each condition. (**d**) Flow sorted $Foxp3RFP^+T\text{-}betAmCyan^-GATA3GFP^-$ T_{reg} cells (harvested from 3 mice) were transferred into *Rag1*$^{-/-}$ mice. T-bet and GATA3 expressing T_{reg} cells from MLN were analyzed by flow cytometry 2 weeks later. Data are representative of two independent experiments with 2 mice each. (**e**) Sorted $Foxp3RFP^+T\text{-}betAmCyan^-GATA3GFP^-$ T_{reg} cells (harvested from 3 mice) were cotransferred with congenic naive $CD45.1^+CD4^+CD25^-CD45RB^{hi}$ T cells into *Rag1*$^{-/-}$ mice. T-bet and GATA3 expressing T_{reg} cells from spleen, MLN and small intestine lamina propria (siLP) were analyzed by flow cytometry 8 weeks later. Data are representative of two independent experiments with 4-5 mice each. (**f**) Graph depicts the relative proportion of $T\text{-}bet\text{-}ZsGreen^+CD25^+Foxp3^+$ T among the $CD25^+$ reg $Foxp3^+$ cells in spleen and lymph nodes of wild type T-bet-ZsGreen, *Stat1*$^{-/-}$-T-bet-ZsGreen, *Stat4*$^{-/-}$-T-bet-ZsGreen and *IFN-gr1*$^{-/-}$-T-bet-ZsGreen mice. Values from wild-type group are set as 1. Data are representative of two independent experiments. Error bars represent standard deviation of

the mean (n=3 each group). Statistical significance was determined by an ordinary one-way ANOVA. ****p<0.0001.

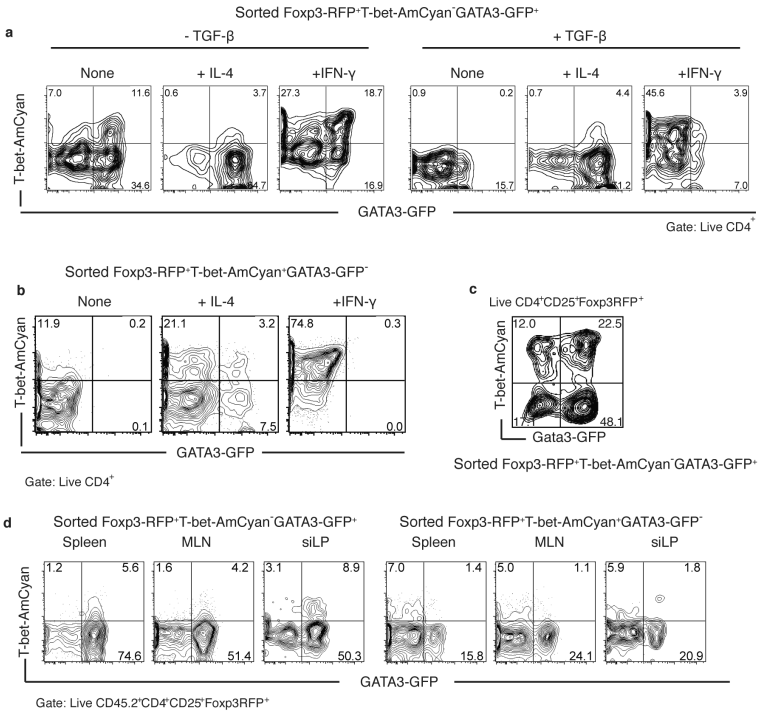

Figure 2.

Dynamic expression of T-bet or GATA3 in T_{reg} cells *in vitro* and *in vivo.* (**a**) Flow sorted Foxp3RFP^{+}T-betAmCyan^{-}GATA3GFP^{+} T_{reg} cells (harvested from 3 mice) were cultured with plate-bound anti-CD3+anti-CD28 and IL-2, in the presence (right panel) or absence of exogenous TGF-β (left panel) for 4 days. IL-4 or IFN-γ was added into some culture as indicated. Flow cytometric analysis of T-bet and GATA3 expressing T_{reg} cells are shown. Data are representative of three independent experiments with a single culture for each condition. (**b**) Flow sorted Foxp3RFP^{+}T-betAmCyan^{+}GATA3GFP^{-} T_{reg} cells (harvested from 3 mice) were cultured in the medium with plate-bound anti-CD3+anti-CD28, and IL-2 in the presence of TGF-β for 4 days. IL-4 or IFN-γ was added in some culture as indicated. T-bet and GATA3 expression in T_{reg} cells was analyzed by flow cytometry. Data are representative of three independent experiments with a single culture for each condition. (**c**) Sorted Foxp3RFP^{+}T-betAmCyan^{-}GATA3GFP^{+} T_{reg} cells (harvested from 3 mice) were transferred into *Rag1*$^{-/-}$ mice. T-bet and GATA3 expressing T_{reg} cells from MLN were analyzed by flow cytometry two weeks later. Data are representative of two independent experiments with 2 mice each. (**d**) Sorted Foxp3RFP^{+}T-betAmCyan^{-}GATA3GFP^{+} (three left panels) or Foxp3RFP^{+}T-betAmCyan^{+}GATA3GFP^{-} (three right panels) T_{reg} cells (harvested from 3 mice) were cotransferred with congenic naive CD45.1^{+}CD4^{+}CD25^{-}CD45RB^{hi} T cells into *Rag1*$^{-/-}$ mice. T-bet and GATA3 expressing T_{reg} cells from spleen, MLN and siLP were nalyzed by flow cytometry 8 weeks later. Data are representative of two independent experiments with 4-5 mice each.

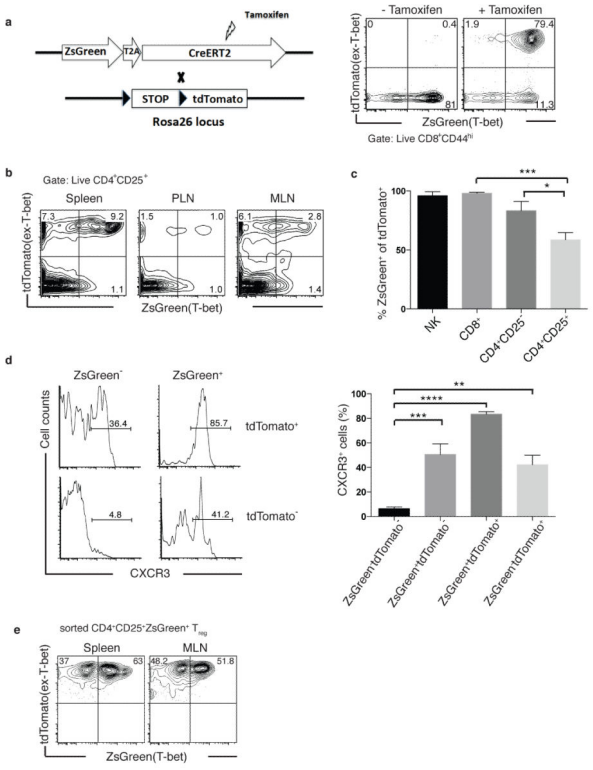

Figure 3.

Dynamic expression of T-bet in T_{reg} cells is confirmed by the T-bet fate-mapping tool. (**a**) Illustration of the tamoxifen-inducible T-bet fate-mapping mice (T-bet-ZsGreen-T2A-CreER^{T2} X Rosa26-loxP-STOP-loxP-tdTomato). (**a-d**) Tamoxifen was injected intraperitoneally (i.p.) into T-bet-fate-mapping mice on day 0, and then cells were analyzed on day 7. Flow cytometric analyses of ZsGreen and tdTomato expression in CD8^{+}CD44^{hi} cells isolated from spleen of tamoxifen-treated and untreated T-bet fate-mapping mice are shown (**a**). Flow cytometric analyses of ZsGreen and tdTomato expression in CD4^{+}CD25^{+} T_{reg} cells isolated from spleen, PLN and MLN of tamoxifen-treated T-bet fate-mapping mice (n=3) are shown (**b**). ZsGreen^{+}tdTomato^{+} indicates T-bet-expressing cells, and ZsGreen^{-}tdTomato^{+} indicates T-bet-expressed cells. The percentages of ZsGreen^{+} cells among the tdTomato^{+} cells within NK, CD8, conventional CD4 and T_{reg} cells in the spleen were plotted (**c**). CXCR3 expression on each T_{reg} population was assessed by surface staining (**d**). (**e**) Sorted CD4^{+}CD25^{+}ZsGreen^{+} T_{reg} cells from three T-bet fate-mapping mice were cotransferred with congenic naive CD45.1^{+}CD4^{+}CD25^{-}CD45RB^{hi} T cells into two *Rag1*$^{-/-}$mice treated with tamoxifen. ZsGreen and tdTomato expression by the transferred T_{reg} cells from spleen and MLN were analyzed by flow cytometry 2 weeks later. Data are representative of three (**b-d**) and two (**e**) independent experiments with 3-4 mice (**b-d**) or 2 mice (**e**) each. Error bars represent standard deviation of the mean (n=3 each group). Statistical significance was determined by a two-tailed unpaired Student' s *t* test (**c and d**). *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

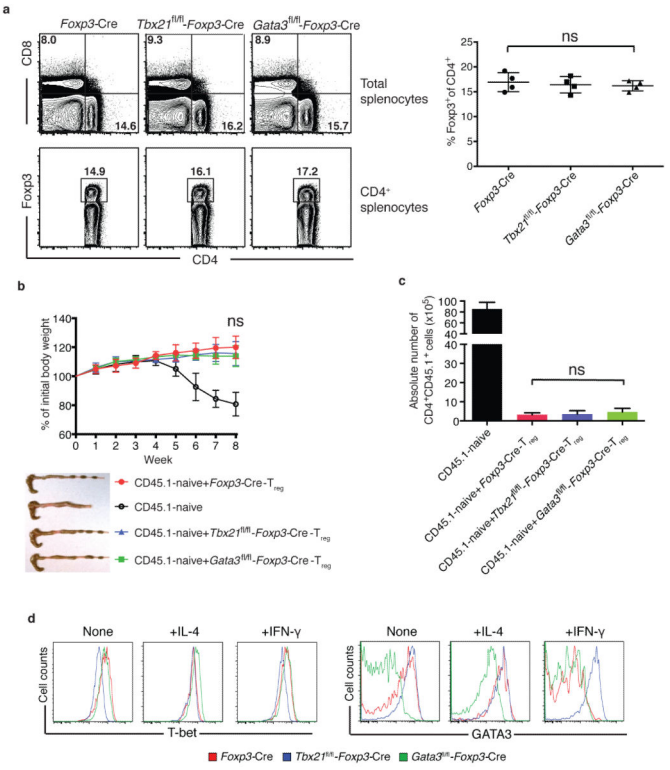

Figure 4.

T_{reg} cells singly devoid of either T-bet or GATA3 are functional in protecting against T cell-mediated colitis. (**a**) Flow cytometric analysis of percentage of $CD4^+$ and $CD8^+$ T cells (upper panel), and percentage of $Foxp3^+CD4^+$ T_{reg} cells (bottom panel; plots are gated live $CD4^+$) in the spleens of 8-week old *Foxp3*-Cre, *Tbx21*$^{fl/fl}$*Foxp3*-Cre and *Gata3*$^{fl/fl}$*Foxp3*-Cre mice (n=4 each group). (**b,c**) *Rag1*$^{-/-}$ mice received congenic $CD45.1^+CD4^+CD25^-CD45RB^{hi}$ (CD45.1-naive) cells alone or in combination with $CD4^+CD25^+YFP^+$ T cells from 8 weeks-old *Foxp3*-Cre, *Tbx21*$^{fl/fl}$ reg *Foxp3*-Cre and *Gata3*$^{fl/fl}$*Foxp3*-Cre mice. Mice were weighed weekly (**b**). Photographs of representative colon thickening from *Rag1*$^{-/-}$ mice 8 weeks after transfer are shown (**b**). Graphical representation of absolute number of congenic $CD45.1^+CD4^+$ effector cells harvested from the siLP (**c**). (**d**) Sorted $CD4^+CD25^+$Foxp3-YFP^+ T_{reg} cells from *Foxp3*-Cre, *Tbx21*$^{fl/fl}$*Foxp3*-Cre, or *Gata3*$^{fl/fl}$*Foxp3*-Cre mice were cultured with plate-bound anti-CD3+anti-CD28 and IL-2 in the presence of either IL-4 or IFN-γ for 4 days. Flow cytometric analysis of T-bet and GATA3 expression was carried out. Data are representative of two (**a-d**) independent experiments. Error bars represent standard deviation of the mean (n=4 for **a**; n=5 for **b** and **c**). Statistical significance was determined by an ordinary one-way ANOVA. ns indicates p value has no significant difference.

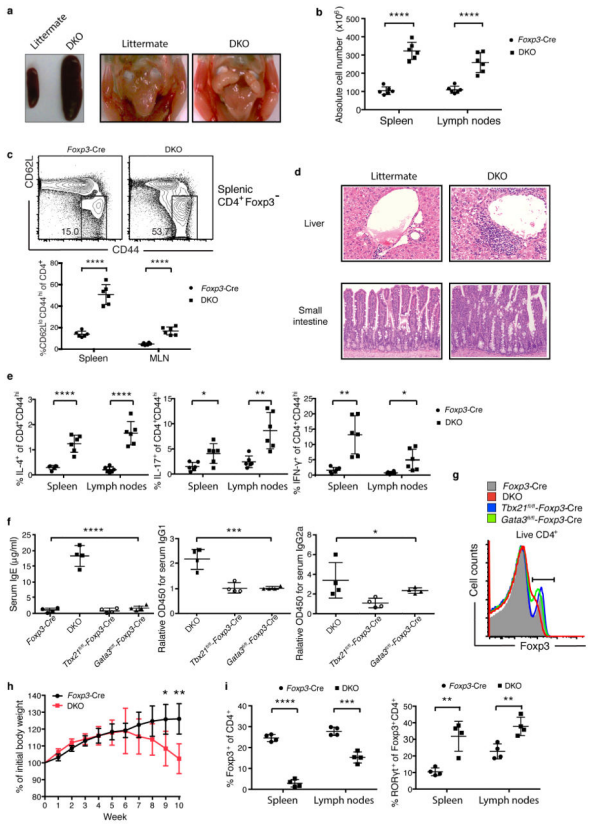

Figure 5.

Ablation of both T-bet and GATA3 in T_{reg} cells results in autoimmune lymphoproliferative disease. (**a**) Splenomegaly and lymphoid hyperplasia in superficial cervical lymph nodes in an 8-week old *Tbx21*$^{fl/fl}$*Gata3*$^{fl/fl}$*Foxp3*-Cre (DKO) mouse in comparison with its littermate (*Tbx21*$^{fl/fl}$*Gata3*$^{fl/+}$*Foxp3*-Cre). Every DKO mouse (more than 20 analyzed at 8-13 weeks of age) displayed splenomegaly and lymphadenopathy. (**b**) Spleen and lymph node cellularity in *Foxp3*-Cre and DKO mice (n=6). (**c**) Flow cytometric analysis of CD44 and CD62L expression on CD4^{+}Foxp3^{-} T cells from spleen and MLN of 8-week-old *Foxp3*-Cre and DKO mice (n=6). (**d**) Representative pictures with hematoxylin and eosin (H&E)-staining of the tissue sections (liver and small intestine) from an 8-week old littermate and DKO mouse are shown. Original magnification, 200X. (**e**) Flow cytometric analysis of IL-4, IL-17A and IFN-γ production by CD4^{+}CD44^{hi}Foxp3^{-} T cells from spleen and lymph node of the *Foxp3*^{Cre} and DKO mice (8-13 weeks old, n=6) stimulated with plate-bound anti-CD3 and anti-CD28. (**f**) Analysis of IgE concentration in the sera of 8-week old *Foxp3*-Cre, DKO, *Tbx21*$^{fl/fl}$*Foxp3*-Cre or *Gata3*$^{fl/fl}$*Foxp3*-Cre mice (n=4). Relative OD450 reads of IgG1 and IgG2a in the sera of DKO, *Tbx21*$^{fl/fl}$*Foxp3*-Cre or *Gata3*$^{fl/fl}$*Foxp3*-Cre mice compared to the *Foxp3*-Cre group are shown. (**g**) Histogram plots show the expression of Foxp3 in splenic CD4^{+} T cells from different mice. (**h,i**) *Rag1*$^{-/-}$ mice received splenocytes of the *Foxp3*-Cre or DKO mice. Mice were weighed weekly (**h**). Percentages of Foxp3^{+}CD4^{+} cells among the total CD4^{+} cells (left panel) and percentages of RORγt^{+} cells among the Foxp3^{+}CD4^{+} T_{reg} cells (right panel) from the spleen and MLN 10 weeks after transfer were plotted (**i**). Data represent three (**b, c, e**) and two (**f-i**) independent experiments. Error bars represent standard

deviation of the mean (n=6 for **b**, **c**, **e**; n=4 for **f**, **h** and **i**). Statistical significance was determined by a two-tailed unpaired Student' s *t* test (**b, c, e, h and i**) or an ordinary one-way ANOVA (**f**). ns indicates p value has no significant difference, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

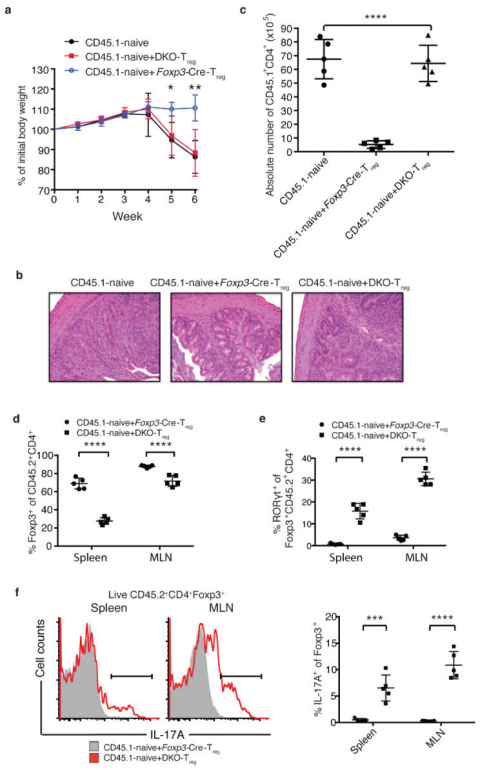

Figure 6.

T-bet and GATA3 double-deficient T_{reg} cells do not suppress colitis. (**a-f**) *Rag1*$^{-/-}$ mice received congenic CD45.1$^+$CD4$^+$CD25$^-$CD45RB^{hi} (CD45.1-naive) alone or in combination with CD4$^+$CD25$^+$YFP$^+$ T_{reg} cells from *Foxp3*-Cre or DKO mice. Mice were weighed weekly (**a**). Colon samples taken for H&E staining (**b**). Original magnification, 100X. Graphical representation of absolute number of congenic CD45.1$^+$CD4$^+$ T cells harvested from the siLP (**c**). Percentages of Foxp3$^+$ cells among the CD4$^+$CD45.2$^+$ population (**d**) and percentages of RORγt$^+$ cells among the Foxp3$^+$CD4$^+$CD45.2$^+$ population (**e**) harvested from spleen and MLN of *Rag1*$^{-/-}$ mice are shown. Histogram plots show IL-17A expression in DKO and control *Foxp3*^{Cre} T_{reg} cells (**f**, left panels) harvested from spleen and MLN of *Rag1*$^{-/-}$ mice received CD45.1-naive in combination with T_{reg} cells from *Foxp3*^{Cre}, and with DKO T_{reg}. Graphical representation of percentage of IL-17A$^+$ cells among the Foxp3$^+$ T_{reg} cells in the spleen and MLN (**f**, right panels). Data are representative of three independent experiments (**a-f**). Error bars represent standard deviation of the mean (n=5). Statistical significance was determined by a two-tailed unpaired Student' s *t* test (**a, d, e and f**) or an ordinary one-way ANOVA (**b**). *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

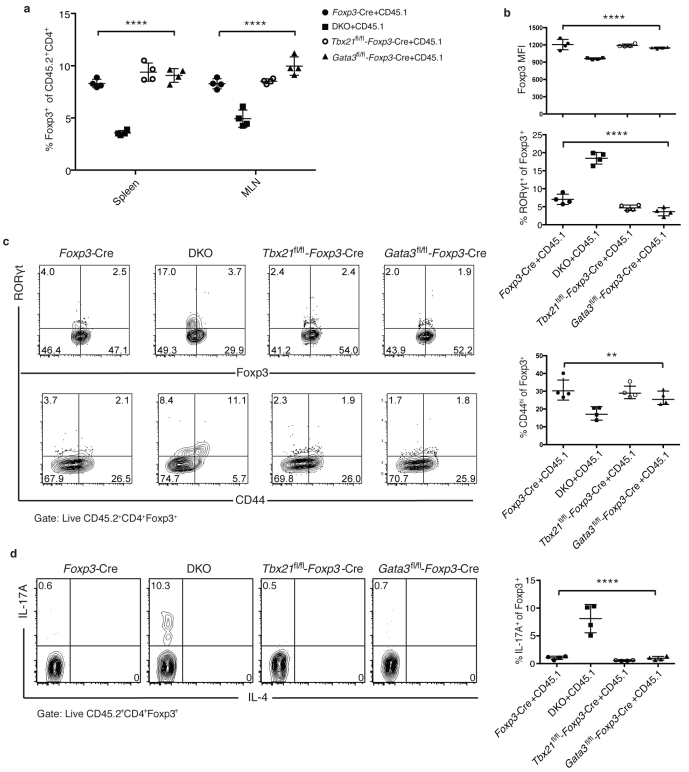

Figure 7.

The dysfunction of DKO T_{reg} cells is cell intrinsic. (**a-c**) Bone marrow cells from *Foxp3*-Cre, DKO, *Tbx21*$^{fl/fl}$*Foxp3*-Cre or *Gata3*$^{fl/fl}$*Foxp3*-Cre mice mixed with bone marrow cells from CD45.1 congenic mice were co-transferred into irradiated *Rag1*$^{-/-}$ mice. 8 weeks after reconstitution, cells were harvested from spleen and MLN of chimeric mice and stained with congenic markers CD45.1 and CD45.2 together with a cocktail of antibodies as indicated. Graphical representation of percentage of Foxp3$^+$ T_{reg} cells among the CD45.2$^+$CD4$^+$ cells in the spleen and MLN (**a**). Graphical representation of Foxp3 MFI (upper panel) and of percentage of RORγt$^+$ cells (lower panel) among the Foxp3$^+$CD45.2$^+$CD4$^+$ T_{reg} cells in the spleen (**b**). Plots show co-expression of RORγt with Foxp3 or CD44 by the Foxp3$^+$CD45.2$^+$CD4$^+$ T_{reg} cells (**c**). Plots show intracellular IL-17A staining of the Foxp3$^+$CD45.2$^+$CD4$^+$ T_{reg} cells after plate-bound anti-CD3 and anti-CD28 stimulation (**d**, left panels). Graphical representation of percentage of IL-17A$^+$ cells among the Foxp3$^+$CD45.2$^+$CD4$^+$ T_{reg} cells from the spleen of chimeric mice (**d**, right panel). Data are representative of three independent experiments (**a-d**). Error bars represent standard deviation of the mean (n=4). Statistical significance was determined by an ordinary one-way ANOVA. **p<0.01 and ****p<0.0001.