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TGFβ1 deficiency does not affect the generation and maintenance of CD4⁺CD25⁺FOXP3⁺ putative T_{reg} cells, but causes their numerical inadequacy and loss of regulatory function

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Abstract

TGFβ1 is considered to be required for peripheral maintenance of CD4⁺CD25⁺FOXP3⁺ T_{reg} cells. However, we demonstrate no reduction in the percentage of such T cells in the spleens and thymi of *Tgfb1*^{-/-} mice. Although putative T_{reg} cells, characterized as CD4⁺CD25⁺FOXP3⁺CD62L⁺ T cells, are increased in *Tgfb1*^{-/-} mice, they may be inadequate to control activated T cells since the ratio of activated T cells:putative T_{reg} cells is several-fold higher in *Tgfb1*^{-/-} mice than in control mice. We further show that whereas *Tgfb1*^{-/-} mice that express a chicken OVA-specific TCR transgene (DO11.10) have an increase in putative T_{reg} cells, there are no detectable CD4⁺CD25⁺ T cells in the spleens of DO11.10 *Rag1*^{-/-} mice suggesting that T_{reg}-cell generation is self-antigen dependent regardless of whether they express *Tgfb1*. Finally, we demonstrate that *Tgfb1*^{-/-} T cells remain responsive to the suppressive effect of TGFβ1 *in vitro*. These data suggest that TGFβ1 is required for the regulatory function of T_{reg} cells to prevent activation of T cells and autoimmunity.

Keywords

Autoimmunity; DO11.10; FOXP3; Inflammation; Knockout mice; TGFβ1; Tolerance; T_{reg} cells

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Introduction

Multi-organ inflammation is a feature of many systemic immune-mediated inflammatory diseases such as systemic lupus erythematosus and Sjogren's syndrome [1]. Systemic inflammation in these diseases is associated with activation of self-reactive T cells and loss of self-tolerance. Peripheral blood cells from patients and splenic T cells from animals with these diseases also produce lower levels of TGF β 1 than do control subjects [2–4]. That TGF β 1 may be involved in the maintenance of self-tolerance and pathogenesis of systemic inflammatory diseases is indicated in studies which show the development of multi-organ inflammation in *Tgfb1*^{-/-} mice [5,6]. We have shown that inflammation in *Tgfb1*^{-/-} mice is dependent on T cells, which undergo massive activation [7,8]. Furthermore, T-cell activation in *Tgfb1*^{-/-} mice requires self-antigen presentation, again indicating loss of self T-cell tolerance as a cause of inflammation [9]. What causes loss of self-tolerance in *Tgfb1*^{-/-} mice remains to be determined.

There are at least three major mechanisms of the induction and maintenance of self-tolerance: negative selection by clonal deletion, clonal anergy, and suppression by CD4⁺CD25⁺ T cells referred to as regulatory T (T_{reg}) cells [10]. Several lines of evidence implicate TGF β 1 in the generation and functioning of such T_{reg} cells [11]. For example, CD4⁺CD25⁺ T_{reg} cells produce TGF β 1, and blocking it with neutralizing antibody prevents T_{reg} cell activity [12,13]. TGF β 1 also induces expression of *Foxp3*, a signature molecule of T_{reg} cells, in CD4⁺CD25⁺ T cells *in vitro* [14]. Other studies have found a decrease in *Foxp3*-expressing T cells in the periphery of *Tgfb1*^{-/-} mice, but no difference in CD4⁺CD25⁺ T cells [15], and a significant decrease in CD4⁺CD25⁺ T cells in *Tgfb1*^{-/-} spleens, but not in thymi [11]. These studies are complicated by the fact that TGF β 1-deficient animals have massive inflammation; therefore, some cellular changes likely result from the secondary effects of inflammation, rather than from the TGF β 1 deficiency. In fact, the proinflammatory cytokine IL-6 that is a primary mediator of T-cell-mediated inflammation in *Tgfb1*^{-/-} mice (our unpublished observation) inhibits FOXP3 expression and T_{reg} cells [16].

To obviate the confounding roles of inflammation and proinflammatory cytokines in the generation and maintenance of T_{reg} cells, we recently generated TCR transgenic *Tgfb1*^{-/-} mice which live longer and develop milder and delayed inflammation [9]. In this article, we took advantage of those animals to address the role of TGF β 1 in the generation and maintenance of T_{reg} cells. We also analyzed CD4⁺CD25⁺ T cells in animals that have no endogenous T cells (*Rag1*^{-/-} DO.11 Tg mice), which allowed us to show that self-antigen recognition is required for the generation and maintenance of CD4⁺CD25⁺ T cells. Finally, we asked whether TGF β 1-deficient T cells remain amenable to suppression by TGF β 1, and showed that TGF β 1 is not required for the generation and maintenance of CD4⁺CD25⁺FOXP3⁺ T cells, but likely confers suppressive function to CD4⁺CD25⁺ T cells.

Materials and methods

Mice

Tgfb1^{+/-} (BALB/c, N7) and DO11.10 mice (gifts from James D. Gorham, Dartmouth Medical School and J. Gabriel Michael, Cincinnati, respectively) were genetically combined with *Rag1*^{-/-} mice. All animal experiments were performed following approved institutional guidelines.

Flow cytometry

Phenotype analysis of splenocytes and thymocytes was determined by flow cytometry as described [9]. FITC-, PE-or APC-KJ1-26 (clonotypic anti-TCR Ab against DO11.10 TCR) and FOXP3 staining kit (clone FJK-16s) were from CALTAG (Burlingame, CA) and

eBioscience (San Diego, CA), respectively. All other antibodies were either from BD Biosciences or eBioscience. Surface-stained cells were fixed and permeabilized using the Fix/Perm buffer overnight at 4 °C before staining for FOXP3.

Assessment of proliferation by tritiated thymidine incorporation

2×10^5 splenocytes were cultured in 200 μ l Aim-V serum-free medium (Gibco/Invitrogen) in the presence of mitogens with and without TGF β 1 in triplicates in flat bottom 96-well tissue culture plates. Cell proliferation was measured using tritiated thymidine, as described previously [8]. Data are represented as the percent of control from triplicate cultures $\pm$ standard deviation. Background incorporation of unstimulated cultures was always <3000 dpm. The optimum concentration of PMA is 1 nM and of ionomycin is 250 nM in Aim-V serum-free medium supplemented with L-glutamine, streptomycin sulfate, gentamicin sulfate and human serum albumin.

Results

CD4⁺CD25⁺FOXP3⁺ T cells are not reduced in the spleen of *Tgfb1*^{-/-} mice

To investigate the effect of TGF β 1 deficiency on the maintenance of CD4⁺CD25⁺ T cells, we analyzed the frequency of these cells in the spleen of *Tgfb1*^{-/-} mice and their wild-type littermates. We found that *Tgfb1*^{-/-} mice have a significant CD4⁺CD25⁺ T-cell population (Fig. 1A). In fact, *Tgfb1*^{-/-} DO11.10 mice that develop milder inflammation and survive longer have a significant increase in CD4⁺CD25⁺ T cells in their spleen at 4–6 weeks of age ($p < 0.01$; Fig. 1B and C). The increase in CD4⁺CD25⁺ T cells in *Tgfb1*^{-/-} DO11.10 mice becomes less profound as the animals age and develop inflammation ($p = \text{NS}$ at 7–8 weeks of age; Fig. 1C). Thus, the minor decrease in CD4⁺CD25⁺ T cells in some non-transgenic *Tgfb1*^{-/-} mice (Fig. 1A, 2 week Ntg) could be due to the inflammatory environment rather than to a TGF β 1 deficiency.

The increase in CD25-expressing T cells in *Tgfb1*^{-/-} DO11.10 mice (Fig. 1C) could simply reflect the presence of activated T cells since CD25 is expressed both on T_{reg} cells as well as activated T cells. Hence, we analyzed the expression of the T_{reg}-cell signature marker FOXP3 [17,18]. In *Tgfb1*^{-/-} DO11.10 mice we observed a 2-fold increase in the proportion of CD4⁺ T cells that express FOXP3 (Fig. 1D). Further analysis shows a 2-fold increase in the FOXP3-expressing CD4⁺ TCR transgenic (KJ1-26⁺) as well as endogenous TCR-expressing (KJ1-26⁻) T cells (Fig. 1D). The proportion of FOXP3-expressing CD4⁺ T cells that recognize self-Ag (KJ1-26⁻ T cells) (Fig. 1D) is higher than that of FOXP3⁺CD4⁺ T cells that express transgenic TCR (KJ1-26⁺) in both WT and KO mice (Fig. 1D), suggesting that T_{reg}-cell generation is dependent upon self-Ag recognition and that decreasing the self-reactive TCR repertoire decreases T_{reg}-cell generation. We have recently shown that transgenic KJ1-26⁺ T cells harbor the hybrid TCR (transgenic as well as endogenous) [9], which may confer to these T cells (KJ1-26⁺) the ability to express *Foxp3* and become T_{reg} cells.

CD4⁺CD25⁺FOXP3⁺ T_{reg} cells induce suppression of effector CD4⁺ T cells both in a cell–cell contact and cytokine-mediated manner. Among the CD25⁺ population CD25^{high} cells are more effective in their regulatory function. In fact, in NOD mice only CD25^{high} but not CD25⁺CD4⁺ T cells were shown to exhibit regulatory function [12]. The most likely explanation for this effect could be the ability of CD25^{high} cells to sequester IL-2 from the vicinity of effector T cells and cause cytokine deprivation [13]. Hence, we further analyzed the frequency of CD25^{high} cells on gated CD4⁺ T cells (Fig. 1E). Results show that CD4⁺CD25^{high} cells were also not reduced in *Tgfb1*^{-/-} mice as compared to control animals.

The spleen is generally smaller and the splenocyte number is lower in older *Tgfb1*^{-/-} mice than in wild-type littermates (2–3 months; data not shown), probably due to activation-induced cell death (AICD) and egress of these cells to other organs [8,9]. Hence, to determine the effect of TGFβ1 deficiency on absolute cell numbers, we analyzed CD4⁺CD25^{+/hi} T cells in younger *Tgfb1*^{-/-} DO11.10 animals and their wild-type littermates that had comparable numbers of CD4⁺ T cells in the spleen (Fig. 1F). Results show that CD4⁺CD25^{+/hi} T cells were higher in *Tgfb1*^{-/-} DO11.10 mice than in control mice, while the total numbers of CD4⁺ T cells were comparable between the two groups.

Characterization of activation markers CD69 and CD62L on CD4⁺CD25⁺ T cells in *Tgfb1*^{-/-} mice

Next, we characterized the phenotype of CD4⁺CD25⁺ T cells in TGFβ1-deficient mice to detect if CD4⁺CD25⁺ T cells express other activation markers. We found that CD4⁺CD25⁺CD69⁻ and CD4⁺FOXP3⁺CD69⁻ T cells are lower in *Tgfb1*^{-/-} (TCR non-transgenic) mice than in their wild-type littermate controls; whereas, CD69⁺-expressing CD4⁺CD25⁺ and CD4⁺FOXP3⁺ cells were higher in *Tgfb1*^{-/-} mice than in controls (Fig. 2A, B). These data suggest that CD4⁺CD25⁺ cells in *Tgfb1*^{-/-} mice are either activated T_{reg} cells or they are simply activated effector T cells which cause early lethality. To test this further, we analyzed CD62L expression in splenic CD4⁺ cells in TGFβ1-deficient mice using 6-week-old *Tgfb1*^{-/-} DO11.10 mice that exhibit a substantial increase in CD4⁺CD25⁺ cells. We found a two-fold increase in both CD62L⁺ and CD62L⁻CD4⁺CD25⁺ cells in *Tgfb1*^{-/-} DO11.10 mice as compared to *Tgfb1*^{+/+} DO11.10 mice (Fig. 2D, E). Similar data were obtained in another set of 7-week-old *Tgfb1*^{-/-} DO11.10 mice, which exhibit increases in both CD69⁺ and CD69⁻CD4⁺CD25⁺ cells as compared to control mice (Fig. 2G). Taken together, CD4⁺CD25⁺ cells in TGFβ1-deficient mice are not simply activated T cells; they likely represent phenotypic CD4⁺CD25⁺ T cells.

CD4⁺CD25⁺ putative T_{reg} cells are numerically inadequate vis-à-vis activated T cells in *Tgfb1*^{-/-} mice

We have previously characterized the activated state of *Tgfb1*^{-/-} T cells in mice by showing an increase in CD69⁺ T cells [8]. Here we analyze CD69 expression on CD4⁺CD25⁺ T cells in these mice. Despite increases in CD4⁺CD25⁺ putative T_{reg} cells, *Tgfb1*^{-/-} mice develop inflammatory disease and exhibit an increase in activated T cells, as shown by markedly increased frequencies of CD69⁺CD25⁻FOXP3⁻CD4⁺ T cells as compared to control animals (Fig. 2A, B). Thus, the ratio of activated T cells (CD4⁺CD69⁺) to T_{reg} cells (CD4⁺CD25⁺CD69⁻ or CD4⁺FOXP3⁺CD69⁻) is markedly higher in TGFβ1-deficient mice than in control animals (Fig. 2C). These data suggest that CD4⁺CD25⁺ T cells might be simply outnumbered and overwhelmed by activated T cells in TGFβ1-deficient mice as compared to normal animals. Such numerical inadequacy of CD4⁺CD25⁺ T cells was less obvious in *Tgfb1*^{-/-} DO11.10 mice (Fig. 2F) which develop milder inflammation and T-cell activation than TCR non-transgenic *Tgfb1*^{-/-} mice [9]. In fact, in one set of *Tgfb1*^{-/-} DO11.10 mice that exhibit minimal T-cell activation (Fig. 2G), the ratio of activated T cells (CD4⁺CD69⁺) to CD4⁺CD25⁺ T cells (CD4⁺CD25⁺CD69⁻) was almost similar between *Tgfb1*^{-/-} and *Tgfb1*^{+/+} control mice. Thus, numerical inadequacy of CD4⁺CD25⁺ T cells in TGFβ1-deficient mice appears to correlate with the level of T-cell activation and inflammation. This is consistent with a recent study in EAE (a mouse model for MS) where it was found that effector T_H17 cells outnumber the T_{reg} cells during the active phase of disease in the target organs [19].

CD4⁺CD25⁺ T cells are not reduced in the thymus of *Tgfb1*^{-/-} mice

To test whether the impaired CD4⁺CD25⁺ T-cell phenotype is due to thymic abnormality that is usually observed in *Tgfb1*^{-/-} mice due to inflammation we have analyzed thymocyte profiles and found that thymocyte profiles are nearly normal in *Tgfb1*^{-/-} DO11.10 mice (Fig. 3A).

Similar to the increase in CD4⁺CD25⁺ T cells in the spleen, FOXP3 and CD25-expressing CD4⁺CD8⁻ thymocytes were several-fold higher in *Tgfb1*^{-/-} DO11.10 mice than in *Tgfb1*^{+/+} DO11.10 mice. This suggests an increased generation of thymic CD4⁺CD25⁺ T cells in the absence of TGFβ1 (Fig. 3B).

Self-Ag recognition induces FOXP3 in T cells independent of TGFβ1

To test whether the increase in FOXP3⁺ T cells in *Tgfb1*^{-/-} mice is due to enhanced self-reactivity, we compared FOXP3 expression in thymocytes and splenocytes from WT, DO11.10 and DO11.10 *Rag1*^{-/-} mice. Since CD4⁺ T cells in DO11.10 *Rag1*^{-/-} mice have no self-Ag to become activated, they should not become T_{reg} cells. Indeed, FOXP3 is expressed only in CD4⁺ T cells in RAG-sufficient strains, but not in RAG-deficient DO11.10 mice (Fig. 4A). This suggests that self-Ag recognition induces FOXP3 expression and T_{reg}-cell generation. However, the early activation marker CD69 is nearly undetectable on these CD25⁺FOXP3⁺ T_{reg} cells (data not shown) suggesting that their generation requires self-tolerance induction and not T-cell activation. Similar upregulation of FOXP3 is observed in mature CD4⁺CD8⁻ thymocytes in older *Tgfb1*^{-/-} mice (3 weeks) suggesting an increased activation of thymic T cells in the absence of TGFβ1 (Fig. 4B). Hence, activation of *Tgfb1*^{-/-} T cells is not due to a CD4⁺CD25⁺ T-cell reduction but rather to a decrease in their suppressor function [15]. Analysis of CD25 expression on CD4⁺ T cells in *Tgfb1*^{-/-} DO11.10 *Rag1*^{-/-} mice reveals insignificant numbers of CD4⁺CD25⁺ T cells, suggesting that self-Ag recognition is essential for their generation, even in the absence of TGFβ1 (Fig. 4C). Since the absence of TGFβ1 leads to responsiveness to self, there are more autoreactive T cells in the mouse in which an autoimmune response is occurring. However, in the DO11 *Tgfb1*^{-/-} *Rag1*^{-/-} mice, there are no cognate antigens for DO11.10 T cells. Consequently, in the absence of cognate antigen, TGFβ1 does not play a role in T_{reg}-cell development.

Tgfb1^{-/-} T cells remain amenable to suppression

The above results raise the question why CD4⁺CD25⁺ T cells that increase in *Tgfb1*^{-/-} mice do not suppress activated T cells that accumulate in *Tgfb1*^{-/-} mice? Several *in vitro* and *in vivo* studies have shown that *Tgfb1*^{-/-} CD4⁺CD25⁺ T cells exhibit suppressor function in a TGFβ1-sufficient environment and that they can induce TGFβ1 in wild-type APCs *in vitro* [11,15,20]. One possibility is that activated *Tgfb1*^{-/-} T cells are no longer amenable to suppression. We show, however, that TGFβ1 very efficiently inhibits the proliferation of splenic lymphocytes from *Tgfb1*^{-/-} mice, suggesting that *Tgfb1*^{-/-} T cells are in fact amenable to suppression by TGFβ1 (Fig. 5). This suggests that CD4⁺CD25⁺ T cells in TGFβ1-deficient mice are unable to inhibit effector T cells, not because the TGFβ1-deficient effector T cells are resistant to inhibition but rather because TGFβ1 is lacking.

Discussion

Several *in vitro* studies have shown that *Foxp3* is induced only in the presence of TGFβ1 and that it requires CTLA-4 and IL-2 [14,21–23]. These studies also showed that *Foxp3* is not induced in the *in vitro*-activated CD4⁺ T cells in the absence of TGFβ1. Previous studies have found a decrease in *Foxp3*-expressing T cells in the periphery of *Tgfb1*^{-/-} mice, but there was no difference in CD4⁺CD25⁺ T cells [15], and there was a significant decrease in CD4⁺CD25⁺ T cells in *Tgfb1*^{-/-} spleens, but not in thymi [11]. In contrast to these findings, deficiency of SMAD3 that mediates TGFβ1 signaling causes an increase in T_{reg} cells [24]. The deficiency of a protein tyrosine phosphatase called Src homology region 2 domain-containing phosphatase-1 (SHP-1) also leads to increased T_{reg}-cell generation [25]. SHP-1 mediates the inhibitory effect of TGFβ1 on *Tbx21* (T box expressed in T cells) that contributes to T_H1-cell differentiation. These studies imply that deficiency in TGFβ signaling could result in increased T_{reg}-cell generation probably as a compensatory mechanism. The discrepancy in

CD4⁺CD25⁺ T-cell frequency between *Tgfb1*^{-/-} mice and *Smad3*^{-/-} or *Shp1*^{-/-} mice could be related to differences in the extent of inflammation in these strains. We have resolved these apparent discrepancies by generating *Tgfb1*^{-/-} DO11.10 TCR transgenic mice that have reduced inflammation and enhanced survival. In this strain, TGFβ1 deficiency is associated with increased frequency of CD4⁺CD25⁺ T cells in the spleen and thymi.

In the present study we show that FOXP3 is expressed in activated T cells (CD69⁺) in *Tgfb1*^{-/-} mice, further confirming that FOXP3 expression is modulated by activation signals and not directly induced by TGFβ1 *in vivo*. Recently Li et al. and Marie et al. found that FOXP3 expression decreases in TGFβ receptor type II-deficient T cells as the *Tbr2* conditional knockout mice progressively develop inflammation. Since there was no defect in the generation of T_{reg} cells in the thymus of these conditional knockout mice, it was argued that TGFβ signaling in T_{reg} cells is important for their maintenance in the periphery [26,27]. Since we found that the generation or maintenance of CD4⁺CD25⁺ T cells is unaffected in the absence of TGFβ1, it is possible that other ligands such as TGFβ2 which signal through the same receptor (*Tbr2*) may be protecting the T_{reg} cells from apoptosis in *Tgfb1*^{-/-} mice.

The activation of *Tgfb1*^{-/-} T cells is not due to a reduction in CD4⁺CD25⁺ T-cell numbers, but rather due to a TGFβ1 deficiency which causes a decrease in their suppressor function [15]. Our results also suggest that FOXP3 is expressed in activated T cells *in vivo* in *Tgfb1*^{-/-} mice, that its expression depends on the quality of signal strength, and that TGFβ1 plays an indirect role in *Foxp3* induction [28]. We have shown that CD4⁺ T cells undergo activation in *Tgfb1*^{-/-} DO11.10 mice, even in the presence of increased numbers of CD4⁺CD25⁺ T_{reg} cells *in vivo*, indicating that the T_{reg} cells do depend on TGFβ1 for their function (rather than development) *in vivo* [9]. Recent studies from two groups independently confirmed the TGFβ-signaling-dependent suppressor functions of T_{reg} cells by showing that wild-type T_{reg} cells fail to prevent autoimmune responses of TGFβ receptor-deficient T cells *in vivo*, thereby confirming a role for TGFβ signaling in effector T cells for inhibition by T_{reg} cells [26,27].

It is possible that T_{reg} cells deliver several suppressor signals and that TGFβ1 is only one of them. T_{reg} cells suppress effector T cells via multiple mechanisms, including: i) cell-cell contact, ii) secretion of suppressor cytokines such as IL-10 and TGFβ1, and iii) cytokine deprivation such as sequestration of IL-2 due to constitutive expression of CD25 (IL-2 receptor α chain) on T_{reg} cells. Hence, *Tgfb1*^{-/-} T_{reg} cells could function as suppressor cells but are not as potent as *Tgfb1*^{+/+} T_{reg} cells [15]. Using CD4⁺CD25⁺ T_{reg} cells from *Tgfb1*^{-/-} mice and CD4⁺ T cells from dnTBR2 tg and *Smad3*^{-/-} mice, Piccirillo et al. have suggested that TGFβ1 is not essential for T_{reg}-cell suppressor function *in vitro* [20]. However, that *in vitro* study used irradiated splenocytes from wild-type mice as APC that can produce TGFβ1. Thus, it is possible that extraneous TGFβ1 produced by wild-type APCs or that is present in FCS in cultures might render a suppression that is being attributed to CD4⁺CD25⁺ T_{reg} cells from TGFβ1-deficient mice. Moreover, others, using both *in vitro* [11] and adoptive transfer studies [15,29], have shown that T_{reg} cells need not produce TGFβ1, but they can acquire it if they are in a TGFβ1-sufficient environment. We have addressed the situation for a TGFβ1-deficient environment, and found that TGFβ1 is important for T_{reg}-cell function *in vivo* because in a completely TGFβ1-deficient environment, CD4⁺ T cells undergo activation in DO11.10 mice, even in the presence of increased numbers of CD4⁺CD25⁺ T cells. Since TGFβ1 is a negative regulator of T-cell activation, the increased CD4⁺CD25⁺ T-cell generation in *Tgfb1*^{-/-} DO11.10 mice could be due to increased signal strength generated during the interaction of TCR or hybrid TCR with self-Ags [30]. The co-existence of activated T cells and FOXP3⁺ T cells in lymphoid organs of TGFβ1-deficient mice suggests that TGFβ1 is required for the regulatory function of T_{reg} cells to prevent activation of T cells and autoimmunity. Since *Tgfb1*^{-/-} T cells respond to TGFβ1 *in vitro*, it is the absence of TGFβ1 production by T_{reg} cells that leads to their loss of regulatory function. Taken together, these data indicate a role of

TGF β 1 in the functioning, but not in the generation and maintenance, of CD4⁺CD25⁺ T_{reg} cells.

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Figure 1.

TGFβ1 deficiency results in increased CD4⁺CD25⁺ T_{reg} cells in the periphery. Splenocytes from 2-week-old *Tgfb1*^{-/-} (TCR non-transgenic [Ntg]) or 2–12-week-old *Tgfb1*^{-/-} DO11.10 (Tg) and littermate control mice were surface-stained for CD4 and CD25. (A and B) Representative dot plots show percentages of CD4⁺CD25⁺ cells in the spleens of 2-week-old *Tgfb1*^{-/-} (Ntg), 2-week-old *Tgfb1*^{-/-} DO11.10 (Tg), and age-matched littermate controls. (C) CD4⁺CD25⁺ T cells are shown as the percentages of CD4⁺ cells in *Tgfb1*^{-/-} DO11.10 and their littermate control mice at different age groups. Data from individual animals are shown as circles. The means of the groups are shown as horizontal grey lines. **p* < 0.05 (Student's *t*-test). (D) Dot plots with percentages of populations expressing FOXP3 and CD4 (top panels) and CD4⁺-gated splenocytes expressing FOXP3 and CD25, and TCR (KJ1-26) (middle and bottom panels) in 7-week-old *Tgfb1*^{-/-} DO11.10 mice. Dot plots with percentages of populations in each quadrant are shown. (E) Gated CD3⁺CD4⁺ T cells from 8-week-old *Tgfb1*^{-/-} mice and their controls were analyzed for CD25^{high} T cells. Data shown are representative of similar data in three mice in each group. (F) Total numbers of CD4⁺ T cells (left panel) and

CD4⁺CD25^{+/high} T cells (right panel) in the spleen of *Tgfb1*^{-/-} mice and their wild-type littermates. Data are mean±SD of three mice for each group (4–7 weeks). Results from *Tgfb1*^{-/-} and wild-type littermates that have comparable numbers of CD4⁺ T cells are shown. *Tgfb1*^{-/-} mice, as compared to wild-type littermates, have higher numbers of CD4⁺CD25^{+/high} T cells in the spleen, although the differences were not statistically significant.

Figure 2.

Phenotypes of CD4⁺CD25⁺ T_{reg} cells in *Tgfb1*^{-/-} mice: increased activation markers and increased ratio of activated T cells: T_{reg} cells. (A–C) Splenocytes from *Tgfb1*^{-/-} (TCR non-transgenic) and control mice were stained for CD4, CD25, CD69 and intracellular FOXP3. (A) Percentages of CD4⁺ T cells expressing these markers are shown on representative dot plots. (B) Subsets of CD4⁺ T cells based on the expression of CD25 and CD69 or of FOXP3 and CD69 in WT and KO mice. (C) Ratio of activated (CD69⁺):T_{reg} (CD25⁺CD69⁻ or FOXP3⁺CD69⁻) CD4⁺ T cells determined from data in (B). (D–G) Splenocytes from 6-week-old (D–F) or 7-week-old (G) *Tgfb1*^{-/-} DO11.10 and littermate control mice were analyzed for CD4, CD25, CD69 and CD62L. (D) Percentages of CD4⁺ cells expressing CD25 and CD62L are shown on representative dot plots. (E) Subsets of CD4⁺ T cells based on the expression of CD25 and CD62L in WT and KO mice are shown. Ratio of activated (CD62L⁺):T_{reg} (CD25⁺CD62L⁺) CD4⁺ T cells is shown in (F). (G) CD25 and CD69 expression was analyzed on KJ1-26⁺ versus KJ1-26⁻ CD4⁺ splenocytes. Results shown represent two experiments.

Figure 3.

TGFβ1 is not essential for the generation of T_{reg} cells. Thymocytes were stained for CD4, CD8, CD25, KJ1-26 and intracellular FOXP3 as described above. (A) Percentages of thymocytes expressing CD4 and CD8 are shown. (B) Percentages of CD4⁺CD8⁻ thymocytes expressing KJ1-26, CD25 and FOXP3 are shown.

Figure 4.

FOXP3 expression and T_{reg}-cell generation is self-Ag specific. (A) Splenocytes and thymocytes from 4-week-old mice were stained for CD4, CD25 and intracellular FOXP3. FOXP3 expression in the TCR NTg control (green) and DO11.10 (red) CD4⁺CD8⁻ SP thymocytes (left panel) and CD4⁺ splenocytes (right panel) but not in DO11.10 *Rag1*^{-/-} mice (blue) because of elimination of endogenous TCR. (B) Analysis of intracellular FOXP3 expression in *Tgfb1*^{-/-} (non-transgenic) thymocytes. (C) CD25 expression on CD4⁺ T cells in *Tgfb1*^{-/-} DO11.10 *Rag1*^{-/-} and littermate control mice.

Figure 5.

TGFβ1 inhibits the proliferation of TGFβ1-deficient T cells *in vitro*. Splenocytes from $Tgfb1^{-/-}$ and littermate control mice were stimulated with 1 nM PMA (P) and 250 nM ionomycin (I), with or without TGFβ1, for 3 days. Cells were pulsed with tritiated thymidine for the last 14 h of the culture. Cells were harvested and radioactivity was counted. Results are shown as the percent inhibition by TGFβ1 in reference to control cultures without any exogenous TGFβ1. Results from 17-day-old animals are shown.