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Molecular antagonism and plasticity of regulatory and inflammatory T cell programs

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Abstract

Regulatory T (Treg) and T helper 17 (Th17) cells were recently proposed to be reciprocally regulated during differentiation. To understand the underlying mechanisms, we utilized a Th17 reporter mouse with a red fluorescent protein (RFP) sequence inserted into the interleukin-17F (IL-17F) gene. Using IL-17F-RFP together with a Foxp3 reporter, we found that the development of Th17 and Foxp3$^+$ Treg cells were associated in immune responses. Although TGF-β receptor I signaling was required for both Foxp3 and IL-17 induction, SMAD4 was only involved in Foxp3 upregulation. Foxp3 inhibited Th17 differentiation by antagonizing the function of the transcription factors RORγt and RORα. In contrast, IL-6 overcame this suppressive effect of Foxp3 and together with IL-1, induced genetic re-programming in Foxp3$^+$ Treg cells. STAT3 regulated Foxp3 downregulation, whereas STAT3, RORγ and RORα were required for IL-17 expression in Treg cells. Our data demonstrate molecular antagonism and plasticity of Treg and Th17 cell programs.

Introduction

Naïve CD4$^+$ helper T (Th) cells, upon encountering their cognate antigens presented on professional antigen-presenting cells (APCs), differentiate into effector cells that are characterized by their cytokine production profiles and immune regulatory functions. In addition to Th1 and Th2 cells (Dong and Flavell, 2000), a third subset of effector Th cells, Th17, has been identified, which produce IL-17, IL-17F and IL-22 and regulate inflammatory responses by tissue cells (Dong, 2008). Th17 differentiation in mouse is initiated by TGF-β and IL-6. Recently, IL-21 was reported as an autocrine factor induced by IL-6 to regulate Th17 differentiation. STAT3, downstream of IL-6 and IL-21, is essential for RORγt and RORα expression and Th17 differentiation (Laurence et al., 2007; Yang et al., 2007). STAT3 may function by regulating the expression of two orphan nuclear receptors RORγt and RORα in developing Th17 cells (Ivanov et al., 2006; Yang et al., 2008b).

Thymus-derived natural regulatory T (nTreg) cells represent a unique subpopulation of CD4$^+$ T cells that inhibit T cell proliferation and autoimmune responses (Wing et al., 2006). The hallmark of nTreg cells is the expression of Foxp3 transcription factor, which is required for maintaining Treg cell function (Williams and Rudensky, 2007). TGF-β has been shown to

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maintain peripheral nTreg; its deficiency leads to development of early lethal autoimmunity (Marie et al., 2005; Shull et al., 1992). Moreover, in the presence of TGF-β, Foxp3 can be also induced in naive T cells in periphery and the resulting inducible Treg (iTreg) cells exhibit a suppressive phenotype similar to nTreg (Wing et al., 2006).

As describe above, TGF-β is required for regulation of nTreg and iTreg cells, and it is also involved in Th17 differentiation. Thus, there is not only functional antagonism between Th17 and Treg cells in autoimmunity, as well as reciprocal regulation in the generation of these cells. Although TGF-β induces Foxp3 expression, IL-6 and IL-21 inhibit this regulation and together with TGFβ drive Th17 differentiation. The molecular mechanisms underlying this differential T cell fate decision initiated by cytokines is unclear. In the present study, we have analyzed the molecular interaction of Treg and Th17 cell genetic programs in response to cytokine regulation. To better address this question, we utilized a Th17 reporter mouse with a red fluorescent protein (RFP) coding sequence inserted into the IL-17F gene. Our data reveal intrinsic association of Th17 and Treg cell differentiation programs in activated T cells *in vitro* and *in vivo*. In addition, we have analyzed the genetic programming and re-programming of Treg and Th17 cells. These results thus have revealed the molecular antagonism of Treg and Th17 cell genetic programs and indicated the plasticity of T cell differentiation programs

Results

Generation and characterization of an IL-17F-RFP reporter mouse

To better characterize Th17 cell differentiation, we generated an IL-17F reporter strain by insertion of a cassette containing an IRES-driven red fluorescent protein (RFP) and a bovine growth hormone polyA tail into the 2nd exon of the IL-17F gene through homologous recombination in murine embryonic stem cells (Yang et al., 2008a). Heterozygous IL-17F-RFP (hereafter referred as *Il17f^{rfp}*) mice were first tested for the fidelity of RFP expression in reporting Th17 cells. Naïve CD4^{+}CD25^{-}CD62L^{hi}CD44^{lo} T cells were isolated from *Il17f^{rfp}* mice by FACS sorting and differentiated under Th1, Th2, Th17 and iTreg conditions. 4 days after activation, RFP was highly expressed in Th17 cells but not in Th1 and Th2 cells (Fig. 1A). Notably, under iTreg condition, weak expression of RFP was also observed (Fig. 1A) although IL-17 protein was not expressed (data not shown).

Because not all cells differentiated under Th17 cell conditions express RFP, we next sorted RFP^{+} and RFP^{-} subsets from the above Th17 culture by flow cytometry and IL-17 and IL-17F expression was evaluated by intracellular staining. In RFP^{+} population, there was a substantial IL-17 and IL-17F double producing subset and an IL-17F single expression subset (Fig. 1B). In contrast, RFP-cells did not express IL-17 or IL-17F. Next, we also assessed the gene expression profiles of RFP^{+} and RFP^{-} cells in Th17 culture by real-time RT-PCR and compared them to Th0 (T cells differentiated under neutral conditions), Th1, Th2 and iTreg cells. The RFP^{+} but not RFP^{-} population highly expressed Th17 signature genes, including *Il17a*, *Il17f*, *Il22*, *Il21*, *Il23r* and *Rorc* (encoding RORγt), and upregulated *Rora* (encoding RORα) gene expression (Supplemental Fig. 1A). Th1-, Th2- and iTreg-specific genes (*Tbx21*, *Gata3* and *Foxp3*, respectively) were not highly expressed in the RFP^{+} cells (Supplemental Fig. 1A).

Because IL-17F-RFP marks Th17 cells, we further utilized T cells from *Il17f^{rfp}* mice to characterize the cytokine regulation of Th17 differentiation. Naïve T cells isolated from *Il17f^{rfp}* mice were activated in the presence or absence of IL-6, IL-21, TGF-β or combination of the cytokines. Unlike neutral condition, IL-6 or TGFβ slightly increased the number of RFP expressing cells (Fig. 1C); in these cells, IL-17F was expressed at low amounts (Supplemental Fig. 1B). The combination of TGFβ with IL-6 or IL-21 greatly increased the frequency of RFP-expressing cells (Fig. 1C). Cytokine staining revealed that IL-17F expression correlated well with RFP expression (Supplemental Fig. 1B). IL-17 expression also correlated with RFP

expression under IL-6, TGF-β + IL-6 or TGF-β + IL-21 conditions but not under TGF-β stimulation (Supplemental Figure 1B).

Lamina propria was previously shown as a site where some T cells constitutively express IL-17 (Ivanov et al., 2006). We thus isolated lamina propria cells from wild-type, *Il17f*^{rfp} and *Il17f*$^{rfp/rfp}$ mice and examined RFP expression in CD4$^+$ T cells. Although T cells from WT mice had no background fluorescence, approximately 2% from *Il17f*^{rfp} and 8% of those from *Il17f*$^{rfp/rfp}$ mice expressed RFP (Supplemental Fig. 2A). To further characterize the RFP$^+$ cells in lamina propria, we sorted RFP$^+$ and RFP$^-$ fractions by flow cytometry and performed gene expression analysis by real-time RT-PCR. The RFP$^+$ population exhibited a Th17 gene expression profile-they highly expressed IL-17, IL-17F and IL-22 as well as upregulated IL-23R, RORα and RORγt expression (Supplemental Fig. 2B).

We also utilized the reporter mice to identify IL-17-expressing cells *in vivo*. In spleen, in contrast to lamina propria, only a minor population of CD4$^+$CD25$^-$CD62L^{lo}CD44^{hi} memory T cells and a very small percentage of NKT cells expressed RFP upon restimulation (Supplemental Fig. 2C). Moreover, a substantial portion of γδ T cells was RFP+ (Supplemental Fig. 2C). These results suggest not only that our reporter mice can be used to characterize various IL-17-expressing T cells, but also that γδ T cells may constitute a major source of IL-17 and IL-17F cytokines *in vivo*.

To analyze Th17 cells generated under pathological conditions, we immunized *Il17f*^{rfp} and WT mice with MOG peptide emulsified in CFA. 5 days later, splenocytes and draining lymph node cells were harvested from the immunized mice and restimulated with MOG peptide for 24 h. As assessed by FACS, RFP was expressed in about 1-2% of the CD4+ cells from *Il17f*^{rfp} mice (Fig. 1D), comparable to the frequency of IL-17/IL-17F secreting cells (data not shown). In WT mice, no RFP+ cells were observed.

To understand the characteristics of pathogenic Th17 cells, we induced EAE in *Il17f*^{rfp} and WT mice. When most of the mice reached clinic score 3, with a symptom of hind leg paralysis, the CNS infiltrates were isolated and analyzed for IL-17F-RFP expression. In the CNS infiltrates from *Il17f*^{rfp} mice, there were approximately 20% of CD4$^+$ cells expressed RFP (Fig. 1E). To further characterize these Th17 cells in the CNS, we sorted RFP$^+$ and RFP$^-$ CD4$^+$ T cells from the CNS infiltrates of *Il17f*^{rfp} mice by FACS and performed gene expression analysis by real-time RT-PCR. The RFP$^+$ cells from the CNS highly expressed mRNA for Th17-specific genes *Il17a*, *Il17f*, *Il22*, *Il23r*, and *Rorc* (Fig. 1F). RFP$^-$ cells highly expressed *Ifng* and *Tbx21*, correlating with a large population of IFN-γ-secreting cells in the CNS of EAE mice (data not shown). Thus, *Il17f*^{rfp} reporter sensitively and faithfully marked Th17 cells *in vitro* and *in vivo*.

Analysis of Treg and Th17 cell development using an IL-17F and Foxp3 dual reporter mouse

To investigate the relationship between Th17 and iTreg during T cell activation, we crossed *Il17f*^{rfp} with *Foxp3*^{gfp} reporter mice (Fontenot et al., 2005) and produced an *Il17f*^{rfp}*Foxp3*^{gfp} dual reporter. Naïve (GFP$^-$CD44^{lo}CD62L^{hi}) CD4$^+$ T cells were activated with anti-CD3 and anti-CD28 in the presence of IL-2 under iTreg (TGF-β, anti-IL-4 and anti-IFN-γ) or Th17 (TGF-β and IL-6) conditions for 1-4 days. IL-17F-RFP *vs* Foxp3-GFP expression was assessed daily by FACS. On day 1, Foxp3 expression was weakly induced in cells activated under either iTreg or Th17 conditions and there was no cell expressing RFP (Fig. 2A). On day 2, GFP$^+$ cells were markedly increased in both conditions and RFP-expressing cells started to appear under Th17 culture condition. Interestingly, under Th17 condition, approximately one third of Foxp3-GFP$^+$ cells also express RFP (Fig. 2A), indicating that iTreg and Th17 differentiation can be simultaneously induced, at least in a portion of the activated T cells. On day 3-4, GFP + cell numbers were drastically reduced whereas percentage of RFP$^+$GFP$^-$ cells was greatly

increased. The presence of exogenous IL-2 did not affect the kinetics of RFP and GFP expression (Supplemental Fig. 3A). Moreover, on days 2 and 3, GFP^+ cells did not exhibit increased apoptosis as examined by Annexin V staining (Supplemental Fig. 3B), suggesting that the Foxp3-GFP^+ cells on day 2 in this culture did not preferentially undergo cell death. Compared with Th17 cell condition, under iTreg cell condition, although IL-17 expression was not detected (data not shown), weak RFP expression was found on day 2 and more substantially on day 3 in both GFP^+ and GFP^- populations but it was greatly reduced on day 4 (Fig. 2A). To further analyze the RFP^+GFP^+ cells associated with iTreg differentiation, GFP^+RFP^- and GFP^+RFP^+ cells were sorted on day 3 from the iTreg culture and examined by real-time RT-PCR for their gene expression. RFP^+ cells expressed similar amounts of Foxp3 as RFP^- cells while markedly upregulated expression of Th17-specific genes IL-17, IL-17F, RORγt and RORα (Fig. 2B), supporting a dual program in these cells. These results indicate that under both Th17 and Treg cell conditions, Foxp3 and IL-17F co-expressors were induced transiently in some T cells before they were terminally differentiated into single producers, supporting the intimate relationship of Treg and Th17 cells during their development.

We then asked whether this RFP^+GFP^+ population or state also exists in the immune response *in vivo*. In naïve *Il17f*^{rfp}*Foxp3*^{gfp} mice, splenic and laminal propria $CD4^+$ T cells contained RFP and GFP single positive cells; very few, if any, double positive cells were observed (Fig. 2C). To analyze the regulation of T cell differentiation in immune responses, *Il17f*^{rfp}*Foxp3*^{gfp} dual reporter and WT mice were immunized with MOG in CFA and IL-17F-RFP and Foxp3-GFP were detected by FACS after MOG *ex vivo* restimulation. In comparison to $CD4^+$ T cells from WT mice, about 1% of RFP^+GFP^- Th17 cells and 4% GFP^+RFP^- Treg cells were observed in the dual reporter mice after the immunization (Fig. 2C). A small but detectable percentage (less than 0.1% of total $CD4^+$ T cells and approximately 7-8% of RFP^+ cells) of T cells expressed both RFP and GFP (Fig. 2C), suggesting that Th17 and Treg cells generated *in vivo* also share intrinsic common programs as those differentiated *in vitro*.

TGFβ signaling requirements during Th17 and iTreg cell differentiation

TGF-β regulates iTreg and, together with IL-6, Th17 differentiation but the underlying signaling mechanism is unclear. To analyze this, naïve cells from C57BL/6 (B6) mice were differentiated towards Th17 cells for 5 days and an inhibitor of TGFβRI kinase activity, SB431542, was added at different time-points during the Th17 differentiation. Inhibition of TGFβ signaling on day 0 or day 1 completely abolished IL-17 production (Fig. 3A). Moreover, a 50% reduction in IL-17 production was observed after addition of the inhibitor on day 2, whereas no substantial inhibition was found on day 3 (Fig. 3A). Real-time RT-PCR analysis also indicated decreased expression of IL-17, IL-17F, RORα, RORγt and IL-23R mRNA in Th cells after addition of SB431542 on days 0 and 1 (Fig. 3B). Similar to Th17 differentiation, TGFβ signaling was also required during the first two days after initiation of iTreg differentiation (Fig. 3C). These data indicate that active TGF-β signaling is required during the first two days of Th17 and Treg cell differentiation.

TGF-β receptor activation induces activation and phosphorylation of Smad2 and Smad3, which then bind to Smad4 and translocate to the nucleus (Feng and Derynck, 2005). Therefore, we next investigated the role of Smad4 by using Smad4-deficient $CD4^+$ T cells. Mice with Smad4 deletion in T cells were generated by breeding mice with floxed *Smad4* (fl) alleles (Chu et al., 2004) with *CD4-Cre* mice (Lee et al., 2001). Deletion of the *Smad4* gene in $CD4^+$ T cells from *Smad4*$^{fl/fl}$*CD4Cre*$^+$ mice was confirmed by PCR (Supplementary Fig. 4A). *Smad4*$^{fl/fl}$*CD4Cre*$^+$ mice exhibit normal populations of $CD4^+$ and $CD8^+$ T cells, as well as nTreg cells in spleen, lymph nodes, and thymus (Supplemental Fig. 4B-C). Moreover, Smad4-deficient nTreg were as suppressive as wild-type (WT) nTreg (Fig. 3D). The proliferation of naïve $CD4^+$ T cells from WT or *Smad4*$^{fl/fl}$*CD4Cre*$^+$ mice was inhibited by nTreg from either

WT or *Smad4*$^{fl/fl}$*CD4Cre*$^{+}$ mice (Fig. 3D). Thus, Smad4 is not required for nTreg cell development and suppressive activity or naïve T cell suppression by nTreg cells. We next investigated whether Smad4 is required for iTreg or Th17 differentiation by stimulating naive Th cells under iTreg or Th17 conditions. Smad4-deficient Th cells showed reduced Foxp3 expression compared to WT T cells upon iTreg induction (Fig. 3E). However, upon Th17 differentiation, comparable numbers of IL-17-producing cells were observed between Smad4-deficient and WT T cells (Fig. 3F), indicating that Smad4 is differentially required for iTreg and Th17 differentiation. Further *in vivo* analysis showed no difference in IL-17, IL-17F and IL-22 production between WT and Smad-4 deficient T cells after KLH immunization (Supp. Fig. 4D). Thus, although active TGFβ signaling regulates both iTreg and Th17 differentiation, different downstream molecules may be utilized to regulate the development of these two T cell lineages.

Regulation of Th17 cell differentiation by Foxp3

Because TGF-β induces the expression of Foxp3, we next assessed Foxp3 function in Th17 differentiation. First, we over-expressed Foxp3 in T cells by retroviral transduction. Naïve CD4^{+} T cells from OT-II mice were activated with Ova peptide and irradiated splenic APCs under Th17 conditions. On day 1, activated T cells were infected with bicistronic retroviruses containing an IRES-GFP. 4 days after infection, IL-17- and Foxp3-expressing cells were determined by intracellular staining. Compared to cells infected with a control virus, Foxp3 overexpression greatly decreased the percentage of IL-17-secreting cells (Fig. 4A). We then sorted GFP^{-} and GFP^{+} cells from the above experiment and their gene expression profiles were assessed using real-time RT-PCR. In comparison with cells infected with the control virus, Foxp3 over-expression greatly reduced IL-17, IL-17F and IL-21 mRNA expression, whereas RORγt and RORα expression remained the same (Fig. 4B). These results indicate that Foxp3 inhibits Th17 differentiation and suggest that it might not inhibit RORα or RORγ mRNA expression but rather interfere with their function.

To analyze the regulation of RORα and RORγt function by Foxp3, EL-4 cells were transfected with an *Il17a*-promoter-*CNS2* (promoter+CNS2) luciferase reporter vector (Yang et al., 2008b) in the presence or absence of RORα or RORγt with or without Foxp3-expressing vector. Whereas RORα or RORγt alone induced luciferase activity, co-expression of Foxp3 markedly reduced their activity (Fig. 4C-F and Supplemental Fig. 5D-F). Because neither IL-17 promoter nor CNS2 element contains a detectable Foxp3 binding site, we analyzed if Foxp3 DNA binding or homo-dimerization is required for its ability to suppress RORα or RORγt function. Foxp3ΔFKH/NLS, a Foxp3 mutant lacking the forkhead domain but with the SV40 nuclear localization sequence (Lee et al., 2008), or Foxp3ΔE250, a Foxp3 mutant that possesses a single amino acid deletion in the leucine-zipper domain and thus cannot homodimerize or bind to DNA well (Chae et al., 2006) were co-expressed with RORγt. These two mutants were found to still inhibit RORγt-driven luciferase activity (Fig. 4C). Foxp3ΔE250 and Foxp3 ΔFKH/NLS also inhibited Th17 differentiation in primary T cells (data not shown). Furthermore, Foxp3 also directly inhibited RORγ activation of a RORE reporter in a dose-dependent manner (data not shown). Our data together indicate that Foxp3 overexpression inhibits RORγt activity independent of Foxp3 homodimerization or DNA binding.

Nuclear receptors bind to co-activators or co-repressors through interaction between the AF2 domains of the nuclear receptors with LxxLL motifs in coactivators and/or corepresors. To understand how Foxp3 inhibits ROR function, we utilized a mammalian two-hybrid system, in which luciferase reporter activity is activated upon binding of RORγ to an LxxLL containing peptide EBIP96 derived from SRC-1 co-activator (Kurebayashi et al., 2004). Interestingly, Foxp3 inhibited, in a dose-dependent manner, the binding of RORγ to the LxxLL-containing peptide (Supplemental Fig. 5A), indicating that Foxp3 may interfere the association of

RORγ with its co-activator. Given that Foxp3 contains an LQALL sequence in its second coding exon, we hypothesized that Foxp3 might interact with both RORα and RORγt and compete with coactivators for ROR binding. We therefore examined the interaction of RORα with wild-type Foxp3 and several Foxp3 mutants using co-immunoprecipitation. Although Foxp3 and RORαwere found to associate when they were co-expressed in 293T cells, a Foxp3 mutant carrying a mutation in LQALL motif (LL-AA mutant) exhibited a greatly decreased association with RORα (Supplemental Fig. 5B). In addition, a mutant Foxp3 containing only amino acids encoded by exons 1 and 2, which include the LQALL motif but no DNA binding or dimerization domain, inhibited RORγ transcriptional activity (Supplemental Fig. 5C), supporting that LQALL in Foxp3 may inhibit RORα or RORγ interaction with a co-activator.

We further tested the function of Foxp3 LQALL motif by use of the LL-AA mutant. In the mammalian two-hybrid system, the Foxp3 LL-AA mutant had reduced ability to inhibit the binding of RORγ to EBIP96, compared to Foxp3 WT (Supplemental Fig. 5A). To evaluate the functional implication of this decreased inhibitory activity by the Foxp3 LL-AA mutant, we analyzed whether lack of the LxxLL domain impairs Foxp3 ability to inhibit activation of IL-17 transcription by RORs. We observed a dose-dependent downregulation of luciferase activity that was not statistically significant by Foxp3 LL-AA mutant when co-expressed with RORγt or RORα (Fig. 4D and Supplemental Fig. 5D). Thus, these results indicate that Foxp3 LxxLL domain is important for binding to RORs, but lack of this domain inhibits but does not completely abolish Foxp3-mediated interference of ROR activity.

Foxp3 has been previously shown to bind to TIP60 and HDAC7 (Li et al., 2007). To further explore which additional domain of Foxp3 might be required for its inhibitory activity on RORs, we utilized a Foxp3 Δ105-190 mutant which does not bind to TIP60-HDAC7 complex (Li et al., 2007). Interestingly, although this mutant binds to RORα well when they were co-expressed in 293T cells (Supplemental Fig. 5B), the ability of Foxp3 to inhibit transcriptional activity of RORγt was partially reversed by lack of TIP60-HDAC7-binding domain (Fig. 4E and Supplemental Fig. 5E). Moreover, lack of both LxxLL and TIP60-HDAC7 domains completely impaired Foxp3 inhibition of RORγt or RORα transcriptional activity (Fig. 4F and Supp. Fig. 5F). To further demonstrate the role of these domains in inhibition of Th17 differentiation, naïve OT-II cells were infected with Foxp3 constructs under Th17 polarizing conditions, and IL-17 production was evaluated by intracellular staining. Overexpression of wild-type, LL-AA or Δ105-190 Foxp3 mutants greatly decreased the percentage of IL-17-secreting cells compared to cells infected with vector alone (Fig. 4G). However, Foxp3 lacking both LxxLL and TIP60-HDAC7 domains was not able to inhibit IL-17 production (Fig. 4G), further demonstrating that Foxp3 inhibits the activity of RORα and RORγ by direct binding with its LQALL domain and/or by recruiting the TIP60-HDAC7 complex.

To further assess the function of Foxp3 in Th17 differentiation, we bred OT-II TCR transgenic mice with Scurfy mice, which have a point mutation in Foxp3 gene. Naïve $CD4^+$ T cells from wild-type or Scurfy OT-II mice were stimulated with Ova peptide and irradiated splenic APC in the presence of different cytokine stimuli. Similar to wild-type T cells, Scurfy OT-II cells did not produce IL-17 in the present of TGFβ alone (Fig. 4H, supplemental Fig. 6A), indicating that Foxp3 deficiency was not sufficient to convert iTreg into Th17 cells and suggesting that IL-6 signaling is required for Th17 differentiation and not just functions by suppressing Foxp3 expression. When activated in the presence of TGF-β and IL-6, reduced production of IL-17 and IL-17F and enhanced IFN-γ was observed in the Scurfy T cells (Fig. 4H, supplemental Fig. 6A). However, addition of blocking antibodies to IFNγ and IL-4 together with TGF-β and IL-6 resulted in comparable amounts of IL-17 and IL-17F production in wild-type and Scurfy Th cells (Fig. 4H, Supplemental Fig. 6A). Real-time PCR analysis also indicated decreased expression of IL-17, IL-17F, RORγt mRNA in Scurfy-deficient Th cells compared to wild-

type counterparts when they were activated by TGF-β and IL-6, whereas blocking antibodies to IL-4 and IFN-γ restored their IL-17, IL-17F and RORγt mRNA expression (Supplemental Fig. 6B). Taken together, these results indicate that lack of Foxp3 expression in T cells leads to increased Th1 differentiation, and does not enhance Th17 differentiation. The latter is probably due to an inhibitory effect by IL-6 on Foxp3 expression and function during Th17 differentiation.

Conversion of iTreg to Th17 cells

Because TGFβ induces Foxp3 on its own whereas IL-6 overrides this differentiation process and skews T cells towards Th17 lineage, we next analyze if IL-6 is able to inhibit pre-committed Foxp3-mediated Treg programs. Naïve $CD4^+$ T cells from Foxp3-GFP reporter mice were activated together with TGF-β, IL-2 and neutralizing antibodies against IL-4 and IFN-γ for 5 days, and Foxp3-GFP^+ cells were sorted and restimulated with various cytokines for 4 days. Foxp3-GFP and IL-17 expression was determined by flow cytometry. Although iTreg cell quickly lost Foxp3 expression in the presence or absence of IL-2 as previously reported (Selvaraj and Geiger, 2007), IL-6 stimulation alone, or in combination with IL-1 and IL-23, enhanced Foxp3 downregulation but only slightly increased IL-17 production (Fig. 5A). However, when we stimulated iTreg with TGFβ, Foxp3 expression was sustained (Fig. 5A). In the presence of TGF-β, IL-6 alone or in combination with IL-1 and IL-23 markedly downregulated Foxp3 expression and increased IL-17 production (Fig. 5A). Real-time RT-PCR analysis of other Th17 specific genes demonstrated substantially enhanced expression of IL-22, IL-23R, and RORγt mRNA in iTreg stimulated with IL-6, IL-1 and IL-23 (Fig. 5B). However, the expression of these genes was not significantly increased in the presence of TGF-β (Fig. 5B). Thus, these results indicate that Treg program is turned off by IL-6 and that iTreg cells can be re-programmed to Th17 cells in the presence of TGFβ and IL-6.

Recently, all-trans retinoic acid (RA) was shown to inhibit the induction of proinflammatory Th17 cells while to promote anti-inflammatory Treg cell differentiation (Mucida et al., 2007). We thus asked whether RA affects re-differentiation of Treg cells towards Th17 cells. Naïve $CD4^+$ T cells from *Il17f*^{rfp}*Foxp3*^{gfp} mice were activated with TGF-β and IL-2 in the presence or absence of RA. As reported, inclusion of RA enhanced Foxp3 induction (data not shown). Three days later, GFP^+RFP^- cells were sorted and restimulated in the presence of TGFβ, IL-6, IL-1 and IL-23. While pretreatment with RA did not affect Foxp3 downregulation, it prevented the upregulation of RFP (IL-17F) and IL-17 expression (Fig. 5C). Thus, although iTreg cells developed in the presence of RA were still susceptible to Foxp3 downregulation, they were resistant to induction of Th17 program.

Cytokine-driven conversion of nTreg into Th17 cells

Because iTreg cells, even 5 days after differentiation, can be re-programmed to Th17 cells, we asked whether nTreg cells could be converted. FACS-sorted $CD4^+CD25^+$ T cells from B6 mice, which contained >99% Foxp3-expressing T cells (data not shown), were activated with plate-bound anti-CD3 and anti-CD28 in the presence of IL-6 for 4 days. Addition of IL-6 to nTreg cells resulted in expression of IL-17 and down-regulation of Foxp3 expression (Fig. 6A). Similarly, when nTreg cells from the IL-17F-RFP reporter mice, which did not express RFP after sorting (data not shown), were cultured as above, RFP was induced (Fig. 6B). This finding indicates that IL-17F was also induced by IL-6 in nTreg cells. Next, to determine if IL-17-IL-17F-expressing T cells were suppressive, RFP^+ and RFP^- cells (Fig. 6B) were FACS-sorted and subjected to a suppression assay. Similar to non-manipulated nTreg cells, RFP^- cells inhibited the proliferation of naïve T cells (Fig. 6C). However, IL-17F-RFP^+ cells exhibited greatly reduced suppressive activity.

The above experiments suggest that nTreg cells can be re-programmed and re-differentiated into Th17 cells. To understand if there exists such a regulation *in vivo*, we purified $CD4^+GFP^+$ Treg cells from Foxp3 reporter mice ($CD45.2^+$) and mixed at 1:10 rations with $CD4^+CD25^-$ cells from $CD45.1^+$ congenic mice before transferred into $Rag1^{-/-}$ recipients. >99% of sorted GFP^+ cells were stained by an anti-Foxp3 antibody (Fig. 6D). Because some nTreg cells reactive with MOG peptides (Korn et al., 2007), the recipients were immunized with MOG peptide in CFA. 5 days later, $CD45.1^+$ and $CD45.2^+$ $CD4^+$ T cells were analyzed for Foxp3 and IL-17 expression after restimulation with PMA plus ionomycin or MOG peptide. As expected, substantially IL-17 expression was detected in $CD45.1^+$ cells, indicating that the immunization protocol was successful (Fig. 6D). However, in $CD45.2^+$ populations, there was significant downregulation of Foxp3 expression (Fig. 6D). In addition, IL-17 expression was detected in $Foxp3^-$ cells (Fig. 6D). When cells were restimulated with PMA plus ionomycin, Foxp3 and IL-17 dual expressers were also observed (Fig. 6D). This result indicates that in the presence of inflammatory signals, nTreg cells can differentiate into Th17 cells *in vivo*.

Regulation of nTreg conversion to Th17 cells

We further examined the regulation of Treg cell differentiation into Th17 cells by other cytokines using Treg cells purified from Foxp3-GFP reporter mice. In addition to IL-6, IL-21 exerted similar regulation but was less potent (Fig. 7A). IL-1 alone induced a small percentage of IL-17-producing cells, but did not markedly change the expression of Foxp3. However, in the presence of IL-6 or IL-21, IL-1 further enhanced the percentages of IL-17 positive cells, without any additional impact on Foxp3 expression (Fig. 7A). No synergistic effect by IL-23 was observed (Fig. 7A). Because nTreg cells produce TGFβ, we next examined whether TGFβ was required for the IL-6-mediated conversion of nTreg to Th17 cells. Addition of a blocking antibody to TGFβ abolished IL-17 expression induced with IL-6, IL-1 and IL-23, but did not enhance Foxp3 expression (Fig. 7A). Thus, although TGFβ, IL-6, IL-21 and IL-1 regulate the induction of IL-17-producing cells, IL-6 seems most potent and unique in downregulating Foxp3 expression.

nTreg from mesenteric lymph nodes could be also converted into Th17 cells upon activation in the presence of IL-6, IL-1 and IL-23 (Supplemental Figure 7A). To rule out the possibility that the converted cells were iTreg generated in the periphery, we isolated $CD4^+CD8^-$ GFP^+ thymocytes from Foxp3-GFP reporter mice and subjected them to conversion by IL-6, IL-1 and IL-23. Similar to their peripheral counterparts, thymic Treg downregulated Foxp3 and expressed IL-17 upon treatment with IL-6, IL-1 and IL-23 (Supplemental Figure 7B). Thus, nTreg cells are developmentally programmed to respond to inflammatory cytokines and undergo re-differentiation into Th17 cells.

To better characterize the conversion of nTreg to Th17 cells, we utilized Treg cells from *Il17f*^{rfp} *Foxp3*^{gfp} double reporter mice. FACS-sorted $CD4^+GFP^+$ cells from these mice were activated with plate-bound anti-CD3 and anti-CD28 in the presence of IL-6, IL-1 and IL-23. Down-regulation of Foxp3 expression was detected on day 1, which was further enhanced during the next 3 days (Fig. 7B). RFP expression was observed on day 2 and optimally induced on day 3 and 4. Interestingly, similar numbers of IL-17F single positive and $IL\text{-}17F^+Foxp3^+$ cells were observed on day 2 and 3; however on day 4, the majority of IL-17F producing cells did not express Foxp3-GFP (Fig 7B). To further characterize these populations, IL-17F and Foxp3 single positive cells were FACS-sorted, and the expression of Th17 and Treg lineage specific genes were analyzed by real-time RT-PCR. IL-17F-RFP single positive cells expressed higher amounts of IL-17, IL-17F, IL-21, IL-23R and RORγt mRNA compared to Foxp3-GFP single positive cells (Supplemental Fig. 8). Interestingly, $Foxp3\text{-}GFP^+$ cells also upregulated RORγt although they did not express Th17 markers, suggesting that Foxp3 repressed the

function of RORγt in these cells. Thus, this result indicates that converted IL-17F-producing cells have the same phenotype as Th17 cells.

To determine whether STAT3, RORγt and RORα are required for conversion of nTreg into Th17 cells, we first bred *STAT*$^{fl/fl}$ mice with *CD4-Cre* mice and efficient deletion of STAT3 was observed in T cells (data not shown). FACS-sorted CD4^{+}CD25^{+} T cells from STAT3-, RORγ- (Kurebayashi et al., 2000) deficient mice (the latter is deficient in all RORγ isoforms including RORγt), *Rora*$^{sg/sg}$, *Rorα*$^{sg/sg}$ *Rorc*$^{-/-}$ mice (Yang et al., 2008b) and their appropriate controls were stimulated with plate-bound anti-CD3 and anti-CD28 in the presence of various cytokines, and Foxp3 and IL-17 expression were determined by intracellular staining. STAT3-deficient nTreg cells failed to downregulate Foxp3 expression or express IL-17 (Fig. 7C). In contrast, *Rorc*$^{-/-}$ nTreg cells downregulated Foxp3 expression, but only expressed very low amounts of IL-17 when compared to their controls (Fig. 7D). Compared to RORγ deficiency, RORα mutant T cells exhibited moderate reduction in IL-17 expression but normal Foxp3 downregulation (Supplemental Fig. 9). T cells defective in both RORα and RORγ were also able to downregulate Foxp3 but had complete IL-17 deficiency (Fig. 7D). Thus, these data indicate that although STAT3, RORγ and RORα are all required for IL-17 expression, Foxp3 downregulation is regulated by STAT3, but not by RORγ or RORα.

Because IL-17F-RFP^{+}Foxp3-GFP^{-} cells expressed IL-21 (Supplemental Fig. 8), we investigated whether this cytokine is required for Th17 induction in nTreg cells. FACS-sorted CD4^{+}CD25^{+} T cells from IL-21-deficient mice and their appropriate controls were stimulated in the presence of various cytokines. IL-21-deficient nTreg cells exhibited normal downregulation of Foxp3 and conversion into IL-17-producing cells (Fig. 7E), indicating that autocrine IL-21 is not required for IL-6-mediated conversion of nTreg cells.

Discussion

In this study, we have investigated the molecular interactions of the Treg and Th17 genetic programs (Supplemental Fig. 10). Using an IL-17F-RFP reporter mouse bred with a Foxp3-GFP reporter, we found the presence of a RFP^{+}GFP^{+} transient phase upon T cell activation *in vitro* and *in vivo*, indicating both Th17 and Treg cell programs could be simultaneously induced in some T cells before they are terminally differentiated into either lineage. On one hand, Foxp3, induced by TGF-β via SMAD4, inhibits Th17 differentiation by antagonizing RORγt and RORα activity. On the other hand, IL-6 induces Foxp3 downregulation in Treg cells and together with IL-1 and TGF-β, reprograms them to become Th17 cells. In nTreg, this process is mediated by STAT3, RORα and RORγt.

TGF-β plays an important role in the development, maintenance, and induction of regulatory T cells. Our mutagenesis analysis reveals that Foxp3 could compete with co-activator binding to RORs via two independent and non-exclusive mechanisms. Moreover, we found that Scurfy T cells, compared to their wild-type counterparts, exhibited reduced expression of Th17 cytokines and RORα and RORγt when stimulated with TGF-β and IL-6, which was associated with increased Th1 differentiation. However, when IFN-γ and IL-4 were blocked under the same conditions, restoration of Th17 cytokines and RORγt was observed. Therefore, lack of Foxp3 expression in T cells results in enhanced Th1 differentiation, but Foxp3, although transiently induced in some T cells undergoing Th17 development is not required for Th17 lineage differentiation. Furthermore, because Scurfy T cells did not spontaneously developed into Th17 cells in the presence of TGF-β, it suggests that TGF-β signaling is not sufficient to drive Th17 differentiation. STAT3 downstream of IL-6 may be also required, which does not merely function by downregulating of Foxp3. Our results thus further support the synergy of TGF-β and IL-6 in Th17 differentiation.

IL-6 inhibits TGF-β-dependent Foxp-3$^+$ Treg cell induction (Bettelli et al., 2006). Our current study also indicates that IL-6 can re-program fully differentiated iTreg and nTreg cells and re-differentiate them towards the Th17 lineage. By using reporter mice for Foxp3 and IL-17F, we found the resulting cells express Th17-specific genes and lack suppressive function. This action by IL-6 is synergized by IL-1 and requires TGF-β. However, although TGF-β, IL-6, IL-21 and IL-1 regulate the induction of IL-17-producing cells, IL-6 seems most potent in downregulating Foxp3 expression. Terminally differentiated cells have been shown to be able to de-differentiate and re-differentiate. For example, it was recently reported that mature B cells, when the Pax5 gene was deleted, were able to re-differentiate into T cells (Cobaleda et al., 2007). Interestingly, it was previously observed that deletion of the Foxp3 gene in mature nTreg cells resulted in loss of suppressive function and upregulation of IL-17 and IL-21 expression (Williams and Rudensky, 2007), suggesting that the suppression of Th17 gene expression and the maintenance of Treg programs both require Foxp3. Unlike these two cases, here we observe pro-inflammatory cytokine milieu in both de- and re-differentiation of Treg cells. Our results are consistent with a recent work by Xu et al who found Treg cells conversion to Th17 cells when cultured with IL-6 in the absence of TGFβ (Xu et al., 2007). These studies not only indicate the plasticity of Th cell differentiation programs but also have important implications. First, they suggest an alternative source of Th17 cells *in vivo*, i.e. derived from Foxp3$^+$ nTreg and iTreg cells. This pathway is potentially problematic because many Treg cells have autoreactive specificities (Hsieh et al., 2004). Second, in future therapy using Treg cells against autoimmune diseases, suppression of inflammatory cytokines especially IL-6 would be needed simultaneously to prevent them from re-differentiating into pathogenic Th17 cells.

How STAT3 mediates Foxp3 downregulation by IL-6 remains to be determined. IL-6 induced Foxp3 mRNA downregulation in nTreg cells 24 and 48 hours after treatment (data not shown). In addition, we also found that in T cells transduced with Foxp3-expressing retroviruses, i.e. with exogenous promoters and untranslated regions, IL-6 downregulated Foxp3 protein expression (data not shown). Regardless which mechanism, this important regulation will unleash the inhibitory function of Foxp3, thus allowing Th17 differentiation to occur. It is of notes that although Foxp3 strongly inhibits Th17 differentiation, substantial number of T cells co-expressed Foxp3 and IL-17/IL-17F in the early phase after IL-6 treatment. IL-17 and IL-17F expression was dependent on RORγt and RORα. How RORα and RORγt overcome Foxp3 expression in these cells remains to be understood. It is not clear at this stage if Foxp3 and IL-17 dual expresser cells are bi-potential.

In summary, we demonstrate in this study, molecular mechanisms of antagonistic regulation of Treg and Th17 programs, both of which depend on TGF-β. In addition, our data indicate the plasticity of nTreg and iTreg cells and the transcriptional pathways that convert them to Th17 program. These results may be beneficial in our further understanding on the genetic programs underlying Th cell differentiation and maintenance, and have implications in immunotherapy. One may consider enhancing Treg generation and function in autoimmunity, while converting regulatory T cells to effector T cells in cancer patients.

Materials and methods

Mice

C57BL/6, Rag1-deficient and B6.SJL (CD45.1) mice were purchased from Jackson Laboratories. *Smad4*$^{fl/fl}$ mice were kindly provided by Dr. Martin Matzuk with permission of Dr. Elizabeth Robertson (Chu et al., 2004), and these mice as well as *Stat3*$^{fl/fl}$ mice were bred with *CD4-Cre* mice provided by Dr. Christopher Wilson. Scurfy mice obtained from Jackson Lab were crossed with OT-II mice and mice at 4 weeks of age were used. IL-21 knockout mice, *Rorc*$^{-/-}$, *Rora*$^{sg/sg}$ and *Rora*$^{sg/sg}$*Rorc*-/- mice, were described previously (Kurebayashi et al., 2000; Nurieva et al., 2007; Yang et al., 2008b). *Il17f*^{rfp} reporter was generated by insertion of

an IRES-mRFP-polyA cassette into the exon 2 of the *Il17f* gene (Yang et al., 2008b) and maintained on a 129xB6 F1 background. Mice were housed in the SPF animal facility at M. D. Anderson Cancer Center and the animal experiments were performed at the age of 6-10 weeks using protocols approved by Institutional Animal Care and Use Committee.

T cell differentiation

$CD4^+CD25^-CD62L^{hi}CD44^{lo}$ cells were FACS-sorted and stimulated and analyzed as described (Nurieva et al., 2007; Yang et al., 2007) *All-trans* retinoic acid (RA) was purchased from Sigma and used at a 100 nM/ml concentration. Gene expression was examined with a Bio-Rad iCycler Optical System using iQ™ SYBR green real-time PCR kit (Bio-Rad Laboratories, Inc.). The data were normalized to *Actb* reference. The primers were previously described (Nurieva et al., 2007; Yang et al., 2007).

MOG immunization and Transfer EAE

Female mice at 5-8 weeks of age were immunized subcutaneously at the dorsal flanks with 150 μg of MOG_{35-55} peptide emulsified in CFA. Five days later, cells from spleens and draining lymph nodes of the immunized mice were isolated and restimulated with MOG for 24 hr, and RFP and GFP expression were assessed by flow cytometry.

Transcription reporter assay

RORγ, RORα, wild-type Foxp3, Foxp3ΔE250, Foxp3ΔFKH/NLS, Foxp3LL-AA, Foxp3Δ105-190 or Foxp3LL-AA Δ105-190 were cloned into bicistronic retroviral vector pGFP-RV provided by Dr. Ken Murphy that contains IRES-regulated GFP. The expression vectors were transfected into EL-4 cells with a luciferase construct containing IL-17 minimal promoter with *CNS2* element (Yang et al., 2008b). The dual-luciferase reporter system (Promega) was used to assay Firefly and Renilla luciferase activity in each sample. Renilla luciferase was used to normalize transfection efficiency and luciferase activity.

Two-hybrid and RORE reporter assay

CHO cells were co-transfected with 0.1 μg of the (UAS)5-Luc reporter, containing 5 copies of the GAL4 upstream-activating sequence (UAS), or the (RORE)3-Luc reporter and 0.05 μg of VP16-RORγ or pZeoSV-RORγ, 0.1 μg pM-EBIP96, encoding an LXXLL motif, and Foxp3 expression vectors as indicated using Fugene 6 transfection reagent (Roche, Indianapolis, IN). Cells were incubated for 40 h and then luciferase activity was analyzed with a luciferase kit (Promega). Transfection efficiency was normalized by β-galactosidase activity.

Co-Immunoprecipitation

Expression vectors encoding RORα, wild-type or mutant Foxp3 molecules were transfected into 293 T cells. After 48 h, cells were washed with ice-cold PBS, and lysed in ice-cold lysis buffer (50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 1% Nonidet P-40, 4 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). Lysates were obtained by centrifugation and pre-cleared with protein A-sepharose (Sigma-Aldrich) for 2 h before 2 μg of anti-FLAG-M2 antibody (Sigma-Aldrich) was added. After incubation, protein A-Sepharose was added and immunoprecipitates were obtained by centrifugation. Equivalent amounts of protein from whole cell lysates or immunoprecipitates were analyzed by Western blot using anti-human/mouse FOXP3 (eBioscience) or anti-FLAG-M2.

Retroviral transduction

Naïve $CD4^+CD25^-CD62L^{hi}CD44^{lo}$ T cells from OT-II mice were FACS-sorted and activated with Ova peptide and irradiated wild-type splenic APCs in the presence of Th17 conditions

(TGF-β, IL-6, IL-23, anti-IL-4, anti-IFN-γ). 24 hours after activation, cells were infected by retroviruses expressing Foxp3 or control empty vector (containing only IRES-GFP) and analyzed 4 days later as previously described (Yang et al., 2008b).

In vivo conversion of Treg cells

$CD4^+GFP^+$ cells from Foxp3-GFP reporter mice ($CD45.2^+$) and $CD4^+CD25^-CD62L^{hi}CD44^{lo}$ cells ($CD45.1^+$) from B6.SJL congenic mice were FACS-sorted and mixed at 1:10 ratio. These cells were intravenously transferred into syngenic *Rag1*$^{-/-}$ mice (5×10^6 cells/mouse). The recipient mice were immunized subcutaneously with 150 μg of MOG_{35-55} peptide emulsified in CFA. Five days later, lymphoid cells from spleens were isolated and restimulated with MOG for 24 hr in the presence of Golgi-stop for last 5 hr or PMA + Ionomycin + Golgi-stop for 5 hr. Cells were stained with PerCP-Cy5.5-conjugated anti-CD45.2 together with APC-conjugated anti-CD4 before they were analyzed using intracellular staining.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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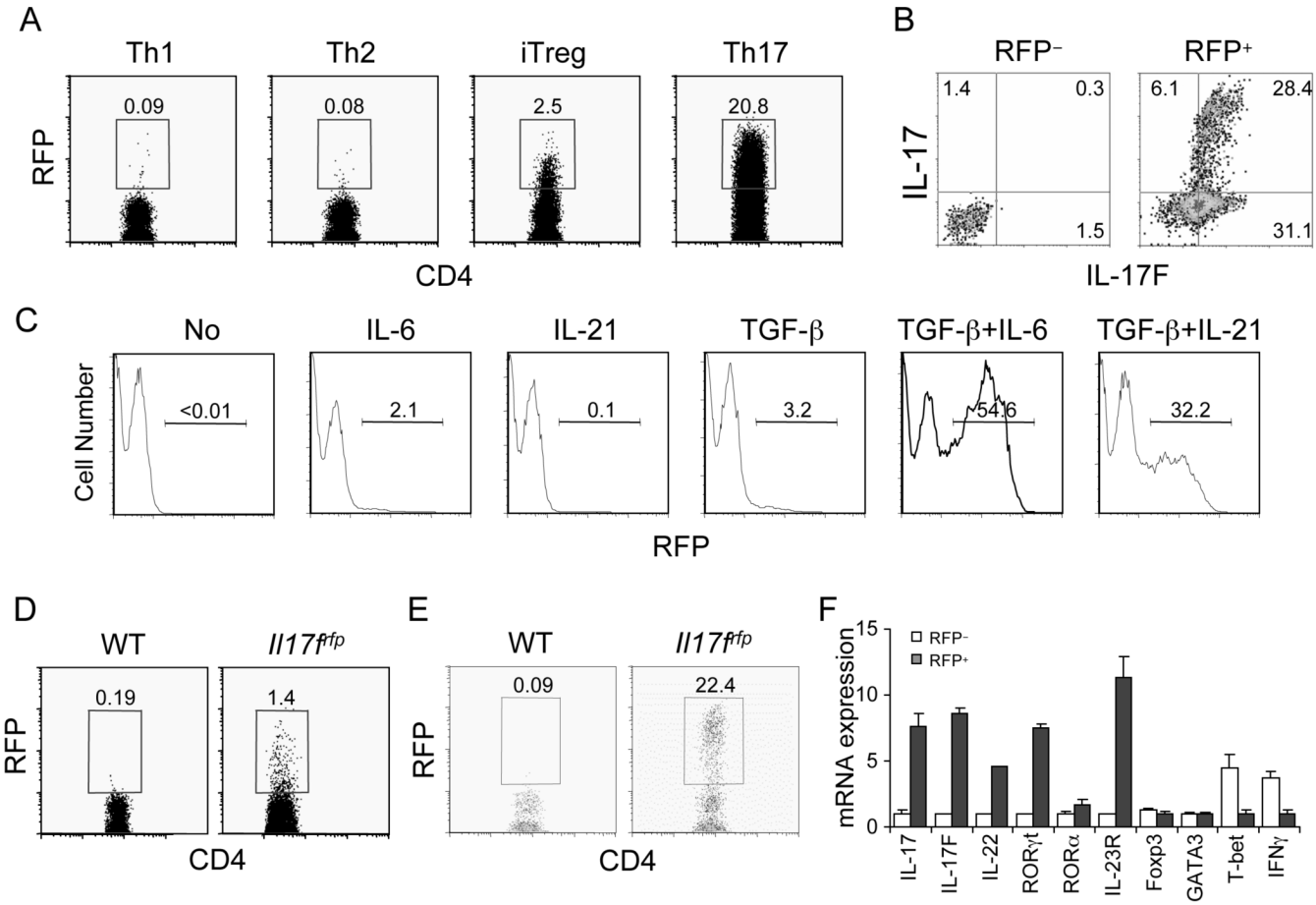

Figure 1. IL-17F-RFP reporter is highly expressed in Th17 cells generated *in vitro* and *in vivo*

(A) FACS-sorted naïve $CD4^+CD25^-CD62L^{hi}CD44^{lo}$ T cells from *Il17f*^{rfp} mice were activated under the Th1, Th2, Th17 and inducible regulatory T cell (iTreg) conditions with plate-bound anti-CD3 and anti-CD28 for 4-5 days and RFP (IL-17F) expression was analyzed by FACS. (B) RFP^+ and RFP^- cells from above Th17 culture were sorted by FACS and rested for 24 h. The cells were then restimulated by PMA and Ionomycin for 5 h and IL-17- and IL-17F-expressing cells were assessed by intracellular staining. Data shown represent at least 3 independent experiments. (C) Naïve T cells from *Il17f*^{rfp} mice were activated in the presence the indicated cytokine stimuli for 3-5 days. The cells were then analyzed for RFP expression by FACS. Data shown were repeated twice with consistent results. Numbers represent percentage of RFP^+ cells. (D-F) IL-17F-RFP reporter expression in Th17 cells generated in EAE. (D) *Il17f*^{rfp} and WT mice were immunized by MOG + CFA for 5 days and RFP expression were analyzed by FACS after *ex vivo* recalled by MOG peptide. Data shown were on a $CD4^+$ gate. (E) IL-17F-RFP expression in $CD4^+$ T cells from CNS of EAE mice. EAE was induced in *Il17f*^{rfp} and WT mice and infiltrates from the CNS of the EAE mice were isolated at the clinic score 3. IL-17F-RFP expression was analyzed by FACS in a $CD4^+$ gate. Data shown represent at least 3 mice from each group with consistent results. (F) RFP^+ and RFP^- $CD4^+$ cells were sorted from CNS of *Il17f*^{rfp} EAE mice and gene expression profile was analyzed by real-time RT-PCR. Data were normalized to a reference gene *Actb*. The lower expression for each gene was referred as 1. Graph shows means ± s.d.

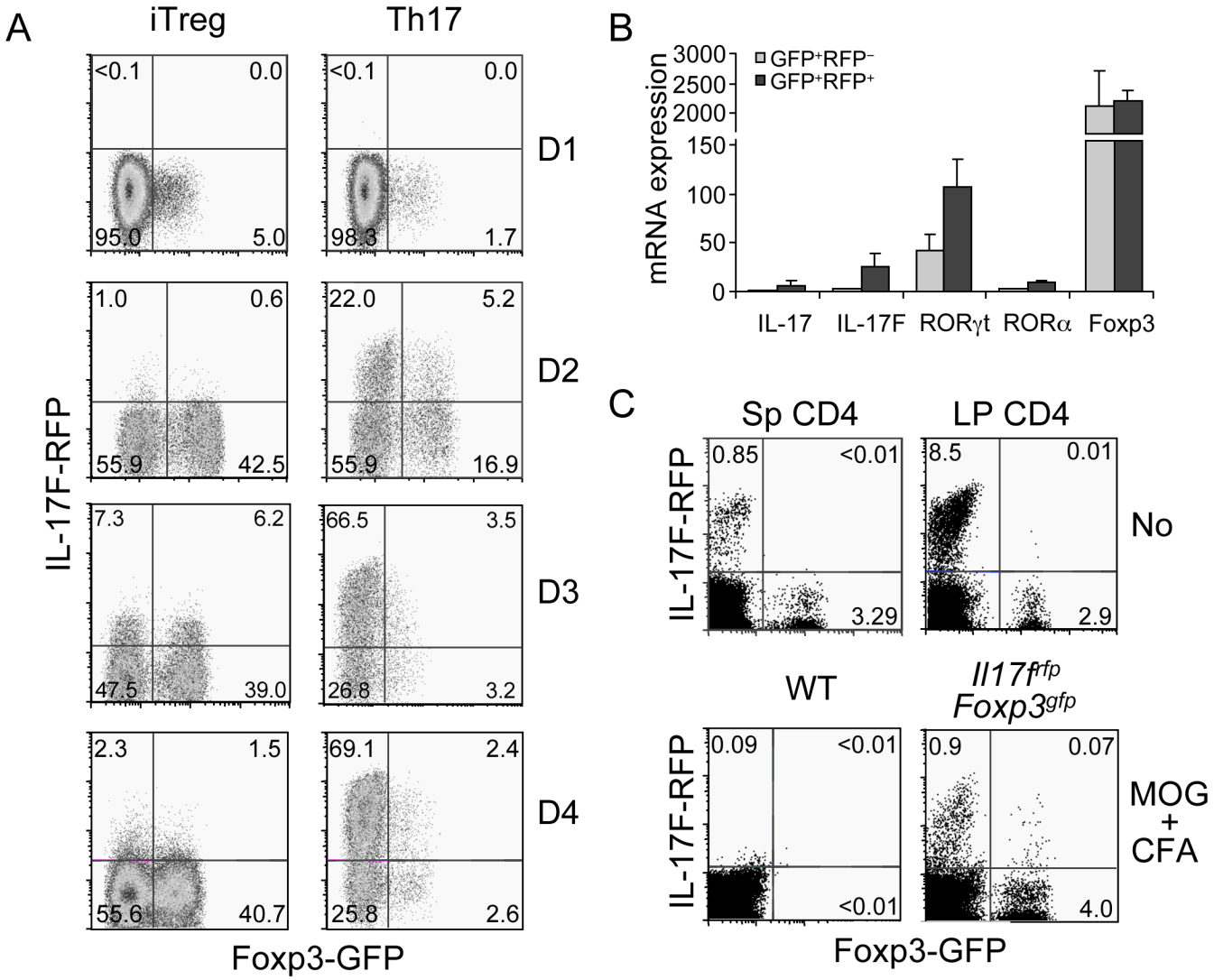

Figure 2. Reciprocal Th17 and iTreg differentiation

(A) Naïve T cells from *Il17f*^{rfp} *Foxp3*^{gfp} mice were activated in the presence of IL-2 under iTreg (TGF-β, anti-IL-4 and anti-IFN-γ) or Th17 (TGF-β and IL-6) conditions for 1-4 days. IL-17F-RFP and Foxp3-GFP expression were assessed daily by FACS. Data shown were repeated twice with consistent results. (B) On day3, RFP^{+} and RFP^{-} subsets were sorted on a GFP^{+} gate from the iTreg culture and gene expression was assessed by real-time RT-PCR. Data shown were normalized to expression of a reference gene *Actb*. The lower expression for each gene was referred as 1. Graph shows means ± s.d. (C) MACS-enriched splenic (SP) and laminal propria (LP) CD4^{+} cells from unmanipulated (No, no immunization) *Il17f*^{rfp}*Foxp3*^{gfp} mice were restimulated with anti-CD3 and IL-23 for 24 h and expression of RFP and GFP was analyzed by flow cytometry. In addition, CD4 cells were enriched by MACS from spleen and draining lymph nodes of the MOG + CFA immunized wild-type (WT) or *Il17f*^{rfp}/*Foxp3*^{gfp} mice and RFP and GFP expression were assessed by FACS after MOG restimulation. (A, C) Numbers in each quadrant represent the percentage of cells.

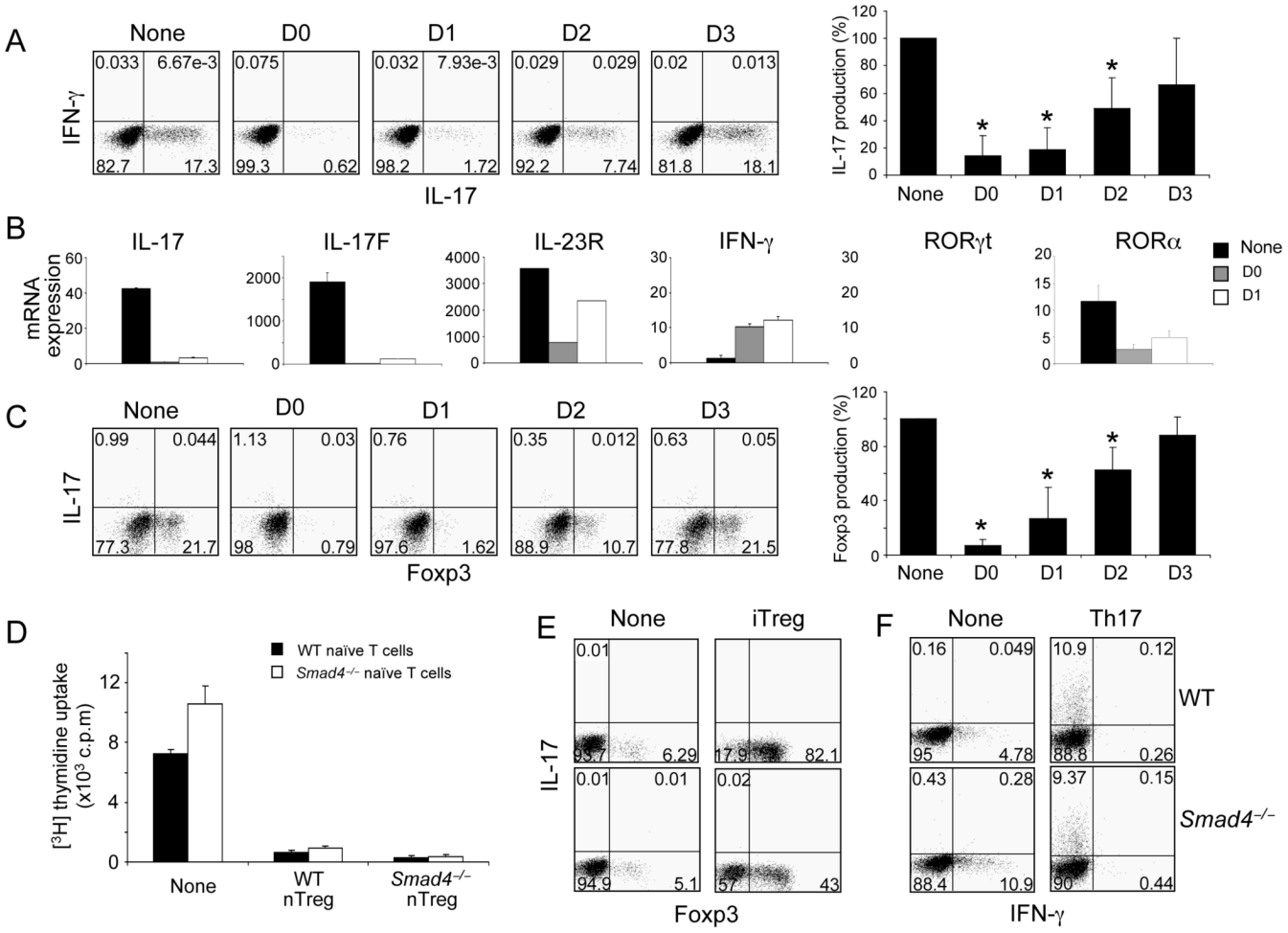

Figure 3. TGF-β signaling requirements in the generation of Th17 or iTreg cells

FACS-sorted naïve T cells from B6 mice were activated under (A-B) Th17 (TGF-β + IL-6 + IL-23 + anti-IFN-γ + anti-IL-4) or (C) iTreg conditions, and a TGF-βRI kinase inhibitor (SB431542, 5μM) was added at different time points as indicated. Cells were assessed for IL-17 and IFN-γ production and Foxp3 expression after 4 days of stimulation using intracellular staining. (A, C) A representative dot plot graph is shown in the left panel, and the numbers in quadrants represent the percentages. In the right panel, the percentage of (A) IL-17$^+$ or (C) Foxp3$^+$ cells for six independent experiments are indicated. *, $p<0.05$, Wilcoxon signed rank test. (B) mRNA expression of indicated genes was analyzed by real-time RT-PCR. The data shown was normalized to expression of a reference gene *Actb*. The lowest expression for each gene was referred as 1. (D) Naïve T cells from *Smad4*$^{fl/fl}$*CD4-Cre*$^-$ (WT) or *Smad4*$^{fl/fl}$ *CD4-Cre*$^+$ (*Smad4*$^{-/-}$) mice were cultured with or without WT or *Smad4*$^{-/-}$ CD4$^+$CD25$^+$ nTreg cells in triplicate wells with irradiated APCs and stimulated with 2 μg/ml of anti-CD3. Proliferation was assayed 72 h after treatment by adding [^{3}H]-thymidine to the culture for the last 8 h. A representative example of three independent experiments is shown. (A-D) Graph shows means ± s.d. (E-F) Naïve Smad4-sufficient or -deficient T cells were activated under (E) iTreg or (F) Th17 conditions, and IL-17, IFN-γ and Foxp3 expression was analyzed by intracellular staining. Numbers in quadrants represent the percentages. The experiments were repeated three times with consistent results.

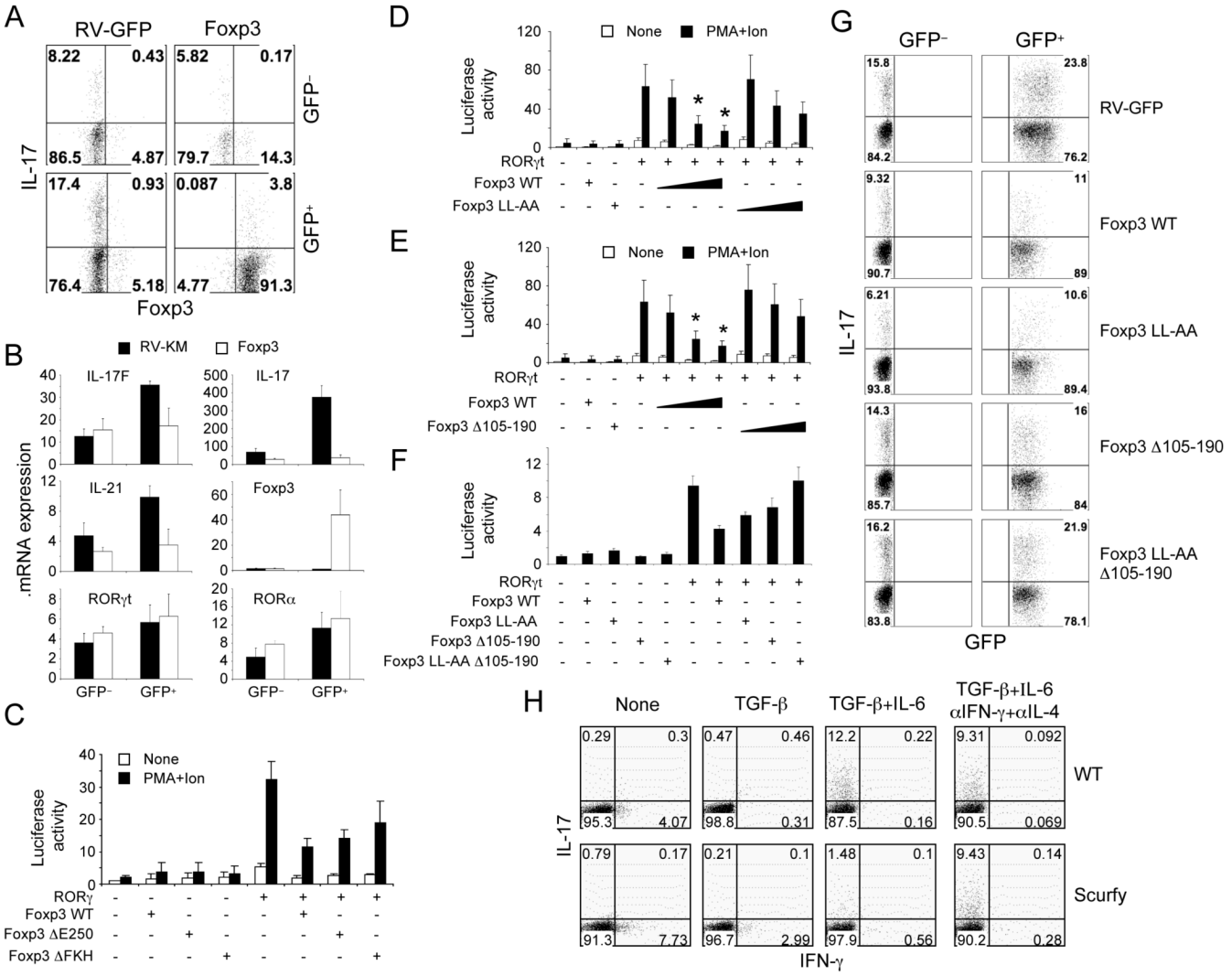

Figure 4. Foxp3 inhibits Th17 cell cytokine induction by antagonizing RORγt function

(A) FACS-sorted naïve OT-II T cells were activated under Th17 conditions and infected with an IRES-GFP-containing bicistronic retrovirus expressing Foxp3 or a vector control virus. IL-17- and Foxp3-expressing cells were measured by intracellular staining on the GFP^- and GFP^+ population. The experiments were repeated at least three times with similar results. (B) GFP^- and GFP^+ cells were sorted from (A) and restimulated for 4 hours with anti-CD3. mRNA expression of indicated genes was analyzed by real-time RT-PCR. The data shown were normalized to expression of a reference gene *Actb*. The lowest expression for each gene was referred as 1. *, $p<0.05$, t test. (C-F) EL-4 cells were transfected with a vector containing the firefly luciferase gene under the control of the *Il17a* promoter-*CNS2* region, a vector expressing Renilla luciferase, and IRES-GFP-containing bicistronic vectors expressing RORγt, Foxp3 wild-type (WT) or various Foxp3 mutants, or vector alone. Luciferase activity was determined and normalized to Renilla luciferase. Values were also normalized to vector alone. The data represent at least four independent experiments with consistent results. *, $p<0.05$, t test. (B-F) Graph shows means ± s.d. (G) Naïve OT-II T cells were activated under Th17 conditions and infected with indicated viruses. IL-17 expression was analyzed by intracellular staining on either GFP^+ or GFP^- gate. (H) Naïve WT or Scurfy OT-II T cells were stimulated with the indicated cytokines and neutralizing antibodies. Four days later, cells were assessed for IFN-

γ and IL-17 production by intracellular staining. The data represent at least three independent experiments with consistent results.

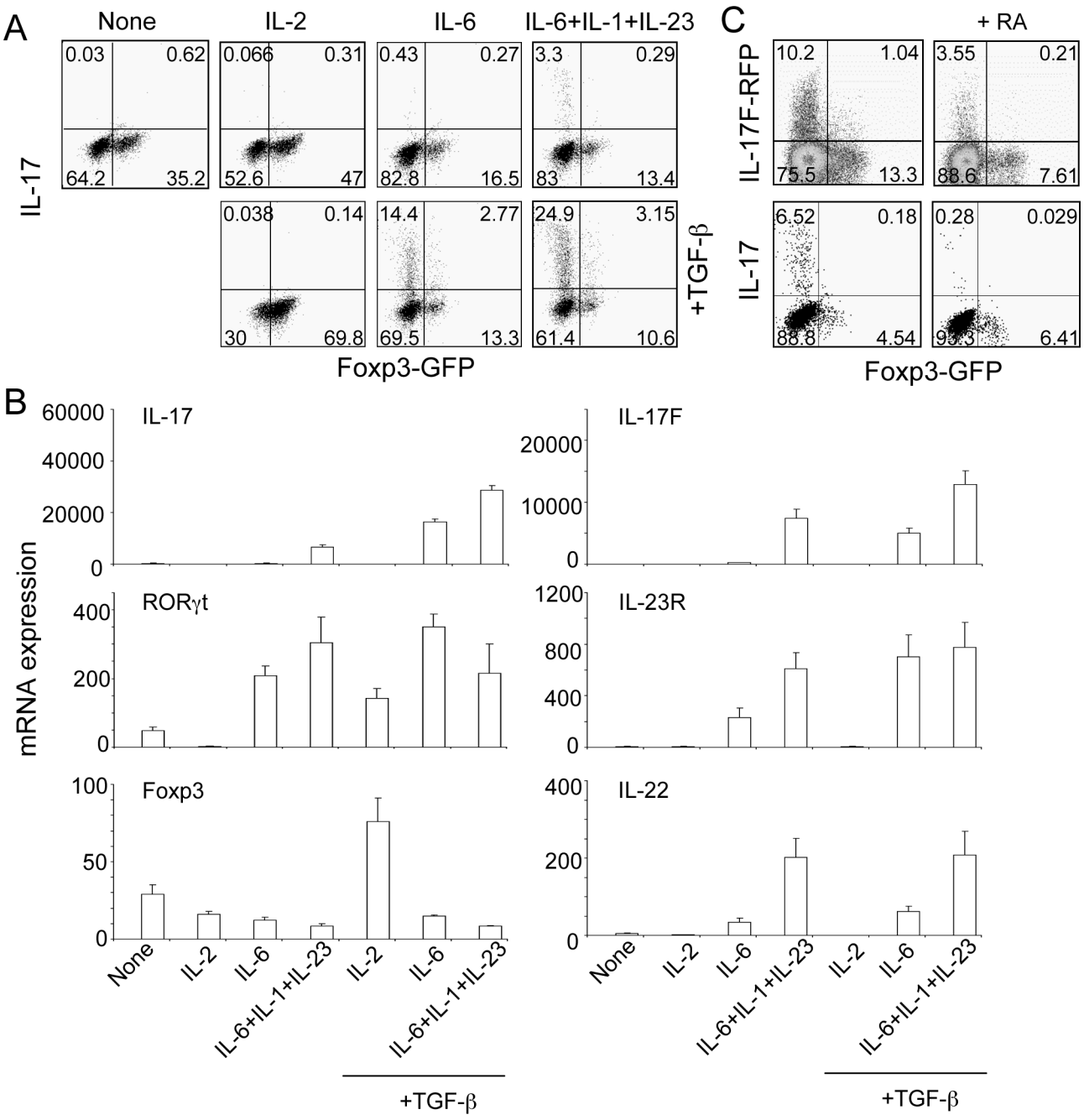

Figure 5. Generation of IL-17-producing cells from inducible regulatory T cells

(A-B) FACS sorted $CD4^+Foxp3\text{-}GFP^-CD44^{low}$ T cells from Foxp3-GFP mice were stimulated under iTreg polarizing conditions (TGF-β, IL-2, anti-IFN-γ and anti-IL-4) for 5 days. Foxp3-GFP^+ cells were FACS-sorted and stimulated with plate-bound anti-CD3 and anti-CD28 in the presence of the indicated cytokines. (A) 4 days later, IL-17 and Foxp3-GFP expression was determined by flow cytometry, and (B) mRNA expression of indicated genes was analyzed by real-time RT-PCR. Numbers in FACS quadrants represent the percentages. The data shown in B was normalized to expression of a reference gene *Actb*. The lowest expression for each gene was referred as 1. Graph shows means ± s.d. The data represent at least three independent experiments with consistent results. (C) Naive T cells from *Il17f*^{rfp}/*Foxp3*^{gfp} mice were

stimulated with plate-bound anti-CD3, anti-CD28, TGF-β, IL-2 in the presence or absence of all-trans retinoic acid (RA). 3 days later, Foxp3-GFP^{+}IL-17F-RFP^{-} cells were sorted and cultured with plate-bound anti-CD3 and anti-CD28 in the presence of TGF-β, IL-1, IL-6 and IL-23. 4 days later, IL-17, IL-17F-RFP and Foxp3-GFP expression was determined by flow cytometry. Numbers in FACS quadrants represent the percentages. The data represent at least two independent experiments with consistent results.

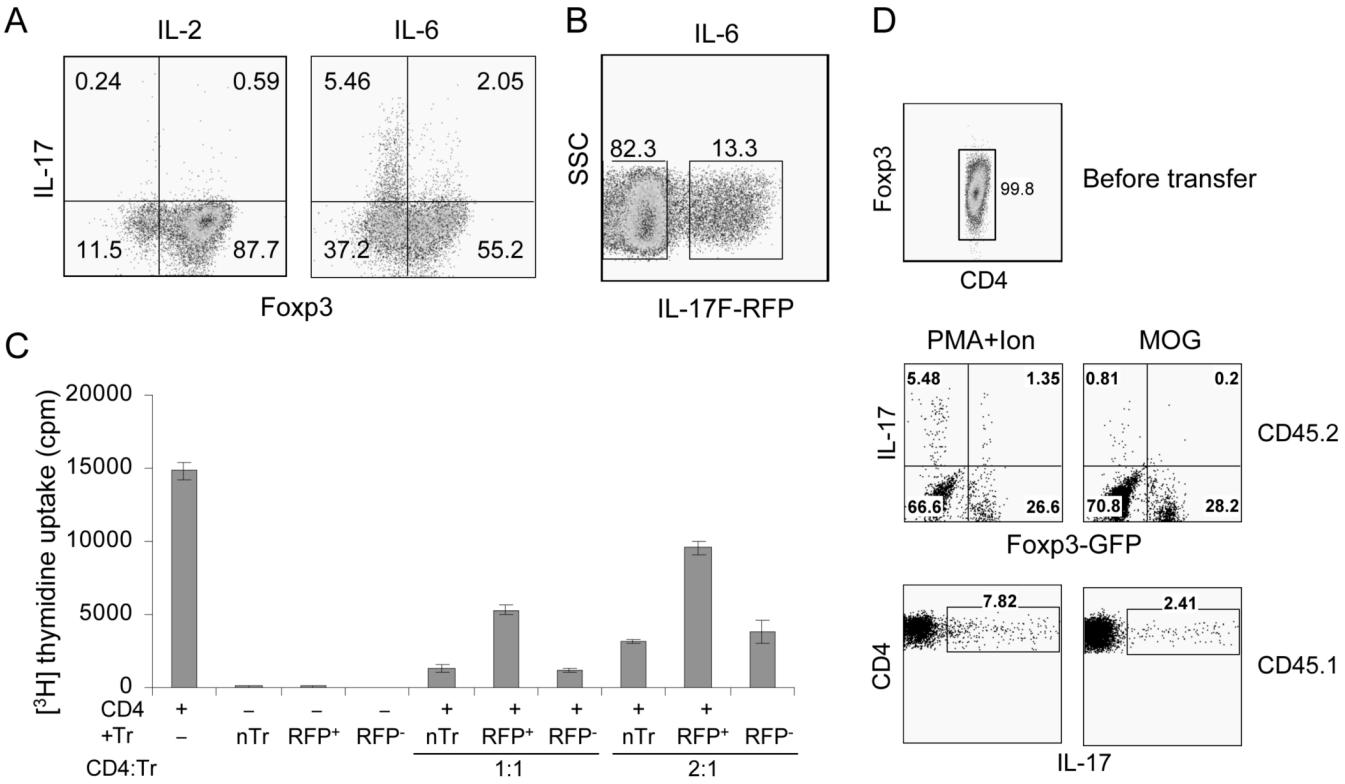

Figure 6. Conversion of natural regulatory T cells into Th17 cells

(A) FACS-sorted $CD4^+CD25^+$ T cells from B6 mice were stimulated with plate-bound anti-CD3 and anti-CD28 in the presence of indicated cytokines. 4 days later, cells were assessed for IL-17 and Foxp3 expression using intracellular staining. (B) FACS-sorted $CD4^+CD25^+$ T cells from IL-17F-RFP reporter mice were activated with anti-CD3, anti-CD28 and with IL-6 for 4 days. RFP expression was analyzed by flow cytometry. (C) RFP^+ and RFP^- cells were sorted from (B) for suppression assays. Naïve T cells from B6 mice were cultured in triplicate wells with or without $CD4^+CD25^+$ T cells from B6 mice or the sorted RFP^+ or RFP^- cells in the presence of irradiated APC and 2 μg/ml anti-CD3. Proliferation was assayed 72 h later by adding $[^3H]$-thymidine to the culture for the last 8 h. Graph shows means ± s.d. The data represent at least two independent experiments with consistent results. (D). $CD4^+GFP^+$ cells from Foxp3-GFP reporter mice ($CD45.2^+$) and $CD4^+CD25^-CD62L^{hi}CD44^{lo}$ cells ($CD45.1^+$) from B6.SJL congenic mice were FACS-sorted and mixed at 1:10 ratio before intravenously transferred into *Rag1*$^{-/-}$ syngenic mice (5×10^6 cells/mouse, n=3). The recipient mice were immunized subcutaneously with 150 μg of MOG_{35-55} peptide emulsified in CFA. Five days later, lymphoid cells from spleens were isolated and restimulated with MOG or PMA + Ionomycin before IL-17 and Foxp3-GFP expression was determined by flow cytometry for each individual mouse. A representative result is shown.

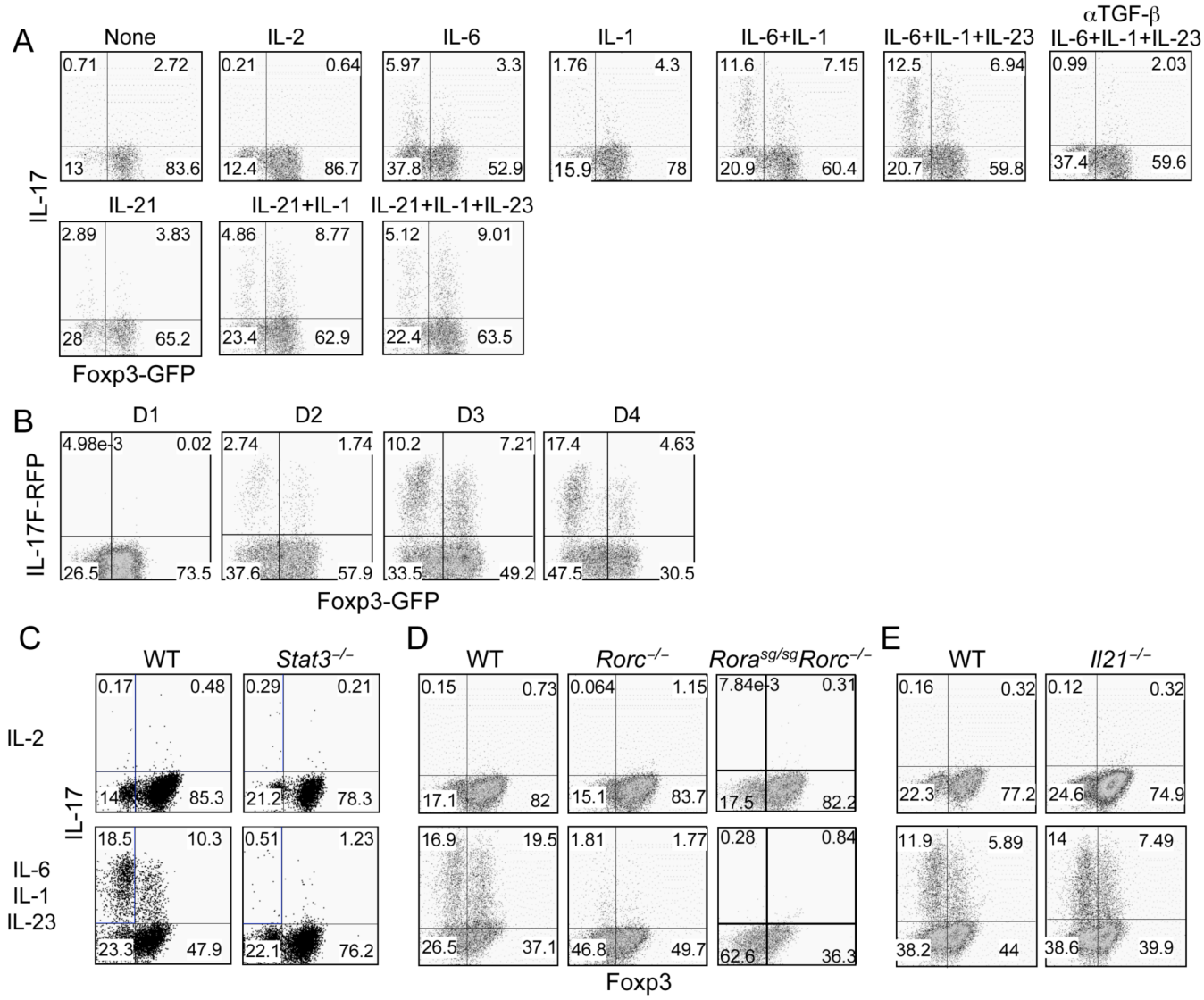

Figure 7. Molecular requirement in nTreg conversion into Th17 cells

(A) FACS-sorted CD4$^+$Foxp3-GFP$^+$ T cells from Foxp3-GFP reporter mice were stimulated with plate-bound anti-CD3 and anti-CD-28 in the presence of the indicated cytokines with or without anti-TGF-β (10μg/ml) for 4 days. Cells were analyzed for IL-17 and GFP expression. (B) CD4$^+$Foxp3-GFP$^+$ T cells from *Il17f^{rfp}-Foxp3^{gfp}* mice were stimulated in the presence of IL-6, IL-1 and IL-23 for indicated days, and IL-17F-RFP and Foxp3-GFP expression was determined by flow cytometry. (C-E) CD4$^+$CD25$^+$ T cells from wild-type (WT) and (C) Stat3-, (D) RORγ- or RORγ/α- or (E) IL-21-deficient mice were stimulated in the presence of indicated cytokines. 4 days later, cells were assessed for Foxp3 and IL-17 expression by intracellular staining. Numbers in quadrants represent the percentages. The data represent at least three independent experiments with consistent results.