HHS Public Access

Author manuscript

Nat Immunol. Author manuscript; available in PMC 2010 December 08.

Published in final edited form as:

Nat Immunol. 2008 December ; 9(12): 1347–1355. doi:10.1038/ni.1677.

Interleukin 4 inhibits TGF-β-induced-Foxp3$^+$T cells and generates, in combination with TGF-β, Foxp3$^-$ effector T cells that produce interleukins 9 and 10

Valérie Dardalhon[1,2,*], **Amit Awasthi**[1,2,*], **Hyoung Kwon**[1], **George Galileos**[1], **Wenda Gao**[3], **Raymond A. Sobel**[4], **Meike Mitsdoerffer**[2], **Terry B. Strom**[3], **Wassim Elyaman**[2], **I-Cheng Ho**[5], **Samia Khoury**[2], **Mohamed Oukka**[1], and **Vijay K Kuchroo**[2]

[1] Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, 65 Landsdowne Street, Cambridge, MA 02139, USA

[2] Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, 77 Ave. Louis Pasteur, Boston, MA 02115, USA

[3] Transplant Research Center, Beth Israel Hospital, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, Massachusetts 02115, USA

[4] Palo Alto VA Health Care System and Department of Pathology, Stanford University School of Medicine, Palo Alto, CA 94304, USA

[5] Division of Pediatric Hematology-Oncology, Children's Hospital, Department of Pediatric Oncology, Harvard Medical School, Boston, MA 02115, USA

Abstract

Foxp3 is a key transcription factor involved in the generation and function of regulatory T (T_{reg}) cells. Transforming growth factor β (TGF-β) induces Foxp3, which generates inducible Foxp3$^+$ T_{reg} cells from naïve T cells, and interleukin 6 (IL-6) inhibits the generation of inducible T_{reg} cells and induces T helper cells that produce IL-17 (T_H-17 cells). However, a role for IL-4 in the generation of TGF-β-induced T_{reg} cells and/or the generation of effector CD4$^+$ T helper cells has not been studied. Here, we show that IL-4 blocked the generation of TGF-β-induced Foxp3$^+$ T_{reg} cells. Instead, IL-4 induced a population of T helper cells that predominantly produce IL-9 and IL-10. The IL-9$^+$IL-10$^+$ T cells did not exhibit any regulatory properties in spite of producing large quantities of IL-10. Adoptive transfer of IL-9$^+$IL-10$^+$producing T cells into RAG-1-deficient mice induced colitis and peripheral neuritis. Interestingly, the severity of tissue inflammation was aggravated when IL-9$^+$IL-10$^+$ T cells were co-transferred with CD45RB^{hi} CD4$^+$ effector T cells into RAG-1-deficient mice, which indicated that IL-9$^+$IL-10$^+$ T cells do not display any suppressive function and therefore constitute a unique population of IL-10-producing helper-effector T cells that promote tissue inflammation.

Correspondence should be addressed to: M.O. (moukka@rics.bwh.harvard.edu) and V.K.K. (vkuchroo@rics.bwh.harvard.edu).
*These authors contributed equally to this work.

Introduction

Upon encounter of specific antigen, naïve $CD4^+$ T cells get activated, expand and differentiate into various effector T cell subsets: T helper (T_H) 1, T_H2 or T_H-17, which are characterized by distinct patterns of cytokine secretion. These subsets of T cells have specific effector functions and recruit different cell types at the site of inflammation by the virtue of cytokines that they produce. T-bet, GATA-3 and ROR-γt are the transcription factors required for the generation of T_H1, T_H2 and T_H-17 cells respectively 1–3. Whereas T_H1 cells clear intracellular pathogens and mediate autoimmune tissue inflammation, T_H2 cells are required for the clearance of extracellular pathogens, and an exaggerated T_H2 response induces asthma, allergy and atopy. On the other hand, T_H-17 cells appear to have a role in controlling both intracellular and extracellular pathogens and in orchestrating autoimmune tissue inflammation4,5. Since exaggerated response of T_H1, T_H2 and T_H-17 cells can induce tissue inflammation, the maintenance of immune homeostasis and prevention of immunopathology requires tight regulatory mechanisms to control these effector T cells by subsets of regulatory T cells. Different types of T cells have been described that mediate these regulatory functions *in vivo* and prevent autoimmunity and tissue inflammation. The $CD4^+CD25^+Foxp3^+$ and IL-10-producing Tr1 cells play a crucial role to regulate effector T cell functions6,7. Natural occurring T_{reg} cells, which are generated in the thymus and express a transcription factor, forkhead box transcription factor (Foxp3) (A002750) http://www.signaling-gateway.org/molecule/query?afcsid=A002750, inhibit effector T cells and play a crucial role in the maintenance of peripheral tolerance8. In addition, emerging data suggest that $Foxp3^+$ T cells can also be generated in the peripheral immune compartment 9–11. However, the *in vivo* factors that generate $Foxp3^+$ induced T_{reg} cells in the periphery are still unclear.

In vitro, TGF-β (A002271) (http://www.signaling-gateway.org/molecule/query?afcsid=A002271) together with TCR and co-stimulatory signals can induce Foxp3 and generate T_{reg} cells from naïve T cells 12. TGF-β appears to be a crucial factor in this process, as mice deficient in TGF-β1 show a defect in the maintenance $Foxp3^+$ T_{reg} cells and develop a lethal lymphoproliferative disorder characterized by enhanced T_H1 and T_H2 effector responses. In addition, $Foxp3^+$ T cells from mutant “FILIG” mice which display attenuated expression of Foxp3, produce increased amounts of T_H2 cytokines13, demonstrating that Foxp3 negatively regulates the T_H2 developmental program. How Foxp3 regulates T_H2 responses, whether it is cell intrinsic where Foxp3 suppresses induction of T_H2 specific transcription factors or whether T_{reg} cells suppress T_H2 cells indirectly has not been directly tested. Initial reports14,15 suggest that Foxp3 can bind to the T_H2 lineage specific transcription factor GATA-3, but the consequence of this interaction is not well understood. One outcome of this interaction, however, clearly is that Foxp3 can directly inhibit the development of T_H2 cells.

In addition to $Foxp3^+$ T_{reg} cells, T regulatory type 1 (Tr1) cells can suppress effector T cell function and tissue inflammation independently of Foxp3 expression. Tr1 cells predominantly produce IL-10 (A001243) (http://www.signaling-gateway.org/molecule/query?afcsid=A001243) and suppress immune responses in a contact-independent manner *via* the production of IL-10. Although IL-10 was initially shown to induce Tr1 cell

differentiation, the Tr1 cells thus generated do not proliferate due to the suppressive nature of IL-10. This observation suggested that factors other than IL-10 might be important for the differentiation and expansion of IL-10-secreting Tr1 cells. Several recent papers, including one from our laboratory, demonstrated that IL-27 together with TGF-β induces the differentiation of Tr1 cells 16,17. IL-10-producing Tr1 cells thus generated have potent immunosuppressive properties in that they not only inhibit autoimmune disease and tissue inflammation, but also contribute to successful engraftment of HLA-mismatched bone marrow transplant6. On the other hand, IL-10-producing T cells are not always anti-inflammatory, and under some circumstances they have been shown to promote autoimmunity 18,19. It has not been elucidated yet whether these IL-10-producing cells co-produce other cytokines that negate their anti-inflammatory function of IL-10 and, what are the differences between these pro- and anti-inflammatory IL-10-producing cells.

In this study we show that IL-4, a T_H2 differentiating cytokine, inhibits TGF-β-induced Foxp3 expression and thus suppresses the *de novo* generation of $Foxp3^+$ T_{reg} cells. Unexpectedly, the two cytokines together (TGF-β plus IL-4) induce the generation of predominantly $Foxp3^-IL\text{-}9^+IL\text{-}10^+$ T cells that, unlike Tr1 cells, do not suppress T cell responses even though they produce IL-10. More surprisingly, the $IL\text{-}9^+IL\text{-}10^+$ T cells not only induce the development of colitis but also induce peripheral neuritis when co-transferred with $CD45RB^{hi}$ $CD4^+$ effector T cells into RAG-1-deficient mice. IL-9 was initially cloned as a T cell growth factor whose receptor shares the common γ-chain with IL-2, IL-4, IL-7, IL-15 and IL-2120, and was shown to be involved in T_H2 type immunity against helminthic infection21. Our data suggest that T cells differentiating in the presence of IL-4 and TGF-β, undergo a unique developmental program characterized by production of IL-10 and IL-9, which may not only subvert the immunosuppressive function of IL-10-producing cells, but also promote tissue inflammation, probably by regulating the expansion and/or function of other effector T cells.

Results

IL-4 inhibits TGF–β-induced $Foxp3^+$ T cells

TGF-β induces the *de novo* generation of $Foxp3^+$ inducible T_{reg} cells (iT_{reg} cells) from naïve $CD4^+$ T cells. We have previously shown that both IL-6 and IL-27 inhibit TGF-β mediated induction of Foxp3 12,16. To test the effect of IL-4 on the induction of Foxp3 by TGF-β, we used GFP^- naïve $CD4^+$ T cells from mice with a bicistronic enhanced green fluorescent protein (EGFP) reporter cloned into the endogenous *Foxp3* locus (Foxp3-GFP mice) 12. As expected, TCR stimulation in the presence of TGF-β, induced Foxp3-GFP^+ expression in up to 80% of T cells, and this was inhibited (reduced to 22%) in the presence of IL-4; thus, IL-4 actively inhibits the induction of Foxp3 in the presence of TGF-β (Fig. 1a). To understand the mechanism by which IL-4 mediates the inhibition of Foxp3 expression, we tested the cytokines produced from T cells activated in the presence of TGF-β plus IL-4. Naïve $CD4^+$ T cells stimulated in the presence of IL-4 differentiated into T_H2 cells and produced IL-10, IL-5, IL-13 and very low levels of IL-9. The combination of TGF-β with IL-4 inhibited IL-4, IL-13 and IL-5 production while sparing the IL-10 production from these T cells. Furthermore, activation in the presence of TGF-β plus IL-4 also induced the secretion of

large amounts of IL-9 (Fig. 1b,c and Supplementary Fig. 1). Importantly, T cells expanded in the presence of TGF-β plus IL-4 produced as much IL-10 as T_H2-differentiated T cells, but instead of IL-4 but they co-produced IL-9 with IL-10 (Fig. 1b and Supplementary Fig. 2a). To study whether APCs contribute to the generation of this unique subset of IL-9^+IL-10^+ cells or whether IL-9 was being produced by APCs, we also activated naïve $CD4^+$ T cells with plate-bound anti-CD3 and anti-CD28 (without any APCs) in the presence of TGF-β together with IL-4. Even in the absence of APCs, IL-4 inhibited the induction of Foxp3 induced by TGF-β and induced T cells that co-produced IL-9 and IL-10 (Supplementary Fig. 2a).

To characterize the source of IL-10 production at the cellular level, we performed intracellular cytokine staining and compared T cells activated in the presence of TGF-β, IL-4 or both. Activation of naïve T cells in the presence of IL-4 did not induce the expression of Foxp3-GFP, but induced intracellular IL-4 and IL-10 production as expected, as a part of T_H2 development. Neither IFN-γ nor IL-17 production was observed from responding T cells. However, addition of IL-4 to TGF-β inhibited Foxp3 expression and induced a predominant production of IL-10 from these cells with a dramatic decrease in other T_H2 cytokines production including IL-4 (Fig. 1c). We could not perform staining for IL-9 at the single cell level due to lack of appropriate antibody reagents. Therefore, co-expression of IL-10 and IL-9 could not be established at the cellular level in the T cells differentiated by TGF-β plus IL-4. However, the T cells differentiated in the presence of TGF-β plus IL-4 produced both IL-9 and IL-10 as assessed by ELISA and quantitative real time PCR (Fig. 1b and Supplementary Fig. 1b–d).

To determine whether IL-9 production is dependent on IL-10 in the TGF-β plus IL-4-differentiated T cells, naïve T cells from IL-10-GFP reporter mice22 were differentiated in the presence of TGF-β plus IL-4. Then, IL-10-GFP^+ and IL-10-GFP^- T cells were sorted by flow cytometry based on GFP expression. We could detect IL-9 mRNA expression in IL-10-GFP^+ and IL-10-GFP^- expressing cells (Supplementary Fig. 2a), although maximal expression was only observed when IL-10-GFP^+ and IL-10-GFP^- cells were together. These TGF-β plus IL-4 differentiated T cells co-produce IL-9 and IL-10 and we could not detect any IL-10^+IL-17^+ or IL-10^+IFN-γ^+ double producing cells (Supplementary Fig. 2b). However, small numbers of IL-4^+IL-10^+ double producers (about 8%) are present in the cells activated in the presence of TGF-β plus IL-4, especially when T cells are activated in presence of low amounts of TGF-β. Titration of TGF-β had an important effect on IL-4 production, the percentage of IL-4^+IL-10^+ cells decreased with the increasing amounts of TGF-β (•••••• •••• •••••). The IL-4^+IL-10^+ double producers probably represent a transitional state since IL-4 is extinguished with increasing amounts of TGF-β (Supplementary Fig. 2b). These results show that like IL-6 and IL-27, IL-4 is also able to inhibit the induction of Foxp3 and induce a predominant production of IL-9 and IL-10 from the responding T cells.

Previous studies have shown that IL-10-producing Tr1 cells do not proliferate well and appear to acquire an anergic phenotype. However, IL-9^+IL-10^+ T cells induced in the presence of TGF-β plus IL-4 were not anergic and vigorously proliferated when activated by CD3 antibody (Fig. 1d). We could not detect, in the T cells differentiated with TGF-β plus IL-4, the expression of T-bet, GATA-3, Foxp3 and ROR-γt, lineage specific transcription

factors necessary for the generation of T_H1, T_H2, T_{reg} and T_H-17 cells respectively (Fig. 1e). We previously hypothesized that TGF-β may act together with other cytokines to induce new T cell subsets 23 as exemplified by the observation that TGF-β in combination with IL-6/IL-21 or IL-27 induced T_H-17 and Tr1 respectively. Similar to TGF-β and IL-6 that cross-regulate each other's function and give rise to unique T_H-17 subset, TGF-β in combination with IL-4 inhibited the expression of T_H1, T_H2, T_H-17 and T_{reg} cells specific transcription factors (Fig. 1e).

IL-4 inhibition of Foxp3-induction depends on STAT6

To understand the role of IL-4 in the active inhibition of TGF-β-induced Foxp3, naïve T cells from IL-4 transgenic mice were cultured in the presence of different concentrations of TGF-β. Surprisingly, TGF-β, ••••••••••••••••••, did not induce the generation of $Foxp3^+$ T_{reg} cells, suggesting that IL-4 may play a dominant role in inhibiting the induction of Foxp3 expression (Fig. 2a). Upon binding to its receptor, IL-4 induces several signaling pathways including insulin receptor substrate (IRS)-2 and STAT6, which form a signaling complex in association with the cytoplasmic tail of the IL-4 receptor 24. However, STAT6 is a dominant pathway of T_H2 differentiation because it induces GATA-3, the master transcription factor for T_H2 differentiation. To study whether STAT6 is involved in inducing IL-4-mediated Foxp3 inhibition, we crossed STAT6-deficient mice with the Foxp3-GFP 'knock-in' mice to generate a STAT6KO.Foxp3-GFP strain.

Naïve $CD4^+$ T cells from Foxp3-GFP and STAT6KO.Foxp3-GFP mice were purified and cultured in the presence of TGF-β, IL-4 or a combination of these two cytokines. Unlike wild-type Foxp3-GFP T cells in which IL-4 inhibited the induction of Foxp3, STAT6KO.Foxp3-GFP T cells expressed higher levels of Foxp3 (80%) even in the presence of TGF-β plus IL-4 (Fig. 2b). Furthermore, when we analyzed the cytokines produced by the STAT6KO cells stimulated in the presence of TGF-β plus IL-4, IL-10 and IL-9 was not induced but these cells continued to express Foxp3 in the presence of TGF-β plus IL-4 (Fig. 2c,d). Since GATA-3 is downstream of STAT6, we also analyzed the induction of IL-9 and IL-10 by TGF-β plus IL-4 in the conditional GATA-3-deficient mice and similar to the STAT6-deficient mice, GATA-3-deficient mice could not produce IL-9 or IL-10 upon differentiation in the presence of TGF-β plus IL-4 (Fig. 2e). The effect of STAT6 and GATA-3 in the induction of $IL\text{-}9^+IL\text{-}10^+$ T cells may not be a direct but an indirect effect, since in the absence of STAT6-GATA-3 signaling, TGF-β-induced Foxp3 can not be antagonized by IL-4 resulting in the predominant induction of $Foxp3^+$, which may not allow differentiation of T cells to produce IL-9 and IL-10. Altogether, these data suggest that the inhibition of Foxp3 expression by IL-4, is mediated through IL-4–STAT6 pathway. Thus, the *de novo* generation of $Foxp3^+$ iT_{reg} cells is likely modulated by IL-4 through the activation of STAT6 GATA-3 pathway. We next explored whether IL-4 affected the function of already established naturally occurring $Foxp3^+T_{reg}$ cells *via* STAT6. When we cultured sorted $Foxp3^-$ T effector cells with either Foxp3-GFP^+ or STAT6KO.Foxp3-GFP^+ T_{reg} cells, Foxp3-GFP^+ T_{reg} cells lost a substantial part of their suppressive ability in the presence of IL-4 whereas STAT6KO.Foxp3-GFP^+ T_{reg} cells still remained partly suppressive despite the presence of IL-4 (40 % vs 70% suppression) (Fig. 2f). Thus, the IL-4–STAT6 pathway not only affects the generation of induced T_{reg} cells but also regulates

the suppressive capacity of natural-occurring T_{reg} cells due to a mechanism that is intrinsic to T_{reg} cells.

Relationship between and Foxp3 and GATA-3

To assess whether GATA-3 could affect Foxp3 expression, we isolated $CD4^+$ T cells from GATA-3 conditional deficient mice and activated them in the presence of TGF-β. On day 4 after activation, T cells were harvested and mRNA was extracted and analyzed for expression of GATA-3 and Foxp3 by quantitative PCR. In GATA-3-deficient $CD4^+$ T cells, TGF-β induced Foxp3 mRNA expression was 3-fold higher than in wild-type $CD4^+$ T cells. On the other hand, the addition of TGF-β decreased GATA-3 expression in wild type $CD4^+$ T cells suggesting a possible inter regulation between these two transcription factors (Fig. 3a).

Because of the correlation between Foxp3 and GATA-3 expression, we sought to determine whether the two transcription factors could physically interact and associate with each other. We used two different approaches to test these possibilities. The first approach was to co-transfect Flag-tagged GATA-3 and renilla-luciferase-tagged Foxp3 cDNA into HEK-293 cells. After lysis of the cells, GATA-3 (or a control protein) was immunoprecipitated using anti-Flag and Foxp3 pull down was assessed in the immunoprecipitate by monitoring the renilla-luciferase expression by luminescence. Precipitation of GATA-3 strongly increased the luciferase activity compared to negative control (empty vector) or other transcription factor such as STAT6 (Fig. 3b) suggesting GATA-3 pulled down luciferase-tagged-Foxp3. We further confirmed this result by performing co-immunoprecipitation assays. For this purpose, Flag-GATA-3 was co-transfected with Myc-Foxp3. After lysis of the cells, immunoprecipitation was performed using anti-FLAG coated beads. To detect whether Foxp3 was co-precipitated with GATA-3, the samples were run on a SDS-PAGE gel and after transfer, the membrane was blotted using anti-myc. GATA-3 was co-immunoprecipitated with Foxp3 showing the direct association of the two proteins (**data not shown**). These assays clearly demonstrated that GATA-3 and Foxp3 could physically associate with each other.

Functional interaction between Foxp3 and GATA-3

Next, we sought to determine the functional significance of this interaction. We assessed whether Foxp3 could affect T_H2 differentiation. Foxp3 was cloned into a retroviral vector (MSCV) and was transduced into activated $CD4^+$ T cells differentiated under T_H2 polarization conditions. T cells expressing Foxp3 were sorted by flow cytometry, RNA was extracted and analyzed for the expression of various cytokines. In parallel, a cohort of cells from each transduction condition was maintained in culture for 2–3 days, re-stimulated and the supernatant was collected for cytokine analysis by ELISA.

Whereas T cells transduced with a control retrovirus vector expressed high levels of IL-10, IL-13 and IL-4 with modest levels of IL-5 transcripts, consistent with T cells differentiated into T_H2 effector cells; T cells transduced with Foxp3 dramatically reduced their expression of IL-4, IL-15 and IL-13 but retained substantial expression of IL-10. Similarly, at the protein level, control-transduced T cells produced IL-4 with low levels of IL-2 whereas

Foxp3-transduced T cells showed a decreased production of IL-4 and an increase in the production of IL-10 (Fig. 3c). This is consistent with our initial observation that activation of T cells in the presence of IL-4 plus TGF-β results in the inhibition of IL-4, IL-5, IL-13 but spares IL-10 production from these cells. This would suggest that Foxp3 interferes with the expression of genes controlled by GATA-3, presumably by binding or interfering with its ability to transactivate specific T_H2 genes. Specifically, these results suggest that the interaction of Foxp3 with GATA-3 inhibits its ability to transactivate promoters that are under the control of GATA-3 such as IL-4, IL-5 and IL-13 but not IL-10, which is most likely controlled by a different transcription factor.

To address this hypothesis, we analyzed the ability of Foxp3 to inhibit GATA-3-mediated transactivation of an *Il5* promoter-luciferase construct. Jurkat cells were transfected with GATA-3, Foxp3 or both GATA-3 and Foxp3 in the presence of the *Il5*-luciferase construct and luciferase activity was measured after activation of the cells with PMA plus ionomycin. As expected, GATA-3 strongly transactivated *Il5* promoter-luciferase activity, whereas this effect was reduced by 50 % when Foxp3 was co-expressed (Fig. 3d). Altogether these results show that Foxp3 can directly interact with GATA-3, and this association inhibits GATA-3-mediated transactivation of *Il5*, which is one of its target genes.

Function of the IL-9$^+$IL-10$^+$Foxp3$^-$T cells

Since IL-10-producing Tr1 cells have been shown to suppress T cell proliferation *in vitro* and to inhibit tissue inflammation *in vivo* 25, we wanted to determine whether naïve T cells differentiated in the presence of TGF-β plus IL-4 that produce IL-9 and IL-10 are also suppressive. Although, Foxp3$^+$ T_{reg} cells are anergic and inhibited effector T cell proliferation *in vitro*, T cells generated in the presence of TGF-β plus IL-4 were not anergic nor suppressive, as they vigorously proliferated on their own and together with effector T cells further enhanced T cell proliferation (Fig. 4a). The addition of anti-IL-9 or anti-IL-10 neutralizing antibodies to the cultures of effector T cells with IL-10.GFP$^+$ T cells generated by TGF-β plus IL-4, decreased proliferation but did not completely abolish it (Fig. 4a). These data suggest that unlike Foxp3$^+$ T_{reg} cells and IL-10-producing Tr1 cells, T cells induced by TGF-β plus IL-4 do not show any suppressive effects *in vitro* but readily proliferate and therefore, behave like effector T cells.

It is clear that Tr1 cells not only suppress proliferation *in vitro* but IL-10-producing cells can suppress induction of colitis *in vivo* as well 26,27. Therefore, to further characterize IL-9$^+$IL-10$^+$ T cells generated in the presence of both TGF-β and IL-4, we assessed their function *in vivo* in a T-cell transfer model of colitis. Naïve T cells purified from IL-10.GFP reporter mice were activated in the presence of TGF-β and IL-4. At day 4, the TGF-β + IL-4 treated IL-10$^+$GFP$^+$ T cells sorted by flow cytometry based on GFP expression. RAG-1-deficient mice were reconstituted with either effector T cells (Foxp3$^-$/CD45RB^{hi} T cells) (Group 1, n = 8), effector T cells together with (TGF-β + IL-4 stimulated IL-10.GFP$^+$ T cells (‘T4-IL-10.GFP$^+$ T cells’) (Group 2 n = 8), (T4-IL-10.GFP$^+$ T cells alone (Group 3, n = 8) or effector T cells together with naturally-occurring T_{reg} cells (Group 4, n = 6), and the development of colitis was followed by monitoring the body weight of the mice and scored as described previously28.

Histopathologic analysis was also performed to evaluate the induction and severity of the disease. All the mice reconstituted with effector T cells did not lose weight significantly but developed severe colitis as observed by histopathology with an average colitis score of 7.5 ± 3.5 (Fig. 4c). As a control to show that effector T cells can indeed be regulated, mice that received both effector T cells and natural-occurring T_{reg} cells were protected from both clinical and histological disease (average colitis score 3.7 ± 1.5). Interestingly, recipient mice of T cells cultured in the presence of TGF-β plus IL-4 showed a weight loss and the development of a more moderate colitis (average colitis score 5.7 ± 2.6). Furthermore, when effector T cells were co-transferred with IL-9^+IL-10^+ T cells generated in the presence of TGF-β and IL-4 (T4-IL-10.GFP^+ cells), the mice showed the greatest weight loss and histopathology revealed the development of a more severe colitis compared to mice transferred with effector T cells alone (average colitis score: 8.8 ± 2.3) (Fig. 4c). Surprisingly, however, the mice receiving either T4-IL-10.GFP^+ T cells or both effector T cells (CD45RB^{hi}) with the T4-IL-10.GFP^+ T cells, additionally showed signs of clinical neurological disease characterized by impaired motor coordination with flaccid monoparesis or asymmetric paraparesis of the hind limbs. Muscular strength and tonus in forelimbs and tail did not appear to be affected. These clinical signs clearly differed from the typical manifestations of experimental autoimmune encephalitis (EAE), *i.e.*, limp tail and ascending paralysis, and suggested rather a peripheral neuropathy. Consistent with this clinical appearance, histopathological analysis showed evidence of peripheral neuritis in the mice that received both effector T cells and T4-IL-10.GFP^+ T cells or T4-IL-10.GFP^+ T cells alone. However, consistent with the clinical picture, no inflammation in brain or spinal cord was observed (Fig. 4c). At week 8–9 after transfer and after the development of colitis, animals were sacrificed, cells were retrieved from lymph nodes and cytokine production was analyzed by intracellular cytokine staining. T cells from the RAG-1-deficient mice that received only the effector T cell (CD45RB^{hi}) produced mostly IFN-γ and very little IL-17 (Supplementary Fig. 3a). However, T cells from the mice receiving both effector (CD45RB^{hi}) and T4-IL-10.GFP^+ T cells showed production of both IL-17 and IFN-γ, but the production of IL-17 predominated over IFN-γ in these cells. And while the expression of both IL-17 and IFN-γ was observed in IL-10.GFP^+ and IL-10.GFP^- cells, over a third of the IL-10.GFP^+ cells co-produced both IL-17 and IFN-γ (Supplementary Fig. 3b). These data suggest that IL-9–IL-10-producing T cells generated in the presence of TGF-β and IL-4 do not act as regulatory T cells but rather they potentiate functions of effector T cells, resulting in additional tissue inflammation not induced by the transfer of effector T cells alone.

Discussion

In this paper we show that when naïve T cells are activated in the presence of both IL-4 and TGF-β, Foxp3 expression is inhibited and a subset of T cells is generated that selectively produces IL-10 and IL-9. Although IL-10-producing cells are known to have regulatory properties, the IL-9–IL-10-producing cells described here do not regulate but induce tissue inflammation.

IL-4 is involved in T_H2 T cell differentiation and it is well described that IL-4 receptor signals are transduced by the transcription factors STAT6 and GATA-3. To date no lineage specific transcription factor(s) have been identified for IL-10-producing T_R1 cells. It has

been suggested that GATA-3 may participate in the transcriptional regulation of IL-10. However, our data demonstrate that IL-9–IL-10-producing cells induced in presence of TGF-β and IL-4, did not express GATA-3 suggesting that the presence of TGF-β could antagonize the induction of GATA-3 yet these cells continued to produce IL-10.

STAT proteins play an important role in T cell differentiation 29. STAT6 is involved in the T_H2 differentiation and a STAT1 binding site has been found in the IL-10 promoter 29,30. In this study, we demonstrate that the inhibition of TGF-β induced Foxp3$^+$ by IL-4 and generation of the IL-9–IL-10-producing T cells was at least partially reversed in a STAT6 deficient and GATA-3-deficient mice, suggesting that STAT6-GATA-3 may play an important role in the generation of IL-9–IL-10-producing cells. However, an alternate interpretation of these data is that in the absence of IL-4 signaling in the STAT6-deficient and GATA-3-deficient mice, there is predominant an unopposed induction of Foxp3 which prevents the differentiation of T cells to produce IL-9 and IL-10. The lack of GATA-3 expression in IL-9–IL-10-producing cells induced by TGF-β plus IL-4 would partly support this interpretation.

In addition to IL-10, IL-9 is produced by the T cells differentiated in the presence of TGF-β plus IL-4, suggesting that TGF-β is not simply scaling back of T_H2 responses, but rather TGF-β and IL-4 together are inducing a new gene program that induces IL-9 production. Whether the combined effects of TGF-β and IL-4 induces another transcription factor not associated with either with T_H2 or T_{reg} cells has not been addressed. Interestingly, Foxp3 and GATA-3 physically associate with each other; this finding is perhaps reminiscent of the interaction between Foxp3 with ROR-γt, which reciprocally regulates induction of T_{reg} or T_H-17 cells 12,31. Binding of Foxp3 to GATA-3 may regulate the molecular mechanism by which TGF-β and IL-4 mutually inhibit the generation of T_{reg} and T_H2 cells. However, this mutual regulation results in the production of IL-9 by the differentiating T cells. During the course of our studies, two other groups reported that Foxp3 and GATA-3 physically associate with each other 14,15. Our results confirm these findings and show that Foxp3 does not only interact with GATA-3 but also inhibits the ability of GATA-3 to transactivate the promoter of one of the target T_H2 genes, *Il5*.

We previously showed that TGF-β together with IL-27 can induce and expand IL-10-producing cells that co-produce IFN-γ ••• show strong immunoregulatory properties•16. IL-10 has been shown to be a potent anti-inflammatory cytokine which inhibits T_H1, T_H-17 and T_H2 mediated immune responses 32–34. The significance of IL-10 in regulating immune responses has been demonstrated in human infectious diseases such as tuberculosis, malaria, leishmaniasis and schistosomiasis 35–37. Besides T cells, IL-10 can be produced by numerous cell types including macrophages, DCs, B cells and NK cells. IL-10 production from T cells plays a major role in down regulating T cell expansion during antigen-specific immune responses. Although Tr1 cells produce high amounts of IL-10, they can also co-produce variable amounts of IFN-γ and are able to suppress *in vitro* T cell responses in a Foxp3-independent manner. These findings have led to a view that IL-10 produced by the T cells have a major role in suppressing inflammation. Here we have shown that IL-10-producing cells can be generated in large numbers when naïve T cells are cultured in the presence of IL-4 plus TGF-β; however these T cells also produce IL-9, do not have

regulatory properties and fail to control inflammation when transferred *in vivo*. The production of IL-10 and TGF-β is associated with the maintenance of peripheral tolerance by T_{reg} cells25. *In vivo*, the absence of IL-10 exacerbates airway inflammation 38. These data argue that production of IL-10 by T cells may not always have suppressive functions and that the context in which IL-10 is produced by T cells may be equally important in determining whether IL-10 producing T cells are anti- or pro-inflammatory.

It is known that IL-10 plays an important role in regulating tissue inflammation in the gut. More specifically, it has been shown that IL-10 produced by the T_{reg} cells is essential for this *in vivo* suppression, since IL-10 deficient T_{reg} cells cannot cure T cell-transfer-induced colitis 27,39. In this study, the IL-10-producing T cells generated in presence of TGF-β and IL-4 induce colitis and when co-transferred with effector T cells, not only not prevent the development of the disease but also induce peripheral neuritis, a disease not induced by effector T cells ($CD4^+CD45RB^{hi}$) alone. These data strongly suggest that the $IL\text{-}9^+IL\text{-}10^+Foxp3^-$ T cells, generated in the presence of TGF-β and IL-4, do not have suppressive functions and therefore represent a unique population of IL-10-producing T cells that promote inflammation. It is not clear at this stage whether IL-9, IL-10 or both cytokines are playing a role in inducing tissue inflammation, but we believe that IL-9 produced by these T cells may be a key factor in promoting their pro-inflammatory activity. This is consistent with the observation that IL-9 in the lung promotes T_H2-mediated allergic pulmonary disease characterized by high level of T_H2-cytokines 40,41. We also have found that IL-9 receptors are expressed on the effector T cells (data not shown) and these cells therefore have the potential to respond to IL-9 and differentiate in the presence of $IL\text{-}9^+IL\text{-}10^+$ cells. Since IL-9 belongs to the IL-2, IL-4, IL-7, IL-15 and IL-21 cytokine family and utilizes the common γ chain for function, we believe that IL-9 produced by T cells stimulated by TGF-β and IL-4 may function as a growth and differentiation factor for other effector T cells. This, we believe, is one of the explanations why $IL\text{-}9^+IL\text{-}10^+Foxp3^-$ T cells are not able to control inflammatory responses but instead synergize with effector cells to potentiate their pro-inflammatory activity. Consistent with this are data from the RAG-1-deficient recipients of a mixture of effector T cells and $IL\text{-}9^+IL\text{-}10^+$ T cells showing that the effector T cells produce IL-17, which is not normally observed if effector cells alone are transferred into the RAG-1-deficient mice.

In addition to IL-9 receptor expression on effector T cells, our preliminary data indicate that IL-9 receptor is also expressed on natural-occurring T_{reg} cells. Therefore IL-9 could exert its effects on both effector T cells and T_{reg} cells. Whether IL-9 inhibits or interferes with the suppressive functions of regulatory cells has not been analyzed. However, IL-9 is also produced by T_{reg} cells themselves 42 which recruits mast cells to the tissue niche and regulates effector T cells during graft rejection. Neutralization of IL-9 *in vivo* under this setting abrogates T_{reg} function and promotes graft rejection. In our experiments we have tested the effects of IL-9–IL-10 producing T cells *in vivo* in the RAG-1-deficient mice, in the absence of T_{reg} cells; under these experimental conditions, IL-9 produced by the IL-9–IL-10 producing T cells may act only on the effector T cells and potentiate their pro-inflammatory functions and thus induce more severe and unusual tissue inflammation (*e.g.*,

peripheral neuritis), which is not normally observed with the transfer of effector T cells alone.

We have identified a novel subset of T cells generated in presence of TGF-β and IL-4 that produces both IL-9 and IL-10, does not suppress T cell responses and promotes inflammation. Thus, depending on the context in which IL-10 is being produced by T cells, the T cells can inhibit inflammation and protect against autoimmunity or they can promote inflammation and mediate tissue destruction.

Methods

Mice

C57BL/6, Balb/c, RAG-1-deficient (*Rag*$^{-/-}$) and STAT6 deficient (*Stat6*$^{-/-}$) mice were obtained from Jackson Laboratories. Foxp3-GFP mice12, IL-4 transgenic mice43 and IL-10.GFP reporter mice 22 have been described previously. GATA-3-conditional-deficient mice (*Gata3*$^{flox/flox}$) and IL-10.GFP mice were provided respectively by Dr. I-Chen Ho (Boston, MA) and Dr. R. Flavell (New Haven, CT). Mice were housed in conventional, pathogen-free facilities at Harvard Institute of Medicine. All experiments were performed in accordance with guidelines prescribed by the standing committee of animals at Harvard Medical School.

In vitro T cell differentiation, proliferation and suppression assays

For T cell differentiation and proliferation, sorted naïve CD4$^+$CD62L$^+$Foxp3-GFP$^-$, CD4$^+$CD62L$^+$GATA-3-KO (cre$^+$) or CD4$^+$CD62L$^+$STAT6KO.Foxp3-GFP$^-$ T cells were cultured with irradiated syngenic APCs in the presence of either TGF-β (3 ng/ml) or IL-4 (20 ng/ml) or both. For suppression assays, 2.0 × 10^4 sorted Foxp3$^+$ T$_{reg}$ cells from wild-type or STAT6-deficient (STAT6KO) mice were co-cultured in triplicate wells at a 1:1 ratio with either naïve wild-type CD4$^+$ T cell or naïve STAT6KO CD4$^+$ T cells (T cell responders) in the presence of soluble anti-CD3 (1 μg/ml) and irradiated syngenic APCs. When indicated anti-IL-9 (10 μg/ml, BD Biosciences) or anti-IL-10 (10 μg/ml, BD Biosciences) was added to the culture. Cells were pulsed with 1 μCi/well of [^{3}H]-thymidine for the last 16 h of incubation and [^{3}H]-thymidine incorporation was measured using a microbeta liquid scintillation counter (PerkinElmer).

Intracellular cytokine staining

Cells were re-stimulated with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml, Sigma), ionomycin (1 μg/ml, Sigma), and GolgiStop (1 μl/1.5ml, BD Bioscience) at 37 °C in 10% CO_2 for 4 h followed by surface and intracellular staining for indicated cytokines according to the manufacturer's (BD Bioscience) instruction. Cells were analyzed on a flow cytometer (BD Biosciences).

In vivo T cell transfer induced colitis

C57BL/6, RAG-1-deficient mice were injected with sorted CD4$^+$Foxp3-GFP$^-$ CD45RB ^{hi} T cell sub populations in PBS as described before44,45. Mice received CD4$^+$Foxp3-GFP$^-$CD45RB^{hi} T cell alone or in combination with CD4$^+$CD62L$^+$ T cells from IL-10.GFP

mice stimulated with TGF-β and IL-4, or $CD4^+Foxp3^+$ naturally occurring T_{reg} cells. Mice were examined for their weight loss for 9 weeks. They were then sacrificed and samples of colonic lymphoid tissues as well as brain and spinal cord were fixed in neutral buffered formalin.

Histopathology

Routinely processed, paraffin-embedded samples of small and large intestine and other tissues were stained with hematoxylin and eosin (H&E). Sections of brain and spinal cord were stained with Luxol fast blue-H&E stain. The presence and severity of colitis and the presence of brain and spinal cord inflammation were evaluated in a blinded manner.

The severity of colitis was graded semiquantitatively from 0 to 3 for the four following criteria: degree of epithelial hyperplasia and goblet cell depletion; leukocyte infiltration in the lamina propria; area of tissue affected; and the presence of markers of severe inflammation such as crypt abscesses, submucosal inflammation, and ulcers. Scores for each criterion were added to give an overall inflammation score for each sample of 0–12. The total colonic score was calculated as the average of the individual scores from the sections of proximal colon, mid-colon, and distal colon 28.

Measurement of cytokines by ELISA and qRT-PCR

Culture supernatant were collected at 48 hours to determine the cytokine production by either ELISA or cytometric bead array (BD Bioscience). RNA was extracted after 48–96 hours of *in vitro* stimulation using RNAeasy columns (Qiagen) and subject to quantitative RT-PCR according to manufacturer's (Applied Biosystem) instructions.

Retroviral transduction of GATA-3 conditional KO T cells and T_H2 polarized T cells

Total $CD4^+$ T cells from GATA-3.conditional KO or wild type were transduced with a MSCV-based retroviral vector (RV-GFP) expressing CRE or Foxp3 respectively. RV-GFP is a bicistronic vector encoding for EGFP which expression monitors the expression of the transgene. The retroviral particles were produced as previously described by co-transfecting the plasmids encoding for gag-pol, the ecotropic envelope and the retroviral vector, using Fugene (Roche) into the HEK-293 cell line. Retroviral supernatant was harvested 48 to 72 hours post-transfection and centrifuged prior to use. Freshly isolated, magnetic-activated cell sorting (MACS)-purified $CD4^+$ T cells were activated by using plate-bound anti-CD3 and anti-CD28 in the presence of mIL-4 (10 ng/ml, R&D Systems) and anti-mIL12 (10 μg/ml, Pharmingen) for T_H2 differentiation. After 24 hours of activation, retroviral supernatant was added to the cells together with polybrene (8 μg/ml). The plates were centrifuged at 2000 RPM for 45 minutes and put back at 37 °C. TGF-β (3 ng/ml) was added when indicated to induce Foxp3 expression. After 48 h of incubation at 37 °C, the GFP^+ cells were purified by flow cytometry and expanded for an additional 48–72 h in the presence of IL-2.

Lumier assay

HEK-293 cell lines were transiently co-transfected with Foxp3-renilla and GATA3-, Foxp1- or STAT6-Flag tagged. Fugene-6 was used for the transfection according to the manufacturer's instructions (Roche). Cell extracts were prepared (Promega) and

immunoprecipation was performed using anti-Flag coated beads (Sigma). Foxp3-renilla expression was assessed by detection of the luminescence.

Luciferase Assays

Jurkat cells were transfected using a Bio Rad electroporator (260 V); 5×10^6 cells in 0.4 ml RPMI were transfected with 5 μg reporter plasmid (*Il5* promoter-luciferase) and 20 μg expression plasmid. Luciferase assays were performed after activating the cells 3 hours with PMA-ionomycin stimulation as previously described.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank D. Kozoriz, R. Chandwaskar, and D. Lee for cell sorting and technical assistance. This work was supported by grants from National Institutes of Health (R01AI073542-01 to M.O. and 1R01NS045937-01, 2R01NS35685-06, 2R37NS30843-11, 1R01A144880-03, 2P01A139671-07, 1P01NS38037-04 and 1R01NS046414 to V.K.K.) and National Multiple Sclerosis Society (RG-2571-D-9 to V.K.K. and RG-3882-A-1 to M.O.), and the Juvenile Diabetes Research Foundation Center for Immunological Tolerance at Harvard Medical School. V.K.K. is a recipient of the Javits Neuroscience Investigator Award from the National Institutes of Health. A.A. and V.D. are supported by a post-doctoral fellowship from National Multiple Sclerosis Society, New York.

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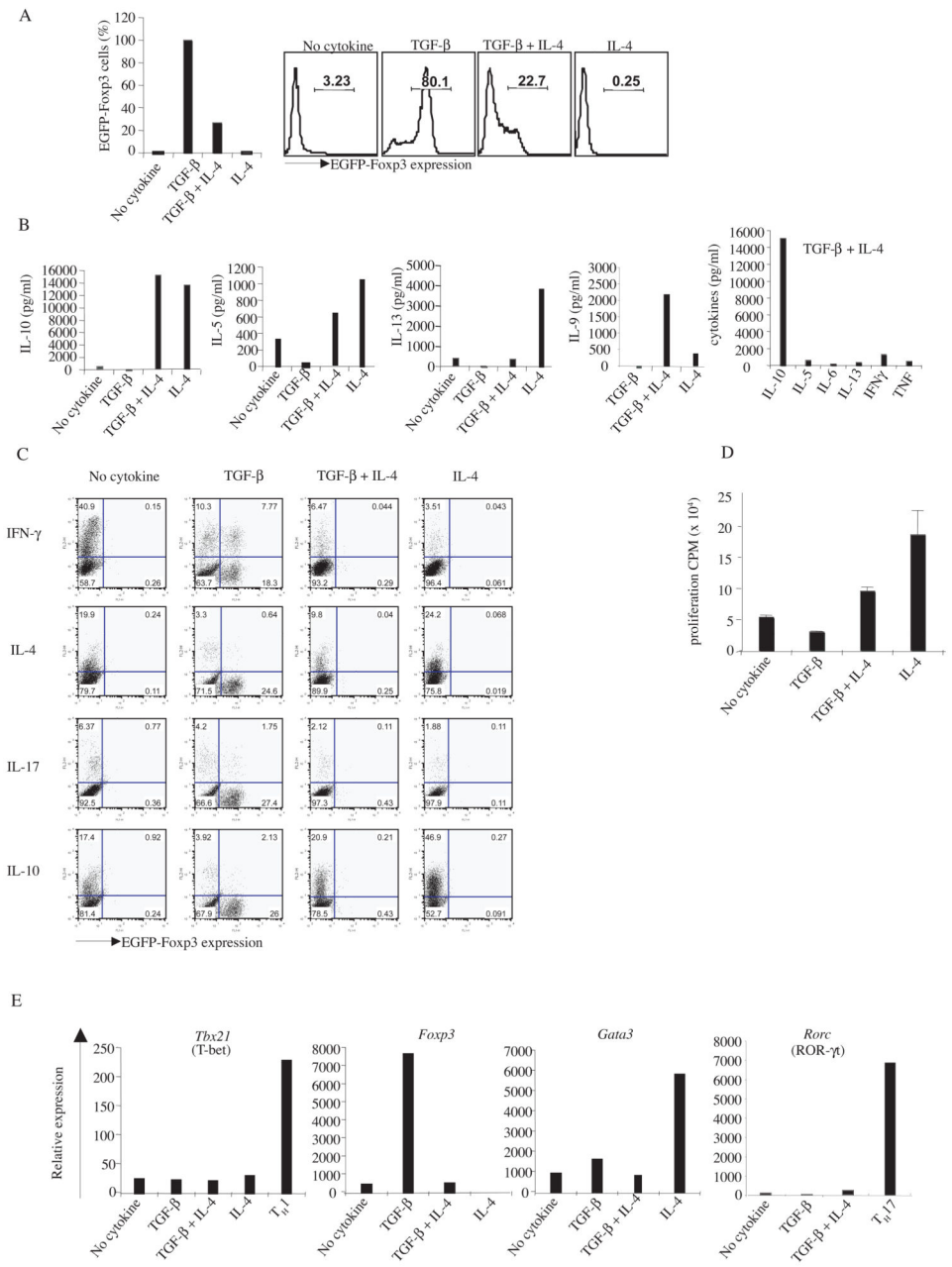


Figure 1. IL-4 inhibits TGF-β–induced Foxp3$^+$ T cells

(**a–e**) Naïve CD4$^+$Foxp3$^-$ CD62L$^+$ T cells from Foxp3-GFP mice activated with or without cytokines in the presence of irradiated syngenic APCs with 1 μg/ml anti-CD3. (**a**) Flow cytometry for GFP expression in CD4$^+$ T cells 72h after activation in the presence of TGF-β, IL-4 or both. Data represent one of three experiments. (**b**) ELISA assay or bead array for the indicated cytokines in 48 h culture supernatants. Data represent one of two experiments. (**c**) Flow cytometry for intracellular expression of IL-10, IL-4, IL-17 and IFN-γ in CD4$^+$ T cells measured at day 4 post-activation. Data represent one of three experiments. (**d**) Proliferation of cytokine-treated WT T cells was measured by ^{3}H-thymidine incorporation in triplicate wells of T cells activated in the presence of irradiated syngenic APCs with 1 μg/ml anti-

CD3. Proliferation was assessed in triplicate wells and is presented as mean ± s.d. Data represent one of three experiments. (**e**) Quantitative RT-PCR for the indicated transcripts for various transcription factors (mRNA expression relative to *Gapdh)*. Data represent mean one of two experiments.

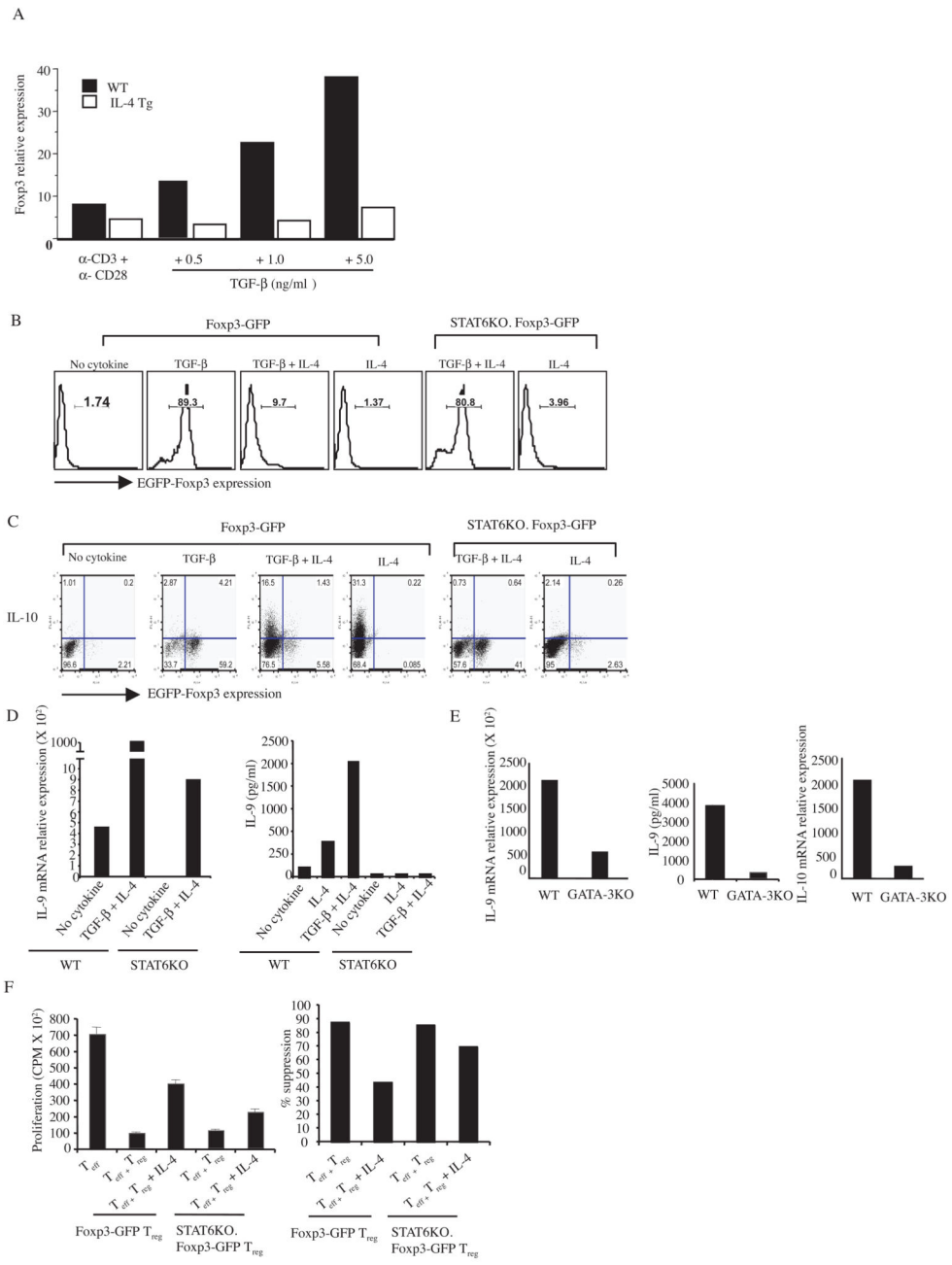


Figure 2. IL-4 inhibition of Foxp3 induction is STAT6-dependent

(**a**) RT-PCR for Foxp3 mRNA induction in IL-4 transgenic T cells (IL-4 Tg) and wild-type T cells (WT) after treatment by anti-CD3 + anti-CD28 and the indicated doses of TGF-β. Data represent one of 2 experiments. (**b–d**) Naïve $CD4^{+}Foxp3^{-}CD62L^{+}$ T cells from STAT6KO mice activated with or without cytokines in the presence of irradiated syngenic APCs and 1 μg/ml of anti-CD3. Flow cytometry for Foxp3-EGFP expression at 72 h (**b**), for intracellular Foxp3-EGFP and IL-10 expression at day 4 (**c**), and RT-PCR for IL-9 mRNA relative expression at day 4 (left) and ELISA for IL-9 protein production at day 2 (right). Data represent one of two experiments (**d**). (**e**) RT-PCR for IL-9 (left) and IL-10 (right)

mRNA expression in naïve $CD4^+CD62L^+$ T cells from GATA-3-deficient.cre^+(GATA-3KO) mice activated with or without cytokines in the presence of irradiated syngenic APCs and 1 μg/ml of anti-CD3; expression was analyzed relative to *HPRT*. ELISA assay for IL-9 production (middle) was also evaluated. (**f**) Suppression assay with naïve STAT6KO responder cells cultured with T_{reg} cells purified from either wild-type (WT) or STAT6KO mice in presence of the indicated cytokines (left). Proliferation was assessed in triplicate wells and is presented as mean ± s.d. Data represent one of two experiments. Percent suppression in each culture was calculated relative to the proliferation of effector T cells alone (T_{eff}) (right).

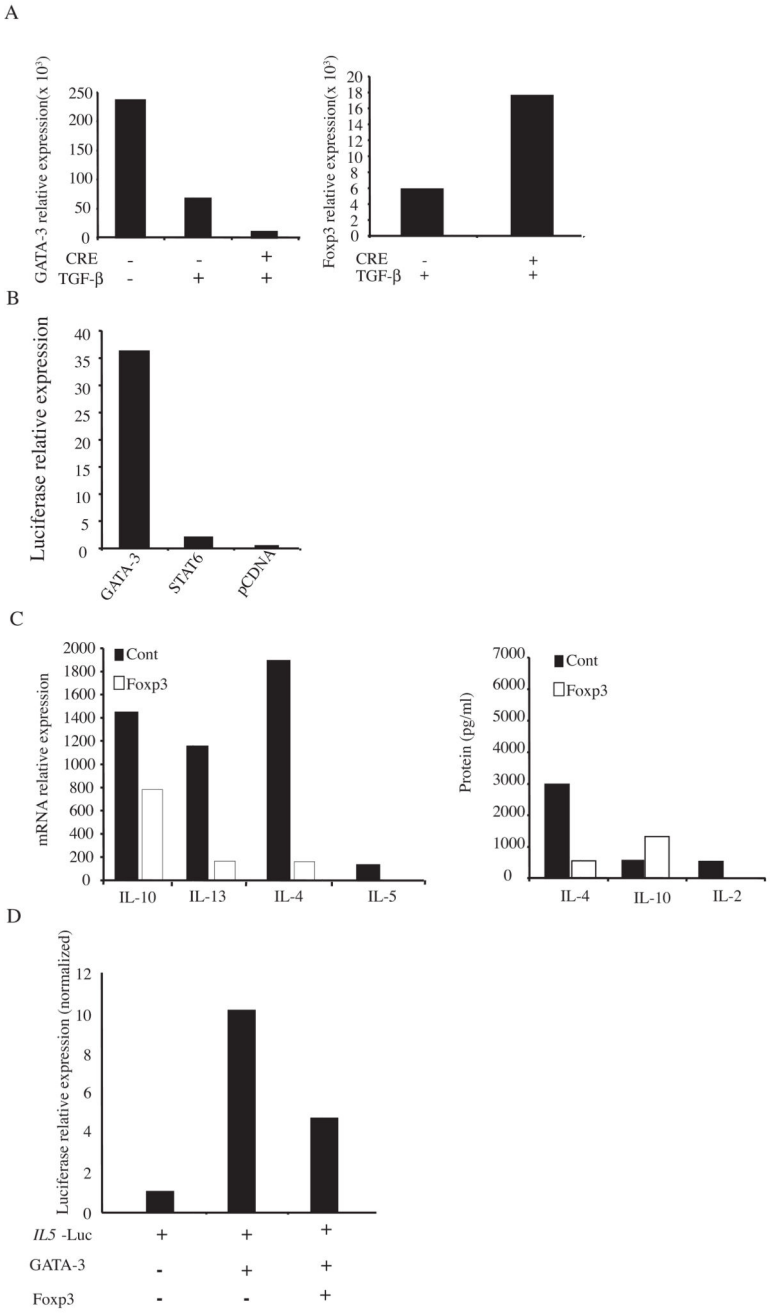
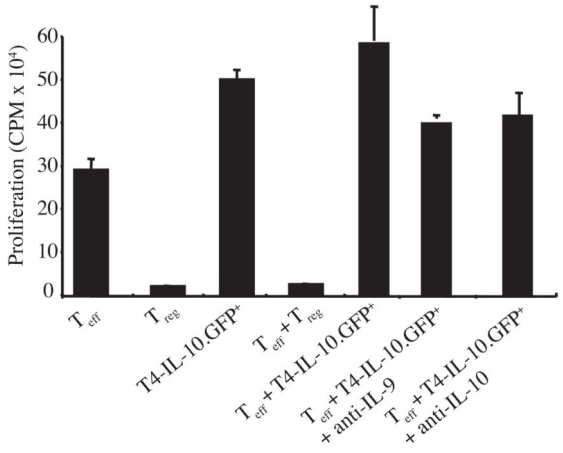


Figure 3. Relationship between and Foxp3 and GATA-3

(**a**) RT-PCR for Foxp3 and GATA-3 expression in CD4^{+}T cells from *Gata3*$^{flox/flox}$ mice transduced with a retroviral vector expressing Cre; the cells were also treated with TGF-β (3 ng/ml), as indicated, to induce Foxp3 expression. RNA was extracted from the cells 48 hr later and expression of mRNAs was measured relative to *HPRT*. Data represent one of two experiments. (**b**) Luciferase assay on HEK-293 cells transfected with a Foxp3-renilla construct and plasmids expressing the indicated Flag-tagged GATA-3 or STAT6 or a control vector (pCDNA). Immunoprecipitation was performed using anti-FLAG coated beads and then the interaction of Foxp3 with GATA-3 and/or STAT6 was determined by monitoring

the luciferase activity. Data are representative of three experiments. (**c**) RT-PCR for expression of the indicate mRNAs in $CD4^+$ T cells activated under T_H2-polarizing condition and transduced with either a control retroviral vector or Foxp3-GFP-expressing vector. Foxp3-GFP^+ cells were sorted 48 hours later and expression of indicated cytokine transcripts was analyzed relative to *HPRT*. Alternatively, the Foxp3-GFP-transduced T cells were cultured for 3 more days in presence of IL-2 and then re-stimulated for 48 hours using plate bound anti-CD3 and anti-CD28. Supernatants were collected and cytokine analysis was performed by ELISA (right graph). Data presented are representative of three experiments. (**d**) Luciferase assay on Jurkat cells transfected with an *Il5* promoter-luciferase construct and plasmids expressing Foxp3, GATA-3 or both, as indicated for 24 hours. The cells were then activated for 3–4 hours with PMA + ionomycin and luciferase activity was measured. Data presented are representative of three experiments.

A



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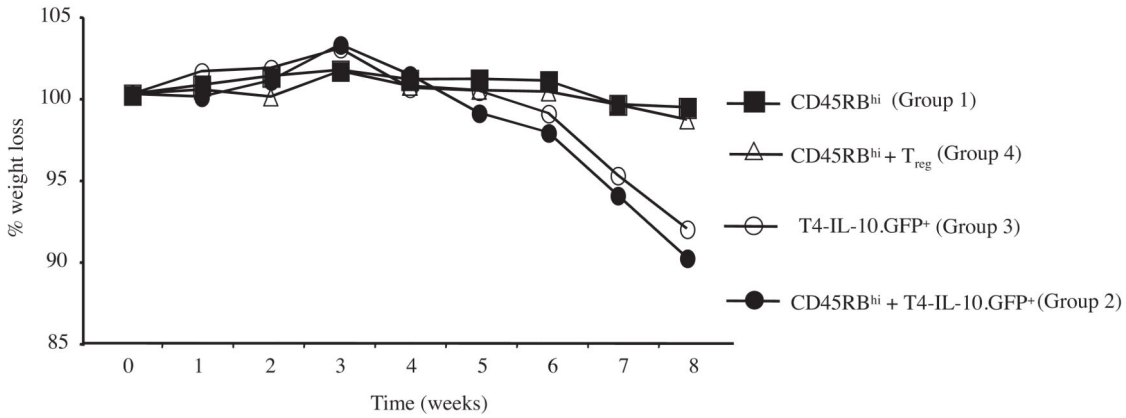

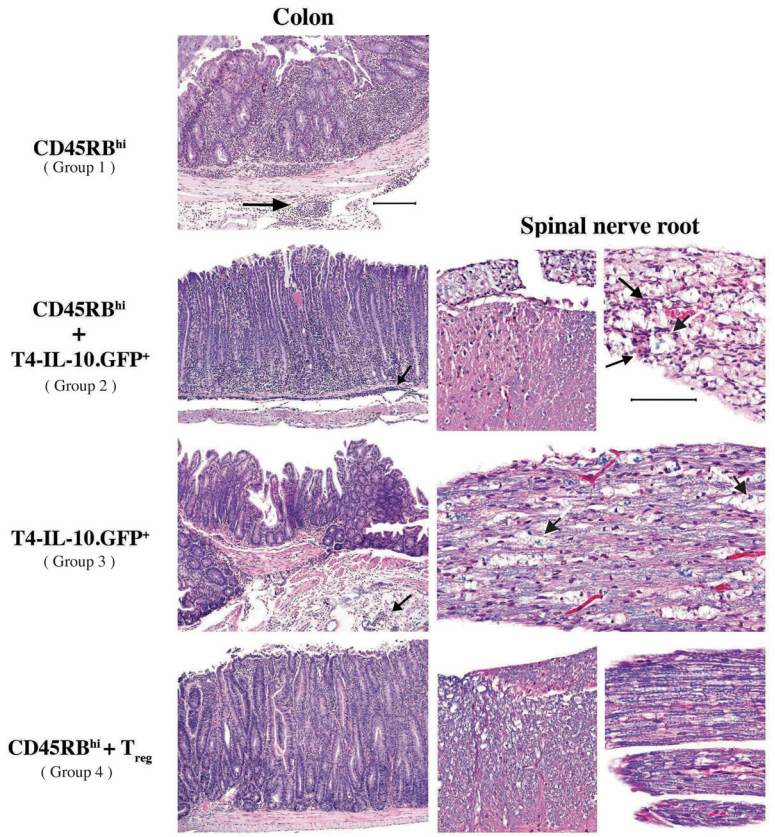

Figure 4. Function of the IL-9^{+}IL-10^{+} Foxp3^{-} T cells generated in presence of TGF-β and IL-4

(**a**) Suppression assay with naïve CD4^{+}CD62L^{+} T cells from IL-10.GFP reporter mice activated with or without cytokines (IL-4 + TGF-β) and non-treated IL-10.GFP^{+} T cells. Naïve wild-type Foxp3^{-} responder T cells were cultured alone, together with natural-occurring T_{regs} or with IL-10.GFP^{+} cells stimulated with TGF-β plus IL-4 (T4-IL-10.GFP^{+} cells); when indicated, anti-IL-9 or anti-IL-10 was added to the culture. Proliferation was assessed in triplicate wells by ^{3}H-thymidine incorporation and is presented as mean ± s.d. Data represent one of three experiments. (**b**) RAG-1-deficient mice were reconstituted with sorted CD4^{+}Foxp3-GFP^{-}CD45RB^{hi}T cell sub populations in PBS. Mice received CD4^{+}Foxp3-GFP^{-}CD45RB^{hi}T cell alone or in combination with IL-10.GFP^{+} cells stimulated with TGF-β plus IL-4 (T4-IL-10.GFP^{+} cells) or CD4^{+}Foxp3^{+} naturally occurring T_{reg} cells. Mice were examined for their weight loss for 9 weeks. Each time point represents an average of the calculated weight of all mice present per group (5–8 mice) of 2 independent experiments. The total colonic score was calculated as the average of the individual scores from the sections of proximal colon, mid-colon, and distal colon28. The mice in Group 1 (CD45RB^{hi} effector T) had a pathology score of 7.5 ± 3.5; Group 2 (CD45RB^{hi} effector T cells + (TGF-β + IL-4) IL-10.GFP^{+} cells): 8.8±2.3; Group 3, (T4-IL-10.GFP^{+} cells): 5.7±2.6 and Group 4, (CD45RB^{hi} T_{eff} cells + T_{reg} cells) : 3.4±1.5. The difference in the mean score was statistically different between Group 2 and 4 (P = 0.01) and Group 3 and 4 (P = 0.03) (Mann-Whitney one tailed U test). (**c**) Representative histopathology of colitis and peripheral neuritis in *Rag1*$^{-/-}$mice reconstituted with the following cell types. Group 1, CD45RB^{hi} effector T cells (mononuclear cell inflammation in

the lamina propria, muscularis and adventitial tissues (arrow)). Group 2, $CD45RB^{hi}$ effector T cells + (TGF-β + IL-4) IL-10.GFP^+ cells (T4-IL-10.GFP^+ cells); inflammation in the lamina propria and submuscularis (arrow) and blue staining of myelin; higher power of a nerve root (middle) with inflammatory cell infiltration (arrows) and myelin breakdown product in vacuoles (right, arrow). Group 3, T4-IL-10.GFP^+ cells alone; inflammation in the lamina propria, muscularis mucosa and adventitial tissues (left, arrow); a spinal nerve root shows vacuolation with myelin breakdown products (right, arrows). Group 4, $CD45RB^{hi}$ T_{eff} cells + T_{reg} cells; intact mucosal architecture and the muscularis (left), intact spinal cord white matter and nerve root (middle) and higher power of intact myelin in cauda equina nerve root (right) **a–d**. Luxol fast blue-H&E, **e–h**. Bar in Group 1 corresponds to 200 μm, in 100 μm in **c** and **h**. Bar in D corresponds to 100 μm in D, F and H.