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LETTERS

TGF-β-induced Foxp3 inhibits T_H17 cell differentiation by antagonizing RORγt function

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T helper cells that produce IL-17 (T_H17 cells) promote autoimmunity in mice and have been implicated in the pathogenesis of human inflammatory diseases. At mucosal surfaces, T_H17 cells are thought to protect the host from infection, whereas regulatory T (T_{reg}) cells control immune responses and inflammation triggered by the resident microflora[1–5]. Differentiation of both cell types requires transforming growth factor-β (TGF-β), but depends on distinct transcription factors: RORγt (encoded by *Rorc(γt)*) for T_H17 cells and Foxp3 for T_{reg} cells[6–8]. How TGF-β regulates the differentiation of T cells with opposing activities has been perplexing. Here we demonstrate that, together with pro-inflammatory cytokines, TGF-β orchestrates T_H17 cell differentiation in a concentration-dependent manner. At low concentrations, TGF-β synergizes with interleukin (IL)-6 and IL-21 (refs 9–11) to promote IL-23 receptor (*Il23r*) expression, favouring T_H17 cell differentiation. High concentrations of TGF-β repress *IL23r* expression and favour $Foxp3^+$ T_{reg} cells. RORγt and Foxp3 are co-expressed in naive $CD4^+$ T cells exposed to TGF-β and in a subset of T cells in the small intestinal lamina propria of the mouse. *In vitro*, TGF-β-induced Foxp3 inhibits RORγt function, at least in part through their interaction. Accordingly, lamina propria T cells that co-express both transcription factors produce less IL-17 (also known as IL-17a) than those that express RORγt alone. IL-6, IL-21 and IL-23 relieve Foxp3-mediated inhibition of RORγt, thereby promoting T_H17 cell differentiation. Therefore, the decision of antigen-stimulated cells to differentiate into either T_H17 or T_{reg} cells depends on the cytokine-regulated balance of RORγt and Foxp3.

When T lymphocytes are exposed to microbial antigens, they acquire diverse effector functions depending on which cytokines are produced by activated cells of the innate immune system[12]. Differentiation of pro-inflammatory T_H17 cells requires the presence of IL-23, which is produced by activated dendritic cells[13–15]. *In vitro*, however, T_H17 cell differentiation is independent of IL-23 and is induced by TGF-β plus IL-6 or IL-21 (refs 6, 9–11). Both *in vitro* and *in vivo* differentiation of the T_H17 cell lineage require the upregulation of the orphan nuclear receptor RORγt[7]. TGF-β is also required to restrain inflammatory autoimmune responses[16]. Among its numerous properties is its ability to induce expression of Foxp3 in naive antigen-stimulated T cells, endowing the cells with regulatory or suppressor function[8]. Thus, TGF-β can induce both regulatory and pro-inflammatory T cells, depending on whether pro-inflammatory

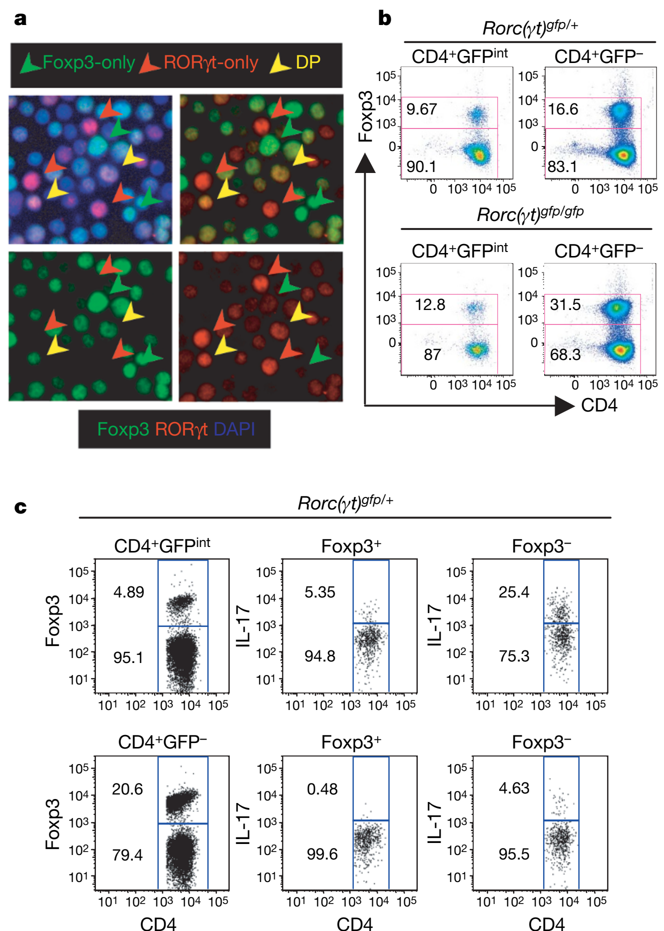

Figure 1 | Co-expression of Foxp3 and RORγt *in vitro* and *in vivo*. **a**, Naive $CD4^+$ T cells were stimulated with anti-T-cell receptor (TCR) and 5 ng ml^{-1} TGF-β for 48 h, and were stained with 4,6-diamidino-2-phenylindole (DAPI; blue nuclear stain; UV channel), anti-RORγ (red; Cy3 channel) and anti-Foxp3 (green; Cy5 channel) monoclonal antibodies. All panels are of the same section. The bottom-left image shows Foxp3-only, bottom-right shows RORγ-only, top-right shows overlay of Foxp3 and RORγ channels, and top-left shows overlay of all three channels. Foxp3, RORγt and double-expressing (DP) cells are indicated with coloured arrows. **b**, Analysis of $Foxp3^+RORγt^+$ cells from the small intestinal lamina propria. $CD4^+GFP^{int}$ and $CD4^+GFP^-$ cells were sorted from lamina propria of *Rorc(γt)*$^{+/gfp}$ and *Rorc(γt)*$^{gfp/gfp}$ mice, and Foxp3 expression was examined by intracellular staining. Results are representative of three experiments. The numbers indicate the percentage of total cells in each gate. **c**, Expression of IL-17 in $Foxp3^+RORγt^+$ and $Foxp3^-RORγt^+$ T cells from small intestine. Foxp3 and IL-17 expression was examined by intracellular staining of sorted $TCRβ^+CD4^+GFP^{int}$ cells from the lamina propria of *Rorc(γt)*$^{+/gfp}$ mice.

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cytokines such as IL-6 and, potentially, IL-23 are present[11,17]. Treatment of antigen-receptor-stimulated T cells with TGF-β alone induces expression of both Foxp3 and RORγt, but not of IL-17 (refs 7, 11). After such treatment, a significant proportion of cells co-expressed the two transcription factors (Fig. 1a and Supplementary Fig. 1a). To determine whether co-expression also occurs *in vivo*, we examined $CD4^+$ T cells from the small intestinal lamina propria of heterozygous RORγt–GFP (green fluorescent protein) knock-in mice, in which IL-17 is produced by TCR^+GFP^{int} lymphocytes[7]. Foxp3 was expressed in about 10% of sorted GFP^{int} ($ROR\gamma t^+$) cells (Fig. 1b and Supplementary Fig. 1b). In addition, Foxp3 was expressed in approximately 17–20% of GFP^- lamina propria $CD4^+$ T cells, consistent with the relatively large proportion of T_{reg} cells in the intestine.

We next performed a fate-mapping analysis to determine the proportion of $IL\text{-}17^+$ small intestinal T cells that had expressed Foxp3 during their ontogeny. Mice expressing Cre recombinase under the regulation of the *Foxp3* locus (Y.P.R. *et al.*, submitted) were crossed with Rosa26-stop–YFP reporter mice[18], and female progeny ($Rosa26^{stop-YFP/+}$; $Foxp3^{cre/+}$) were analysed for expression of yellow fluorescent protein (YFP). When inactivation of X-linked *Foxp3* was taken into account, we found that approximately 15% of $IL\text{-}17^-$ cells and 25% of $IL\text{-}17^+$ cells had expressed Cre at some stage of development (Supplementary Fig. 2). The former represent $Foxp3^+$ T_{reg} cells, whereas the latter are the minimal proportion of T_H17 cells that had expressed Foxp3 at some stage of their differentiation. These data suggest that $Foxp3^+$ T cells can differentiate into T_H17 cells *in vivo* in the presence of pro-inflammatory cytokines.

Examination of IL-17 expression in heterozygous RORγt–GFP knock-in mice revealed that $ROR\gamma t^+Foxp3^+$ lamina propria T cells produced much less IL-17 than $ROR\gamma t^+Foxp3^-$ cells, suggesting that Foxp3 may interfere with the ability of RORγt to induce IL-17 (Fig. 1c). This is consistent with findings showing a >1,000-fold increase in *Il17* messenger RNA, but little change in *Rorc(γt)*, in T_{reg} lineage cells that differentiate in the absence of Foxp3 (ref. 19). To investigate how Foxp3 may influence T_H17 cell differentiation, we asked whether its induction would influence the expression of IL-17 in TGF-β-stimulated T cells. In naive T cells that had been transduced with a retroviral vector encoding RORγt, we found that, whereas IL-6 augmented the proportion of $ROR\gamma t\text{-IRES-GFP}^+$ cells that expressed IL-17, TGF-β had a profound inhibitory effect even when added one day after transduction (Fig. 2a, b). Addition of TGF-β was followed by a sharp increase in expression of Foxp3 in the $CD4^+$ T cells, and both the level of *Foxp3* mRNA and proportion of $Foxp3^+$ cells were not affected by the expression of RORγt (Fig. 2c).

To determine whether the inhibitory effect of TGF-β on RORγt is mediated by Foxp3, we knocked down expression of Foxp3 by using a short hairpin RNA (shRNA) vector. TGF-β-induced Foxp3 expression was reduced by the *Foxp3*-specific shRNA vector, but not by control hairpin vectors (Fig. 2c). Accordingly, TGF-β-mediated inhibition of RORγt-directed IL-17 expression was partially reversed by Foxp3 knockdown (Fig. 2d). Consistent with the idea that this inhibition was mediated by Foxp3 upregulation, the most pronounced rescue of IL-17 expression occurred in cells that had lost the most Foxp3 expression (Supplementary Fig. 3). Thus, Foxp3 induced by TGF-β inhibits the function of RORγt.

These results prompted us to ask whether Foxp3 interacts with RORγt to inhibit its function. Using a yeast two-hybrid screen, we previously found that human FOXP3 interacts with RAR-related

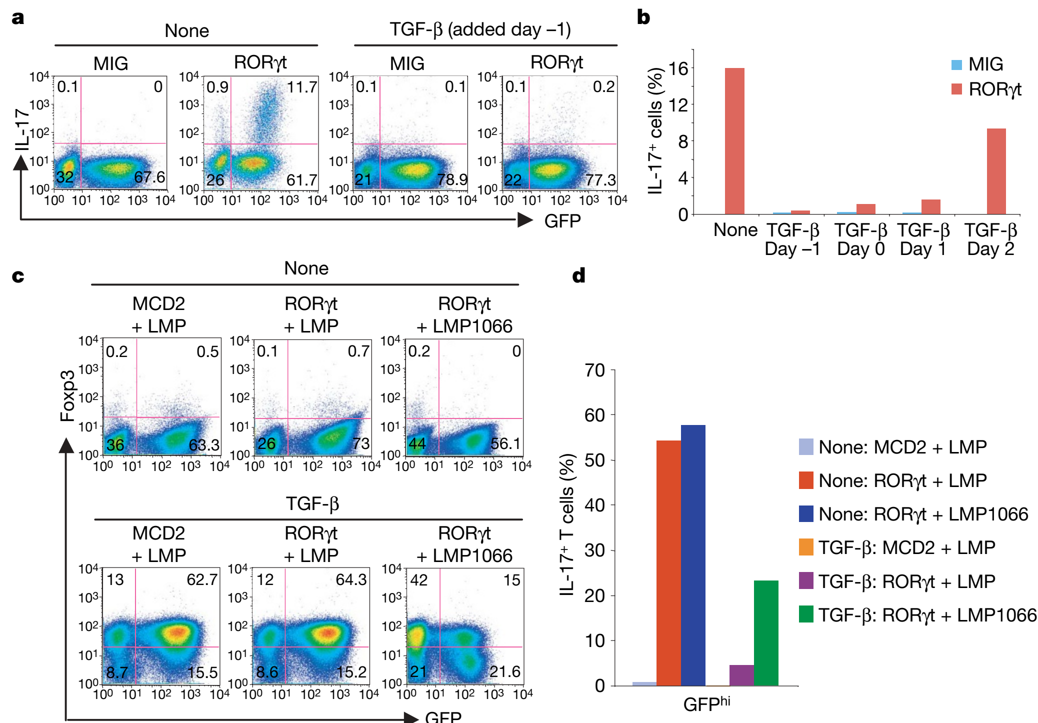

Figure 2 | TGF-β inhibits RORγt-directed IL-17 production by upregulating Foxp3. **a**, The effect of TGF-β on IL-17 expression after transduction of RORγt. Naive $CD4^+$ T cells incubated with TGF-β from day −1 were transduced with control vector MSCV-IRES-GFP (MIG) or RORγt-IRES-GFP (RORγt) on day 0 (24 h after TCR activation), and IL-17 intracellular staining was performed on day 5. **b**, The inhibitory effect of TGF-β when included at different times relative to transduction of RORγt. The percentage of $IL\text{-}17^+$ cells among GFP $(ROR\gamma t)^+$ cells is shown. **c**, Knockdown of TGF-β-induced Foxp3 expression with an shRNA against *Foxp3* (LMP1066). Naive $CD4^+$ T cells were stimulated as in **a** and co-transduced on days 0 and 1 with control retroviral construct MSCV-IRES-hCD2 (MCD2) or RORγt-IRES-hCD2 (RORγt) and the specific shRNA vector (LMP1066) or control vector (LMP). After transduction, the cells were cultured with or without TGF-β, and Foxp3 expression was measured by intracellular staining on day 5. **d**, Restoration of RORγt-induced IL-17 expression on knockdown of Foxp3. IL-17 expression was assessed in cells co-transduced as in **c** and gated for the level of GFP expression. The percentage of $IL\text{-}17^+$ T cells in GFP^{hi} cell populations is shown. Results with additional shRNA vectors that failed to downregulate Foxp3 expression were similar to those with the control LMP vector. Representative data from three experiments are shown.

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orphan receptor A (RORA), and that an alternatively spliced isoform of FOXP3, lacking exon 2 (ref. 20), was deficient in this interaction[21]. We therefore examined whether mouse and human Foxp3 could similarly bind to RORγt, and whether such interaction was necessary for inhibition of the RORγt-mediated induction of IL-17. When Flag-epitope-tagged mouse Foxp3 was co-expressed with mouse RORγt in 293T cells, the two proteins were co-immunoprecipitated (Fig. 3a), even in the presence of DNase I or ethidium bromide, suggesting that the interaction does not involve DNA. A similar interaction was observed between human RORγT and FOXP3 (Fig. 3b). However, both mouse and human Foxp3 lacking the conserved exon 2-encoded sequence (Foxp3ΔEx2) had a substantially reduced association with RORγt (Fig. 3a, b). We examined the localization of the two proteins by confocal microscopy of HeLa cells transfected with Flag-tagged mouse Foxp3 constructs with or without mouse RORγt. Both Foxp3 and RORγt were localized in the nucleus, but Foxp3 lacking the DNA-binding forkhead domain (Foxp3ΔFKH) remained in the cytoplasm due to deletion of the nuclear localization signal in FKH[22] (Supplementary Fig. 4). However, Foxp3ΔFKH translocated to the nucleus when it was co-expressed with RORγt, indicating that the Foxp3–RORγt interaction is independent of FKH. Accordingly, Foxp3ΔFKH co-immunoprecipitated with RORγt in extracts of transfected 293T cells (data not shown). A combined Foxp3ΔEx2/ΔFKH mutant remained in the cytoplasm even when it was co-expressed with RORγt, further indicating that Foxp3 interacts with RORγt by way of the exon 2-encoded sequence (Supplementary Fig. 4).

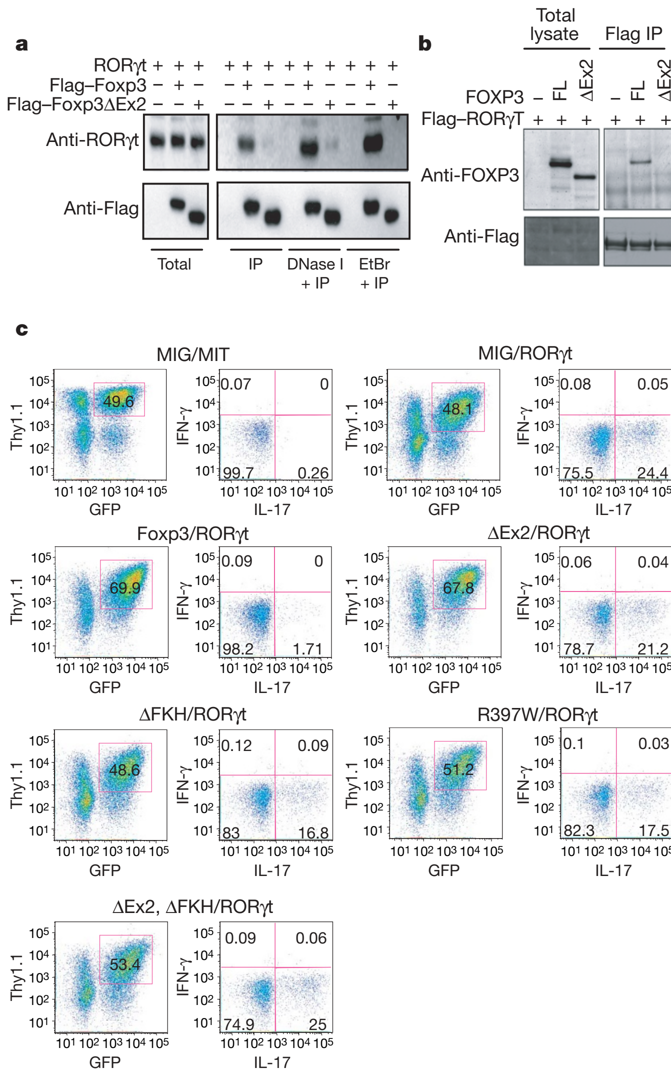

Figure 3 | Foxp3 interacts with RORγt and inhibits RORγt-directed IL-17 expression. **a**, Co-immunoprecipitation of Foxp3 and RORγt from extracts of co-transfected 293T cells with or without DNaseI or ethidium bromide (EtBr). Cells were transfected with mouse RORγt and Flag-tagged wild-type (Flag–Foxp3) or exon 2-deleted (Flag–Foxp3ΔEx2) Foxp3. Anti-Flag immunoprecipitates (IP) and total lysates were immunoblotted with anti-RORγt antibody and anti-Flag antibody. **b**, Cells were transfected with Flag-tagged human RORγT and full-length (FL) or the exon 2-deleted isoform of human FOXP3 (ΔEx2). Anti-Flag immunoprecipitates and total lysates were probed with anti-FOXP3 and anti-Flag antibodies. **c**, Naive CD4$^+$ T cells were co-transduced with retroviruses encoding RORγt (MIT vector, Thy1.1 reporter) and various mouse Foxp3 constructs (MIG vector, GFP reporter). IL-17 expression was assessed on day 4 in cells gated for expression of both Thy1.1 and GFP. Representative data from at least three experiments are shown in each of the panels.

To investigate the role of the interaction between Foxp3 and RORγt in the repression of RORγt-induced transcription, we co-expressed these transcription factors in naive CD4$^+$ T cells and examined expression of IL-17. Both mouse and human Foxp3 blocked RORγt-directed IL-17 expression, but full suppression required the presence of the exon 2-encoded sequence in Foxp3, suggesting that the interaction between Foxp3 and RORγt is essential (Fig. 3c and Supplementary Fig. 5). The ability of both mouse and human Foxp3 to repress RORγt-induced IL-17 expression was abrogated by deletion of the FKH domain or a point mutation in this domain (R397W) that impairs FOXP3 DNA-binding activity and was identified in X-linked immunodeficiency, polyendocrinopathy, enteropathy (IPEX) syndrome in humans[23,24] (Fig. 3c and Supplementary Fig. 5). Therefore, Foxp3 can block the activity of RORγt at least in part through an interaction involving a sequence encoded by exon 2, but the requirement for an intact FKH domain suggests that its DNA-binding activity also contributes to inhibition of IL-17 expression. Thus, Foxp3 may inhibit RORγt-directed transcription through a mechanism similar to that proposed for its inhibition of IL-2 expression, involving its association with NFAT1 and Runx1 (refs 25 and 26). However, Foxp3ΔEx2 was as effective as the full-length protein in suppressing expression of IL-2 and interferon-γ (IFN-γ) in primary mouse T cells, indicating that, like the naturally occurring human spliced isoform[20], it retains regulatory functions and can, presumably, associate with both NFAT1 and Runx1 (Supplementary Fig. 6).

Our results suggest that Foxp3 may inhibit RORγt activity on its target genes during T_H17 cell differentiation. To extend our analysis from *Il17* to other potential RORγt transcriptional targets, we examined the effect of TGF-β-induced Foxp3 on *Il23r* expression, which also requires the activity of RORγt[10,11]. Forced expression of wild-type mouse Foxp3 inhibited IL-6/IL-21-induced *Il23r* expression, whereas Foxp3ΔEx2 had less inhibitory activity (Fig. 4a); this is consistent with the notion that Foxp3 inhibits the function of RORγt through an interaction involving the sequence encoded by exon 2. Similar results were observed with *Il22* expression in response to IL-6 or IL-21 (data not shown). Expression of *Il22* and of *Il23r* in response to either IL-6 or forced expression of RORγt was also inhibited by high concentrations of TGF-β (refs 11, 27 and data not shown). However, at low concentrations, TGF-β synergized with IL-6 and IL-21 to enhance expression of *Il23r* mRNA (Fig. 4b). As a consequence, addition of IL-23 to cultures containing high concentrations of TGF-β had no effect on IL-17 expression, but significantly increased the number of IL-17$^+$ cells and the level of IL-17 expression per cell when low concentrations of TGF-β were used (Figs 4c, d and Supplementary Fig. 7). In contrast, induction of T_{reg} (Foxp3$^+$) cells was optimal at high concentrations of TGF-β, but there was little induction at TGF-β concentrations at which IL-23 had a synergistic effect on expression of IL-17 (Fig. 4e).

TGF-β-induced Foxp3 expression is inhibited by IL-6 (ref. 17), IL-21 (ref. 10) and IL-23 (Supplementary Fig. 8). However, a substantial number of Foxp3$^+$ cells differentiated in response to TGF-β, even in the presence of IL-6, and many of these cells also expressed IL-17 (Supplementary Fig. 9a). Conversely, many of the IL-17$^+$ cells also

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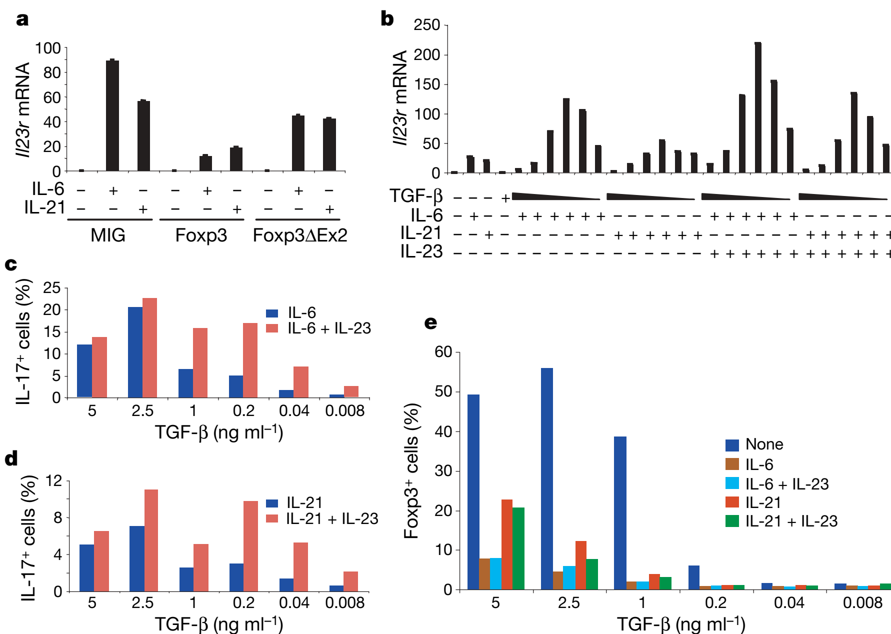

Figure 4 | TGF-β concentration influences *Il23r* expression and levels of IL-17 in response to T_H17-inducing cytokines. a, Foxp3-mediated inhibition of IL-6/IL-21-induced *Il23r* expression. This was measured in arbitrary units relative to expression of transcripts encoding actin. Naive $CD4^+$ T cells were transduced with MIG, full length Foxp3 or Foxp3ΔEx2 viruses, and were treated with the indicated cytokines. RNA was isolated from GFP^+ cells at day 2. *Il23r* expression was measured by real-time RT–PCR and was normalized to the actin level. Error bars represent standard deviations obtained using the standard curve method. **b,** Induction of *Il23r* mRNA in response to cytokines. Naive $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 throughout the culture period in the presence of the indicated combinations of cytokines. TGF-β was titrated into the cultures at the concentrations of 5 ng ml^{-1}, 2.5 ng ml^{-1}, 1 ng ml^{-1}, 200 pg ml^{-1}, 40 pg ml^{-1} or 8 pg ml^{-1}. *Il23r* mRNA expression was measured after 48 h by real-time RT–PCR and was normalized to the actin expression level. **c, d,** IL-23 enhancement of IL-17 expression at low concentrations of TGF-β. The percentage of IL-17^+ cells at 96 h of stimulation with the indicated cytokines is shown. Results in histogram format are shown in Supplementary Fig. 7. **e,** Induction of Foxp3 at different concentrations of TGF-β. Representative data from at least three experiments are shown for each set of panels.

expressed Foxp3. Thus, RORγt-dependent IL-17 expression can occur in the presence of Foxp3, but the level of Foxp3 may be insufficient to block RORγt function or, alternatively, IL-6 may overcome the inhibitory function of Foxp3. To examine this possibility, we added IL-6 or IL-21 to cultures of cells transduced with both RORγt and Foxp3. Under these conditions, the inhibitory effect of Foxp3 on IL-17 induction was largely circumvented, even though the level of Foxp3 protein was not affected (Supplementary Fig. 9b and data not shown); this suggests that IL-6 and IL-21 may have an additional post-translational effect on either Foxp3 or RORγt.

Our data collectively suggest that T cells receiving a TGF-β signal can acquire the potential to develop into either the T_{reg} or the T_H17 lineage. Foxp3 induction restrains the differentiation of inflammatory T_H17 cells in response to TGF-β in the absence of other pro-inflammatory cytokines by inhibiting the activity of RORγt. In the presence of pro-inflammatory cytokines, the suppression of Foxp3 expression and inhibitory function, together with the concurrent upregulation or stabilization of RORγt expression, leads to full progression towards the T_H17 lineage (Supplementary Fig. 10). This process may be especially relevant in the intestinal lamina propria, in which TGF-β can promote either T_H17 or T_{reg} cell lineage differentiation, depending on its local concentration. In this setting, a fine balance between RORγt and Foxp3 may be critical for immune homeostasis. In line with the observation that more $Foxp3^+$ T_{reg} cells were present in the gut of RORγt-deficient mice (Fig. 1b), these mutant mice were also protected from autoimmune disease (ref. 7 and data not shown). Conversely, a decrease of Foxp3 expression and function and an increase of RORγt expression tips the T_{reg}/T_H17 balance towards the T_H17 cell lineage. This may occur in some autoimmune diseases, as suggested by the finding that an *Il23r* polymorphism correlates with protection from Crohn's disease[28]. These results therefore have important implications for how peripheral tolerance is maintained in the presence of potentially pro-inflammatory cytokines.

METHODS SUMMARY

Mice. C57BL/6 mice (Taconic), mice with a *GFP* reporter cDNA knocked in at the RORγt translation initiation site[29], mice with an *IRES-YFP-Cre* cDNA knocked into the 3′ UTR of the *Foxp3* locus (Y.P.R. *et al.*, submitted) and *Rosa26*$^{stop-YFP}$ (ref. 18) mice were kept in specific pathogen-free (SPF) conditions at the animal facility of the Skirball Institute. All animal experiments were performed in accordance with approved protocols for the NYU Institutional Animal Care and Usage Committee.

Cell culture. Naive $CD4^+$ T cells were purified and cultured as described previously[7]. In brief, 1.5×10^6 naive $CD4^+$ T cells were cultured in wells of 24-well plates (or 0.7×10^6 cells per well in 48-well plates) containing plate-bound anti-CD3 (5 μg ml^{-1}) and soluble anti-CD28 (1 μg ml^{-1}). Cultures were supplemented with 2 μg ml^{-1} anti-IL-4 (BD Pharmingen), 2 μg ml^{-1} anti-IFN-γ (BD Pharmingen) with or without 80 U ml^{-1} human IL-2 (a gift from S. Reiner), 20 ng ml^{-1} IL-6 (eBioscience), 5 ng ml^{-1} TGF-β (PeproTech), 50 ng ml^{-1} IL-21 (R&D Systems) and 10 ng ml^{-1} IL-23 (eBioscience). Viral transduction was performed as described previously, unless indicated otherwise in the text[7]. T cells were isolated from the small intestinal lamina propria as described previously[7].

General. All DNA constructs were generated by PCR-based methodology and confirmed by sequencing. Retroviral production and transduction were performed as described previously[7]. Protein–protein interaction was detected by co-immunoprecipitation and confocal microscopy in 293T cells and HeLa cells. Gene expression analysis was monitored by real-time PCR with reverse transcription (RT–PCR) using gene-specific primers and probes. IL-17 and Foxp3 protein expression were examined by intracellular staining performed according to the manufacturer's protocol. Co-expression of RORγt and Foxp3 was examined by immunofluorescence using anti-RORγ[30] and anti-Foxp3 antibodies.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Plasmids and retrovirus production. MIG, MIT and MCD2 are retrovirus-based vectors containing GFP, Thy1.1 and human CD2, respectively, under the regulation of an internal ribosome entry site (IRES). The *Rorc(γt)* cDNA was amplified by PCR and cloned into MIG (RORγt-IRES-GFP), MIT (RORγt-IRES-Thy1.1) and MCD2 (RORγt-IRES-hCD2). The wild-type full-length *Foxp3* cDNA and various *Foxp3* mutant cDNAs were amplified by PCR with a 5′ Flag-tagged primer and a 3′ corresponding primer, and were cloned into MIG (Foxp3-IRES-GFP). *Il23r* cDNA was PCR-amplified and cloned into MIG (IL-23R-IRES-GFP). Human *FOXP3* cDNAs were PCR-amplified and cloned into MIG. MSCV-LTRmiR30-PIG (LMP) is a commercial vector from Openbiosystems. A double-stranded DNA oligonucleotide that targets the coding region of *Foxp3* was cloned into LMP (LMP1066) according to the manufacturer's protocol (the target sequence of *Foxp3* is 5′-GGCAGAGG-ACACTCAATGAAAT-3′). Retrovirus production was as described previously[7].

Surface and intracellular staining, and CFSE labelling. For intracellular staining, cells obtained from *in vitro* culture or isolated from the small intestinal lamina propria were incubated for 4–5 h with 50 ng ml^{-1} PMA (Sigma) and 500 ng ml^{-1} ionomycin (Sigma), plus 2 μg ml^{-1} Brefeldin A (Sigma) during the last 2 h. The cells were kept in a tissue-culture incubator at 37 °C. Surface staining was performed for 15–20 min with the corresponding cocktail of fluorescently labelled antibodies. After surface staining, the cells were resuspended in a Fixation/Permeabilization solution (BD Pharmingen), and intracellular cytokine staining was performed according to the manufacturer's protocol. For intracellular staining of Foxp3, the Foxp3-Staining Buffer Set (fixation/permeabilization and permeabilization buffers) was used (eBioscience) according to the manufacturer's protocol. For carboxyfluoroscein succinimidyl ester (CFSE)-labelling, sorted naive $CD4^+$ T cells were washed twice with Hank's Buffered Salt Solution (HBSS; Invitrogen), and labelled with 5 μM CFSE (Sigma) in HBSS for 10 min at 20 °C. The labelling was then stopped by adding 1/5 volume of FCS. The labelled cells were washed twice with the T cell culture medium before they were seeded and stimulated as described in the text.

Real-time RT–PCR. Complementary DNA was synthesized and analysed by real-time quantitative PCR as described previously[7]. The starting quantity of the initial cDNA sample was calculated from primer-specific standard curves by using the iCycler Data Analysis Software. The expression level of each gene was normalized to the expression level of actin using the standard curve method. The primer sets and probes for real-time PCR were described elsewhere[7,11].

Co-immunoprecipitation and western blot. Cells (293T cells) were transfected with the indicated constructs using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, whole-cell extracts were made in the lysis buffer, which contained 50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 4 mM EDTA, 1% NP-40, 50 mM NaF, 1 mM Na_3VO_4 and protease inhibitors. After the insoluble material was removed by centrifugation, the lysate was immunoprecipated for 12–16 hours at 4 °C with anti-Flag M2 agarose beads (Sigma). After extensive washes with the lysis buffer, samples were resolved in an SDS–polyacrylamide gel electrophoresis (SDS–PAGE) gel and transferred to a nitrocellulose membrane. Western blotting was performed with an anti-Flag monoclonal antibody (Sigma), an anti-Foxp3 monoclonal antibody (eBioscience) and an anti-RORγt hamster monoclonal antibody[30].

Confocal microscopy. HeLa cells were plated on 8-well glass slides (Lab-Tek II Chamber Slide System) before transfection with the indicated constructs using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, cells were washed once in PBS, fixed for 15 min in 2% paraformaldehyde in phosphate buffer (PBS without saline), and then washed twice in PBS. Cells were blocked and permeabilized in PBS-XG (10% goat serum (Sigma) in PBS containing 0.1% Triton X-100) for 1 h at 20 °C. The cells were then incubated for 12–16 hours at 4 °C with anti-RORγt hybridoma supernatant[30] (1:2 dilution in PBS-XG). After two washes in PBS, the cells were incubated for 1 h at room temperature with Cy3-conjugated goat anti-hamster antibody (Jackson ImmunoResearch Laboratory) at 1:400 dilution in PBS-XG. The cells were then washed three times in PBS and incubated for 1 h at 20 °C with anti-Flag M2 monoclonal antibody (Sigma) at 1:1,000 dilution in PBS-XG. After two washes in PBS, the cells were incubated for 1 h at 20 °C with anti-mouse Alexa 633 (Molecular Probes) at 1:200 dilution in PBS-XG. The cells were then washed twice in PBS and incubated for 5 min at 20 °C with 1 μg ml^{-1} DAPI (Sigma), washed two more times in PBS and mounted with Fluoromount-G (Southern Biotechnology Associates). The cells were examined with a Zeiss ZMD510 microscope with a CCD camera, and images were processed with Zeiss LSM Image Browser 4.0 and Adobe Photoshop 7.0.

Immunofluorescence. Naive T cells were sorted as described previously and stimulated in the presence of the indicated cytokines as described[7]. Lamina propria lymphocytes from *Rorc(γt)*$^{gfp/+}$ small intestines were isolated as described[7], and $CD4^+GFP^{int}$ and $CD4^+GFP^-$ cells were sorted on a MoFlo cytometer (DAKO Cytomation). Naive T cells or sorted lamina propria T cells were then cytospinned on glass slides and fixed in 2% paraformaldehyde in phosphate buffer (PBS without saline) for 20 min at 20 °C. After blocking, immunofluorescence staining was performed by incubating the cells consecutively with the anti-RORγ antibody[30] (hybridoma supernatant 1:4) for 12- 16 hours at 4 °C and biotin anti-mouse/rat Foxp3 monoclonal antibody (eBioscience clone FJK-16, 1:200 dilution) for 1.5 hours at 20 °C. The blocking solution contained PBS, 0.1% Triton-X100 and 10% goat serum. Secondary goat anti-Armenian-hamster Cy3 conjugated antibody (Jackson Immunoresearch) and streptavidin–APC (eBioscience), both at 1:400 dilution, were used at 20 °C for 1.5 hours to detect the RORγ and Foxp3 primary antibodies, respectively.

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