The induction and function of the anti-inflammatory fate of TH17 cells

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TH17 cells exemplify environmental immune adaptation: they can acquire both a pathogenic and an anti-inflammatory fate. However, it is not known whether the anti-inflammatory fate is merely a vestigial trait, or whether it serves to preserve the integrity of the host tissues. Here we show that the capacity of TH17 cells to acquire an anti-inflammatory fate is necessary to sustain immunological tolerance, yet it impairs immune protection against S. aureus. Additionally, we find that TGF-β signalling via Smad3/Smad4 is sufficient for the expression of the anti-inflammatory cytokine, IL-10, in TH17 cells. Our data thus indicate a key function of TH17 cell plasticity in maintaining immune homeostasis, and dissect the molecular mechanisms explaining the functional flexibility of TH17 cells with regard to environmental changes.
The consequences of dysfunctional immune adaptation are clear: the immune cells either fail to mount a protective response to lethal infections, or they overreact to non-harmful antigens and in doing so, induce disparate immune-mediated inflammatory diseases (IMIDs). It is therefore both fundamentally and clinically relevant to reveal the molecular mechanisms underlying this phenomenon of immune adaptation. 

**T**h17 cells—a subset of CD4+ T cells—exemplify immune adaptation. On the one hand, T17 cells can have a full effector function protecting the host from pathogens with the associated risk of developing IMIDs1–3. On the other hand, T17 cells can also acquire an anti-inflammatory fate characterized by secretion of the anti-inflammatory cytokine, IL-104–6. However, the relevance of this T17 anti-inflammatory fate and the consequences of its impairment have not yet been fully explored. T17 cells are abundant in the intestine6, but it remains to be tested whether by acquiring an anti-inflammatory fate, they contribute to intestinal immune homeostasis by blocking the development of a pathological response and preserving the tissue integrity. Furthermore, whether this anti-inflammatory fate negatively impacts T17-mediated immunity to pathogens is also unknown.

Finally, the exact molecular mechanism behind this cellular adaptation also remains to be clarified. Even though the key molecular pathways for the differentiation of CD4+ T cells into T17 cells have been studied extensively9–12, investigation into the mechanisms that promote the anti-inflammatory fate of mature T17 cells has just begun13,14. On the basis of in vitro screenings, we proposed that TGF-β controls the expression of IL-10 in CD4+ T cells during their in vitro differentiation into T17 cells15,17. However, whether TGF-β also plays a key role in vivo and on already matured T17 cells, and through which molecular signalling, remain to be studied.

It is known that upon engagement of TGF-β with TGFBR1/R2 on CD4+ T cells, TGFBR2 trans phosphorylates TGFBR1, which propagates signalling by phosphorylating Smad2 and Smad3. Once Smad2 and Smad3 form a heteromeric complex with the common mediator Smad4, they translocate to the nucleus to trans-activate several genes. It has been shown that while Smad2/3 and common mediator Smad4, they translocate to the nucleus to trans-activate several genes. It has been shown that while Smad2 and Smad3 form a heteromeric complex with the common mediator Smad4, they translocate to the nucleus to trans-activate several genes.

**R**esults

**T**h17 derived IL-10 contributes to intestinal homeostasis. We previously showed that some T17 cells can express IL-10 (10-+ T17 cells) and eventually fully convert into bona fide T8 cells (T8exT17 cells): the anti-inflammatory fate of T17 cells6,7. Of note, the identity of these T8exT17 cells has been previously shown at both the transcriptional and functional levels7. However, the spatial distribution of these different cell statuses (i.e. IL-10+ T17 cells and T8exT17 cells) remained to be defined. We used the Fate+ (Il17aCreKatushka, Rosa26-STOPFlpFloxFloxF and Il10FloxFloxF, Il10GFP, Foxp3RFP) mice and assessed the presence of T17 cells (Foxp3RFP– IL-17A Katushka+, IL-10GFP– R26YFP–), IL-10+ T17 cells (Foxp3RFP– IL-17A Katushka+, IL-10GFP+ R26YFP–) and T8exT17 cells (Foxp3RFP– IL-17A Katushka+ IL-10GFP+ R26YFP+) in lymphoid and in intestinal tissues. We observed that the majority of these two T17-derived IL-10 producing cell populations (i.e. IL-10+ T17 cells and T8exT17 cells) reside in the Peyer’s Patches (PP) and in the ileum (Fig. 1a). Considering that the small intestine and PP host several other types of anti-inflammatory cells, such as Foxp3+ Treg and T8 cells, which also secrete high levels of IL-1025–28, we wondered whether the anti-inflammatory fate of T17 cells is redundant for the intestinal homeostasis. To this end, we crossed Il17aCre mice with Il10FloxF and R26YFP mice (from here on referred to as Il17aCre Il10FloxF) (Supplementary Fig. 1a). First, we confirmed the efficiency of IL-10 deletion in T17 cells in vivo and in vitro (Supplementary Fig. 1b–e). Then we investigated whether intestinal homeostasis in these mice was altered. Although we did not observe any obvious intestinal tissue damage microscopically (Supplementary Fig. 1f), many pro-inflammatory genes such as Il1b, Il17f, Cxcl1, Ifng and Tnfa are elevated in the intestinal tissues of Il17aCre Il10FloxF mice, compared to their littermate control mice (Il17aCre Il10WtWt) under steady state conditions (Fig. 1b). Furthermore, a more detailed cellular analysis revealed that under steady state conditions, there was an accumulation of T17 and T17/T17 cells in the small intestine of the Il17aCre Il10FloxF mice compared to their wild-type littermate controls. Of note, the T17 cell population was comparable in frequency and number between the Il17aCre Il10FloxF and control mice, suggesting that T17 cell derived IL-10 has a specific capacity to regulate T17 cell expansion in the intestine (Fig. 1c–e).

Next, we profiled the IL-10 expression in different types of immune cells to assess the different potential contribution to the phenotype observed in the Il17aCre Il10FloxF mice. We observed that more than 90% of the cells that co-express IL-10 and YFP (indicating IL-17A production) in the small intestine are CD4+ T cells (Supplementary Fig. 1g). Finally, we tested whether Il17aCre Il10FloxF mice acquired an extra intestinal spontaneous immune dysregulation, but we could not observe any immune abnormality in the thymus, spleen and other peripheral lymphoid organs (Supplementary Fig. 2). These data reveal the distribution of the IL-10+ T17 cells and T8exT17 cells along the small intestinal tract. Moreover, these data suggest that the anti-inflammatory fate of T17 cells plays a non-redundant role in maintaining the cellular and molecular immune homeostasis in the small intestine.

**IL-10 deletion in T17 enhances antibacterial immunity.** We next hypothesized that IL-10 deletion in IL-17A producing cells may lead to more efficient immunity at the expense of immunological tolerance. To test this hypothesis, we first used...
IL-10 deletion in TH17 impairs intestinal immune regulation. Next, we tested whether IL-17A-producing cell derived IL-10 plays an essential role during the resolution of inflammation in the small intestine. Thus, we challenged mice with anti-CD3 monoclonal antibody (mAb) treatment, a mouse model that we and others have previously established to induce a transient inflammation in the small intestine followed by the re-establishment of immunological tolerance. We found that Il17aCre Il10Flox/Flox mice were more susceptible to inflammation induced weight loss compared to control mice (Il17aCre Il10^WT/WT) (Fig. 3a). Furthermore, the small intestines of the Il17aCre Il10^Flox/Flox mice showed more severe tissue damage, represented by disrupted villi structures and edema compared to control mice (Fig. 3b). In addition, we observed a selective accumulation of Th17 cells and Th17/Th1 cells in the intestine of the Il17aCre Il10^Flox/Flox mice (Fig. 3c–e). We next questioned whether a different expression of IL-10R among the Th17, Th17/Th1 and Th1 cells could justify the selective accumulation of Th17 cells and Th17/Th1 but not of Th1 cells in the Il17aCre Il10^Flox/Flox mice. In line with what we published previously, we indeed observed that
Fig. 2 IL-10 deletion in Th17 impairs antibacterial immunity. Th17 derived IL-10 impairs the development of an efficient immunity against S. aureus. a A schematic depiction of the experimental plan. b Colony-forming units (CFUs) of S. aureus from different organs. Data are cumulative of two independent experiments. Each dot represents one mouse (n_wild type = 7, n_KO = 7). Mean ± SEM; *P < 0.05 by Mann-Whitney U test. c Flow cytometric analysis of small intestinal CD4+ T cells after S. aureus infection. Small intestinal lymphocytes were isolated from the indicated mouse lines and intracellular staining for both IL-17A and IFN-γ was performed to identify Th17 (IL-17A+ IFN-γ+), Th1/Th17 (IL-17A+ IFN-γ−) and Th1 (IL-17A− IFN-γ+) cells. A pre-gate on CD4+ T cell is applied. One representative experiment out of three is shown. d, e Statistical analysis of frequencies (d) as well as numbers (e) are reported. One representative experiment out of three is shown. Each dot represents one mouse (n_wild type = 5, n_KO = 5). Mean ± S.D.; ns, not significant; *P < 0.05 by Mann-Whitney U test. Source data are provided as a Source data file.

Fig. 3 IL-10 deletion in Th17 impairs intestinal immune regulation. a Percentage of initial body weight after anti-CD3 mAb. The arrows indicate the injection of anti-CD3 mAb. Data are cumulative of three independent experiments. Mean ± SEM; *P < 0.05, by two-way ANOVA with Bonferroni’s post test. b Representative histological pictures of H&E stained small intestines after anti-CD3 mAb treatment. For the statistical analysis on the right, one representative experiment out of two is shown. Each dot represents one mouse (n_wild type = 3, n_KO = 3). Mean ± S.D.; *P < 0.05 by Welch’s t-test. c Flow cytometric analysis of small intestinal CD4+ T cells after anti-CD3 mAb treatment. Small intestinal lymphocytes were isolated from the indicated mouse lines and intracellular staining for both IL-17A and IFN-γ was performed to identify Th17 (IL-17A+ IFN-γ+), Th1/Th17 (IL-17A+ IFN-γ−) and Th1 (IL-17A− IFN-γ+) cells. A pre-gate on CD4+ T cell is applied. d, e Statistical analysis of frequencies (d) and numbers (e) are shown. One representative experiment out of three is shown. Each dot represents one mouse (n_wild type = 5, n_KO = 5). Mean ± S.D.; ns, not significant; **P < 0.01 by Mann-Whitney U test. Source data are provided as a Source data file.
**TGF-βRII is required for mature Th17 cells to produce IL-10.**

Despite the fact that the molecular signals inducing IL-10 in naïve CD4+ T cells have been largely explored, the molecular mechanisms that control IL-10 production in mature Th17 cells in vivo remain unknown. TGF-β is an obvious candidate considering its effect on naive CD4+ T cells. To this end, we first differentiated Th17 cells in vitro, FACs sorted IL-17A producing CD4+ T cells and then re-stimulated these cells in the presence of increasing doses of TGF-β. These results confirmed previous findings and further showed that TGF-β promotes IL-10 production also in mature Th17 cells in a concentration-dependent manner (Supplementary Fig. 5a). Next, we tested whether Tgfb2-deficient Th17 cells have any impairment in up-regulating IL-10 expression when cultured in vitro. We found that both the IL-10+ Th17 population and Tgfb2+/Th17 cells have a better IL-10 production compared to Thgfb2 YFP+ cells isolated from littermate controls (Fig. 4a, b). Of note, we didn’t observe an obvious defect of primary Th17 cell differentiation with Tgfb2+ cells (Supplementary Fig. 5c, d), which is in line with the previous observation that IL-17A+ cells do not affect primary Th17 cell proliferation and differentiation (Supplementary Fig. 5e). Finally, we examined whether Th17 cells require TGF-βRII in order to produce IL-10 in vivo. We observed that under steady state conditions, intestinal CD4+ T cells marked by YFP (indicating Tgfb2 deletion) from Tgfb2+/Floxed/Foxp3+ mice had an obvious impairment in IL-10 and IL-17A production compared to those isolated from Tgfb2+/Floxed/Foxp3+ mice (Supplementary Fig. 5e–g). Since TGF-β promotes the expression of Foxp3, as a control we tested whether the small expression of Foxp3+ among the intestinal R26+CD4+ T cells was dysregulated in the Tgfb2+/Floxed/Foxp3+ mice. However, we did not observe any statistically significant dysregulation of Foxp3 and more importantly no difference in the number of Foxp3+ YFP+ cells between the Tgfb2+/Floxed/Foxp3+ and Tgfb2+/Floxed/Foxp3+ mice (Supplementary Fig. 5h, i).

We then injected anti-CD3 mAb to induce a transient intestinal inflammation in the small intestine of these mice. Similar to what we observed by using the Il17aCre Il10Floxed/Foxp3+ mice, Tgfb2+/Floxed/Foxp3+ mice were more susceptible to intestinal inflammation compared to the relative control. The Tgfb2+/Floxed/Foxp3+ lost more weight and had a more severe intestinal pathology than control Tgfb2+/Floxed/Foxp3+ mice (Supplementary Fig. 6a, b). We then harvested the intestinal tissues and analysed the lymphocyte composition. We observed that both the frequency and the amount of IL-10+ Th17 cells and Tgfb2+/Th17 cells from the intestines of Tgfb2+/Floxed/Foxp3+ mice were significantly lower than those from WT Fate+ mice (Fig. 4c–e). Of note, similar to what is seen under steady state conditions, we did not observe statistically significant changes in the expression of Foxp3 among R26+CD4+ T cells, nor did we observe differences in the number of Foxp3+ R26+/Th17 cells between the Tgfb2+/Floxed/Foxp3+ and Tgfb2+/Floxed/Foxp3+ mice (Supplementary Fig. 6c, d).

To exclude the possibility that reduced IL-10 production from the CD4+ YFP+ cells of Tgfb2+/Floxed/Foxp3+ mice was a consequence of more severe inflammation, we co-transferred CD4+ T cells from both CD45.2 Tgfb2+/Floxed/Foxp3+ mice and CD45.1/2 Tgfb2+/floxed/Foxp3+ mice into Rag1−/− mice. After we engrafted the cells, we injected anti-CD3 mAb and finally harvested the cells. We found that even when the cells resided in the same environment, Tgfb2-deficient Th17 cells lost their ability to express IL-10 compared to wild-type cells, which still produced IL-10 (Fig. 4f–h). We also observed that Tgfb2-deficient R26+/Th17 cells expanded and failed to preserve the expression of IL-17A (Supplementary Fig. 6e), suggesting a role of TGF-β signalling in controlling proliferation and IL-17A maintenance in this particular experimental setting. Taken together, these data indicate that mature Th17 cells intrinsically require signalling via TGF-βRII to promote the expression of IL-10 in vitro and in vivo.

**TGF-β signals via Smad3/4 to regulate IL-10 in Th17 cells.**

The next step was to explore the signalling pathway that drives IL-10 expression upon TGF-β engagement with its receptor on mature Th17 cells. After TGF-β engages its receptor, Smad2 and Smad3 complex is phosphorylated and by binding to either TIF1-γ or Smad4, translocates into the nucleus to activate gene expression (21). Whether either Smad4 or TIF1-γ controls TGF-β-mediated IL-10 expression in mature Th17 cells remained to be tested.

We first examined the role of TIF1-γ in the expression of IL-10 in Th17 cells. To this end, we made Tif1γ−/Floxed/Foxp3+ mice, which similar to the Tgfb2+/Floxed/Foxp3+ mice described above, in order to specifically delete Tif1γ in IL17a producing cells (Supplementary Fig. 7a). After inducing intestinal inflammation in these mice, we observed that both IL-10+ Th17 cells and Tgfb2+/Th17 cells remain comparable between Tif1γ−/Floxed/Foxp3+ mice and their wild-type littermate controls, suggesting that TIF1-γ is not required for IL-10 production in Th17 cells in vivo (Supplementary Fig. 7b–d).

We then evaluated whether Smad4 is important for TGF-β-mediated IL-10 expression. To address this, we first created Smad4−/Floxed/Foxp3+ mice in order to delete Smad4 in Th17 cells (Supplementary Fig. 8a). Smad4-deficient CD4+ T cells had a slight, but significant impairment in IL-10 production when cultured under Th17 conditions in vitro (Supplementary Fig. 8b, c). These mice were then injected with anti-CD3 mAb and IL-10−/Th17 and Tgfb2+/Th17 cells populations were analysed. In line with the in vitro observations, both types of cells were reduced in the small intestines of Smad4−/Floxed/Foxp3+ mice compared with the wild-type control mice (Fig. 5a–c). Finally, we also determined the role of Smad3 in regulating IL-10 production in Th17 cells. We disrupted the function of Smad3 by using either Crispr/Cas9 mediated gene depletion (Fig. 5d, e), or small chemical inhibitor SIS3 in vitro differentiated Th17 cells (Supplementary Fig. 9a, b). In both cases, we observed that the induction of IL-10 in in vitro differentiated Th17 cells by TGF-β was impaired when Smad3 was deleted/blocked. These data suggest that Smad3 is also required for TGF-β induced IL-10 production in Th17 cells in vitro.

Taken together, these data show that while TIF1-γ is dispensable, Smad4 and Smad3 are necessary for TGF-β mediated IL-10 induction in mature Th17 cells.
Smad3 and Smad4 activate Il10 transcription in TH17 cells. We next sought to test whether Smad3 and Smad4 bind to the Il10 promoter in mature TH17 cells. First, we tested whether phospho-Smad3 translocates into the nucleus shortly after TGF-β stimulation. Nuclear extracts of TH17 cells were immunoblotted and we observed phospho-Smad3 in the nuclei of these cells (Fig. 6a). Next, we tested whether Smad3 and Smad4 are able to induce Il10 gene transcription. We first cloned Il10 proximal promoter (−1444 to +1) in the pGL2 vector upstream of Luciferase reporter gene, and Smad3 and Smad4 genes in pCMV and
Fig. 5 TGF-β signals via Smad3/4 to regulate IL-10 in TH17 cells. a Flow cytometric analysis of small intestinal CD4+ T cells after anti-CD3 mAb. The indicated reporter mouse lines were used. Top panels are pre-gated on Foxp3−, CD4+ T cells. Bottom panels are pre-gated on YFP+ cells, and the indicated populations are identified on the basis of the reporter molecules as follows: TregFox3TH17 cells: IL-10eGFP− IL-17AKatushka; TH17 cells: IL-10eGFP− IL-17AKatushka. b, c Frequencies (b) and numbers (c) of TregFox3TH17, IL-10+ TH17 and TregFox3TH17 cells in A are one representative experiment out of three. Each dot represents one mouse (nwild type = 5, nB6 = 5). Mean ± S.D.; ns, not significant; *P < 0.05, **P < 0.01 by Mann–Whitney U test. d Experimental design of knocking out Smad3 in in vitro differentiated mature TH17 cells by using CRISPR/Cas9 technology. e Flow cytometric analysis of in vitro cultured mature TH17 cells after knocking out Smad3 by using gRNAs targeting Smad3. Cells were pre-gated on viable CD4+ T cells. NT, non-targeting control. Each dot represents an individual gRNA (nNT = 2, nSmad3 = 3). Mean ± S.D.; **P < 0.01 by Welch’s t-test. Source data are provided as a Source data file.

pCMVβ mammalian expression vectors respectively. Co-transfection of Il10 proximal promoter with increasing concentrations of either only Smad3 or only Smad4 expression plasmids did not result in significant upregulation of luciferase activity. However, when increasing concentrations of Smad4 plasmid were co-transfected with a constant amount of Smad3 plasmid, a significant increase in Il10-luciferase activity was observed (Fig. 6b). These data suggest that Smad3 and Smad4 synergistically activate the Il10 promoter.

In order to show that Smad3 and Smad4 directly bind the Il10 promoter in mature TH17 cells, we performed Chromatin Immunoprecipitation (ChIP) experiments with FACS sorted TH17 cells stimulated with or without TGF-β for 30 min. We found that 30 min after TGF-β treatment, both Smad3 and Smad4 accessed and bound the Il10 promoter on the −929/−756 element. Of note, between −929/−756 and −286/−283 base pairs on Il10 promoter lie predicted Smad3/4 binding sites (Fig. 6c). As a negative control, we interrogated the GAPDH TATA box promoter region where Smad3 and Smad4 proteins do not bind, and as expected we did not see any signal. Finally, in order to show that Smad3 and Smad4 proteins form a complex in TH17 cells, we performed Proximity Ligation Assay (PLA) in FACS sorted TH17 challenged for 30 min with TGF-β, using a plus and a minus ligation probe specific for anti-mouse Smad3 and anti-rabbit Smad4 antibodies, respectively. Confocal microscopic images reveal that the Smad3/4 complexes are only visible in the nucleus of TH17 cells challenged with TGF-β (Fig. 6d). Interestingly, this protein complex forms 3–5 bright, closely spaced foci per cell. The PLA spots were located in the rim of the TH17 cell nucleus associated with the nuclear membrane. Taken together, these results show that Smad3 and Smad4 proteins are instructed by TGF-β signal, form a nuclear complex that bind to the proximal Il10 promoter and transactivates Il10 transcription in TH17 cells.

TGF-β promotes IL-10 expression in human TH17 cells. We tested whether human TH17 cells isolated from the intestine can also produce IL-10. We observed that a small but consistent frequency of CD4+ T cells isolated from healthy colon tissues from 14 human donors, co-produce IL-17A and IL-10 (Fig. 7a).
Next, we wondered whether human TH17 cells can also acquire the expression of IL-10 when stimulated with TGF-β. We therefore isolated circulating CD45RA low CD25−IL-17A+(TH17) cells from human blood using the IL-17A secretion assay and cultured the isolated TH17 cells in the presence of increasing TGF-β concentrations. The purity of the TH17 cells isolated from human blood was always more than 95% (for a representative purity plot see Supplementary Fig. 10a). Upon examining the CD4+ Foxp3− T cell compartment (see Supplementary Fig. 10b for the gating strategy), we observed that approximately half of the sorted cells, that were formerly IL-17A+, ceased to produce IL-17A (i.e. they become exTH17 cells) while the other half retained IL-17A expression. However, this phenomenon was not affected by TGF-β (Fig. 7b). In contrast, TGF-β promoted IL-10 expression in both TH17 cells and exTH17 cells in a concentration-dependent manner (Fig. 7b). Finally, increasing TGF-β concentrations appeared to slightly reduce the frequency and the numbers of IL17A+ IFN-γ+ and IFN-γ+ producing cells (Supplementary Fig. 10c, d). Furthermore, increasing the TGF-β concentrations did not have a reproducible effect on Foxp3 expression in human peripheral blood-isolated TH17 cells (Supplementary Fig. 10e). Collectively, these results show that human

Fig. 6 Smad3 and Smad4 activate IL10 transcription in TH17 cells. a Western blot of TH17 cell-total/nuclear extracts challenged with or without TGF-β for 30 min. One representative experiment out of two is shown. b Schematic representation of the proximal murine IL10 promoter (from −1444 to +1 bp) driving the expression of the luciferase reporter gene (luc). The predicted binding sites for Smad3 and Smad4 as well as the IL10 TSS are depicted (top). Relative Luc activity driven by Smad3 and Smad4 on proximal IL10 promoter (bottom). Mean ± SEM (n = 3). *P < 0.05, **P < 0.01, and ***P < 0.001 by ordinary two-way ANOVA and Tukey’s multiple comparisons test. c Schematic representation of the proximal murine IL10 promoter (from −1444 to +1 bp) indicating the predicted binding sites for Smad3 and Smad4 and the depicted amplicons A (from −416 to −278 bp), B (from −929 to −756 bp), and C (from −365 to −171 bp) (top) that were used to amplify the chromatin Immunoprecipitated DNA. Smad3 and Smad4 Chromatin immunoprecipitation (ChIP) of sorted TH17 cells challenged with or without TGF-β (bottom). One representative experiment out of three is shown. d Proximity Ligation Assay (PLA) using sorted TH17 cells challenged with or without TGF-β. White arrows indicate positive Smad3/Smad4 interaction signal (red dots) superimposed on nuclei stained with DAPI (blue). Scale bar represents 10 μm. Number of PLA spots per cell and the number of PLA positive cells are reported in the box. One representative experiment out of two is shown. ***P < 0.001 for comparison by χ² test. Source data are provided as a Source data file.
T_{H}17 cells from the intestine and from peripheral blood, are able to produce IL-10 and furthermore, that TGF-β is able to promote dose dependent IL-10 expression in human peripheral blood derived T_{H}17 cells.

Discussion

T_{H}17 cells are usually found in the inflamed tissues of IMID patients such as the central nervous system, the kidney, the joints and the heart. However, they are also abundant in the small intestine of healthy individuals without causing any overt diseases\(^{30-32}\). The T_{H}17 cell plastic behaviour allowing them to acquire both pro- and anti-inflammatory fate can explain such ubiquitous presence and consequences: pathological or physiological. Our data show that it is in fact mainly in the ileum and ubiquitous presence and consequences: pathological or physiological. Our data show that it is in fact mainly in the ileum and intestinal in the intestine of healthy individuals without causing any overt dis-

Despite the above, we did not observe macroscopic tissue damage in the \( \text{II17a}^{\text{Cre}} \, \text{II10}^{\text{Floox/Floox}} \) mice. One explanation is the compensatory mechanism which could be exerted by the presence of other regulatory T cells, such as Foxp3\(^+\) Treg cells and TR1 cells able to produce IL-10\(^{23,25}\). Indeed, it has to be considered that only a relatively small fraction of T_{H}17 cells acquire an anti-inflammatory function. Finally, our data show that also human T_{H}17 cells acquire the regulatory phenotype when exposed to TGF-β1.

Another explanation is the composition of the intestinal microbiota of our mice. It is possible that under different specific pathogen free conditions, also \( \text{II17a}^{\text{Cre}} \, \text{II10}^{\text{Floox/Floox}} \) mice develop microscopically evident intestinal inflammation.
Interestingly, we observed a specific expansion of intestinal T17i and T117i/T117g cells, but not of T117i cells in our Il17a<sup>Cre</sup> Il10<sup>Flox</sup>Flox mouse model. One possible explanation of this cell-selective control mediated by IL-10 is that both T117i and T117i/T117g cells express higher level of IL-10R compared to T117i cells. Alternatively, one could consider that this is due to a dysregulation of Rorgt<sup>+</sup> Foxp3<sup>+</sup> Treg cells in Il17a<sup>Cre</sup> Il10<sup>Flox</sup>Flox<sup>Flox</sup> mice. These cells are indeed known to be able to specifically regulate T117i cells, especially in the colon<sup>34</sup>. However, only a small fraction of Il17α<sup>Cre</sup>-activated cells are Foxp3<sup>+</sup> and the large majority of Rorgt<sup>+</sup> Foxp3<sup>+</sup> Treg cells are known to express little to no IL-17A and thus are not mainly affected in the small intestine of the Il17a<sup>Cre</sup> Il10<sup>Flox</sup>Flox<sup>Flox</sup> mice<sup>35,36</sup>. Therefore, these data suggest that a possible dysregulation of Rorgt<sup>+</sup> Foxp3<sup>+</sup> Treg cells is not the explanation for the selective increases in the number of T117i cells observed in this study.

T17i cells also play a key role in mediating host defence against bacteria<sup>2</sup>. Through evolution, pathogens have developed mechanisms to evade the immune system of the host. For example, it was shown in mice and humans that S. aureus, a nosocomial and potentially multi drug resistant bacteria, promotes the anti-inflammatory fate of T17i cells<sup>7,26</sup>. Our data add to this because they show that the T117i cell anti-inflammatory fate can indeed favour dissemination of the pathogens. Given that this anti-inflammatory fate has been preserved throughout evolution, we wondered what its benefit is, and at what cost it comes. Considering that S. aureus bacteria only becomes a lethal threat when it disseminates through the blood stream, we propose that the benefit is the T117i cell anti-inflammatory fate’s ability to constantly maintain the immune homeostasis. We also propose that the cost of the T117i cell anti-inflammatory fate is rather low, considering that occurrence of a bacterial infection through the blood is relatively unlikely.

The role of TGF-β during the differentiation of T117i cells has been widely studied and also debated<sup>9,27</sup>. It is currently accepted that TGF-β is sufficient, but not necessary for the induction of these cells and in addition, that it favours the development of T117i with regulatory potential. Our experiments aimed to expand the understanding of TGF-β function from the early differentiation phase to the effector phase of T117i cell biology. We showed for the first time directly in vivo that Tgbfr2-deficient T117i cells had an impaired capacity to produce IL-10. In addition, the Tgbfr<sup>2</sup>Flox<sup>Flox</sup> Fae<sup>+</sup> mice phenocopied the impairment in immune regulation observed in the Il17a<sup>Cre</sup> Il10<sup>Flox</sup>Flox<sup>Flox</sup> mice. These results broaden the state of knowledge of TGF-β, revealing the role of TGF-β in plasticity and function of T117i cells upon maturation.

Furthermore, we noticed that under steady state conditions and after anti-CD3 mAb treatment, the frequency of YFP<sup>+</sup> cells increase when TGF-βRII signalling is impaired. This suggests that the expansion of T117i cells, which could otherwise lead to pathogenicity, is controlled by TGF-β. This is in line with the known anti-proliferative effect of TGF-β on CD4<sup>+</sup> T cells<sup>38</sup>. Furthermore, we observed that the large majority of TGF-βRII deficient YFP<sup>+</sup> cells lose the expression of IL-17A under steady state conditions and upon transfer into Rag1<sup>−/−</sup> mice, while they maintain the expression of IL-17A during intestinal inflammation. On the one hand, this is in keeping with the concept of a ”sufficient but not necessary” role of TGF-β during the differentiation of T117i cells: under certain conditions, TGF-β is necessary to maintain IL-17A expression, but during inflammation it becomes unnecessary. On the other hand, this also suggests that the fraction of T117i cells which do lose IL-17A, but express IL-10 in wild type mice during inflammation have restrained the TGF-β signalling towards Sma3<sup>−/−</sup>Il10<sup>−/−</sup> thus avoiding the re-activation of RORyt/IL-17A pathway. It has indeed been shown that TGF-β blocks RORyt activity via Smad3<sup>19</sup> and that TGF-β can restrain IL-17A by a post-translational deubiquitination of RORyt<sup>28</sup>. Of note, TGF-β appears to impact IL-10 production also in other effector CD4<sup>+</sup> T cells such as T1i1 cells, although the current evidence is controversial. For example, TGF-β has been shown to inhibit IL-10 production in T1i1 cells by blocking the expression of Blimp-1<sup>35</sup>, while others reported a positive effect of TGF-β on IL-10 production by T1i1 cells<sup>40,41</sup>. Therefore, TGF-β seems to have different effects on T1i1 and T17i cells regarding IL-10 production, but further in vivo studies on T1i1 cells are required to confirm this.

Downstream of TGF-β receptor, our results suggest that the canonical TGF-β mediators Sma3 and Sma4—but not TIF1-γ —promote the expression of IL-10 in mature T117i cells. Apparently in contrast to our data, it has been shown that TIF1-γ blocks the expression of IL-10 in CD4<sup>+</sup> T cells while differentiating into T17i cells<sup>32</sup>. However, while this study used the CD4<sup>Cre</sup> Tff1γ<sup>Flox</sup>Flox<sup>Flox</sup> mouse model, we used an Il17a<sup>Cre</sup> Tff1γ<sup>Flox</sup>Flox<sup>Flox</sup> mouse model instead. One possible explanation is that during T117i cell differentiation, TIF1-γ constrains IL-10 expression by obstructing Smad-recognized cis-elements from the Sma4-Sma2/3 complexes, and that this becomes dispensable in mature T17i cells when the Il10 gene is already accessible. In keeping with the different function of TIF1-γ in the two different phases, this is also evident regarding the regulation of IL-17A expression: TIF1-γ plays a key role in the expression of IL-17A during T117i cell differentiation<sup>32</sup>. However, our data suggest that its deletion in mature T17i cells becomes dispensable for IL-17A expression. Therefore, these datasets are -probably- not incompatible with each other, but rather they reveal different molecular mechanisms operating during the differentiation and maturation phases of T17i cells.

Cell clones of blood derived human T117i cells are capable of acquiring the expression of IL-10 in vitro<sup>26</sup>. In line with this data, here we show that human primary T117i cells are also able to produce IL-10 and are present in the human intestine. It has recently been suggested that IL-27 promotes the expression of IL-10, via the transcription factor c-Maf, in human T17i clones<sup>16</sup>. However, whether IL-27 alone or in combination with TGF-β can also induce the expression of IL-10 in freshly isolated human T117i cells remains to be tested. Nevertheless, our data serve as proof of principle to show that it is possible to turn freshly isolated human T117i cells into IL-10 producing cells.

The transcription factor Blimp1 has also been proposed as being able to promote the conversion of T117i cells into T1i1 cells<sup>14</sup>. However, it is also known that TGF-β blocks Blimp1 activation<sup>39,42</sup>. Because of this, we did not investigate the role of Blimp1 in our system. However, our data do not exclude a role of Blimp1 in the anti-inflammatory fate of T17i cells and it could be possible that TGF-β and IL-27 are two complementary pathways to induce the conversion of T117i cells into T1i1 cells in different microenvironments.

Collectively, our data show that the anti-inflammatory fate of T117i cells is not a mere consequence of immune homeostasis, but that it actually plays an indispensable role in the maintenance of this equilibrium. TGF-β mediates the expression of IL-10 via Sma3 and Sma4 and consequently, it is the fundamental environmental signal to tighten the balance between the pro- and anti-inflammatory fates of mature T17i cells. We propose that the T117i cell plasticity between pro and anti-inflammatory fates is a key feature of immune system adaptation and when impaired, immunity and tissue integrity are compromised. Exploring the molecular mechanisms of this biological phenomena will help to identify therapeutic targets, such as Sma3 and Sma4 for new therapies that aim to steer the immune response according to the
clinical need, rather than globally suppress it, as the majority of the current therapies have been based upon.

Methods

Mice. C57BL/6 Rag1−/− mice (Stock #: 002216) and Rosa26-Cas9 knock-in mice (Stock #: 028555) were purchased from the Jackson Laboratories. Il7A−/− mice (kindly provided by Axel Roers) were further crossed with Il17A−/− mice (kindly provided by Dr. Brigitta Stockinger). Tgβ2Fluc/Fluc (purchased from JAX, Stock #: 012603), Smad3Fluc/Fluc (kindly provided by Dr. Elizabeth Robertson) and TgfβIlluc/Iluc (kindly provided by Dr. Vesa Kaartinen) mice were further crossed with mice expressing YFP and in the meantime, lose the expression of Tgfβr2, Smad3 or Tgfβr genes. All mice were kept under specific pathogen-free (SPF) conditions, on a 12/12 on/off light cycle, maintained at 72 °F with 70% humidity in the animal facility at Yale University. Age- and sex-matched littermates between 8 to 24 weeks of age were used for experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Yale University. Preliminary experiments were tested to determine sample sizes, taking available resources and ethical use into account.

Lymphocyte isolation from mouse small intestine. Methods were described before in detail. Briefly, intestinal tissues were first digested in 1 mM DTE at 37 °C for 30 min to get the IEL fraction. Remaining tissues were then digested in Bouin’s Fixative Solutions for 1 h. Tissues were then embedded in paraffin for sectioning. Pictures were taken by optical microscope equipped with camera systems and analysed.

Flow cytometry antibodies and intracellular cytokine staining. Mouse T cells were stained with monoclonal antibodies to CD4 (GK1.5, Cat #: 100428 or RM4-5 Cat #: 100797), CD3e (Cat #: 109723), CD45R (Cat #: 109713), CD19 (6D5 Cat #: 115508), CD11b (M1/70 Cat #: 101216), CD11c (N418 Cat #: 117318), CD8 (53-6.7 Cat #: 100722), NK1.1 (PK136 Cat #: 108713), CD19 (eBio108713), CD11b (100536), CD8 (100722), NK1.1 (108713), CD19 (117318), CD11b (101216), and CD11c (117318) individually or combinations to detect step 3 to 5 YFP and to deplete Tgfβ2r, Smad3 or Tgfβr genes. All mice were kept under specific pathogen-free (SPF) conditions, on a 12/12 on/off light cycle, maintained at 72 (+/− 2) °F with 70% humidity in the animal facility at Yale University. Age- and sex-matched littermates between 8 to 24 weeks of age were used for experiments. Unless they came with special instructions, mice were randomly assigned to different experimental groups and each cage contained animals of all different experimental groups. Both male and female mice were used in experiments. Animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Yale University. Preliminary experiments were tested to determine sample sizes, taking available resources and ethical use into account.

Anti-CD3 mAb treatment and intestinal histology. Anti-CD3 mAb (Clone #: 2C11) were injected intra-peritoneally three times (15, 15, and 30 μg per mouse) every other day. Mice were euthanized with CO2 24 h after the last injection. Different parts of small intestines (duodenum, jejunum and ileum) were then fixed in 3.7% formaldehyde for 20 min at room temperature. After washing, the cells were then re-suspended in PBS, 0.5% FBS, 5 mM EDTA for 20 min at room temperature. After washing, the cells were then re-suspended in PBS, 0.5% FBS, 5 mM EDTA for 20 min at room temperature. The beads were washed in low (150 mM NaCl), intermediate (300 mM NaCl), and high (500 mM NaCl) salt buffers. Supernatant containing virus was collected and polybrene was added to achieve a final concentration of 4 μg/mL for retroviral transduction. Cas9-expressing T cells were first activated for 24 h and then spin transduced with viral supernatant.

Chromatin Immunoprecipitation. Retired Tgfβr2−/− mice were challenged or not with 1 μg/mL TGF-β1 for 30 min. The cells were fixed with 1% Formaldehyde for 10 min, washed, and re-suspended in 10 mM Tris pH 7.5, 10 mM NaCl, 3 mM MgCl2, 1 mM CaCl2, 4% NP40 supplemented with protease inhibitors, at a final concentration of 4 × 105 cells/mL. Subsequently, 0.2 units MNase (Sigma) were added for 5 min at 37 °C, followed by 3 mM EGTA and the cells were centrifuged at 1500 g for 5 min at 4 °C. The lysates were extensively dialysed in 10 mM Tris pH 7.5, 5 mM glycerol, 1 mM EDTA, 0.5 mM EGTA, 0.1%SDS, 0.1% Na-deoxycholate, supplemented with protease inhibitors and sonicated 3 times for 2 sec. 1% Triton X-100 was added and the samples were centrifuged for 15000 g for 15 min at 4 °C. The lysates were extensively dialysed in 10 mM Tris pH 7.5, 5 mM glycerol, 1 mM EDTA, 0.5 mM EGTA. For immunoprecipitation, the cell lysate was mixed with depletion beads (Mini-PBTE; Invitrogen®) and proteins were transferred to PVDF membranes. The membranes were immunoblotted with the indicated antibodies and developed according to standard protocols. The protein extract equivalent corresponding to 8 × 107 Tgfβr2−/− cells in each well.

Retroviral preparation and transduction. For retroviral preparation, 1 μg of individual Smad3 sgRNA vector with 0.5 μg of EcoHlpLl helper plasmid were employed to transfect HEK293T cells. In all, 12 h later, media was replaced and virus was used for another 48 h. Supernatant containing virus was collected and polybrene was added to achieve a final concentration of 4 μg/mL for retroviral transduction. Cas9-expressing T cells were first activated for 24 h and then spin transduced with viral supernatant.

Chromatin Immunoprecipitation. Retired Tgfβr2−/− mice were challenged or not with 1 μg/mL TGF-β1 for 30 min. The cells were fixed with 1% Formaldehyde for 10 min, washed, and re-suspended in 10 mM Tris pH 7.5, 10 mM NaCl, 3 mM MgCl2, 1 mM CaCl2, 4% NP40 supplemented with protease inhibitors, at a final concentration of 4 × 105 cells/mL. Subsequently, 0.2 units MNase (Sigma) were added for 5 min at 37 °C, followed by 3 mM EGTA and the cells were centrifuged at 15000 g for 5 min at 4 °C. The lysates were extensively dialysed in 10 mM Tris pH 7.5, 5 mM glycerol, 1 mM EDTA, 0.5 mM EGTA. For immunoprecipitation, the cell lysate was mixed with depletion beads (Mini-PBTE; Invitrogen®) and proteins were transferred to PVDF membranes. The membranes were immunoblotted with the indicated antibodies and developed according to standard protocols. The protein extract equivalent corresponding to 8 × 107 Tgfβr2−/− cells in each well.

Real-time quantitative PCR. qPCR was performed using chromatin immunoprecipitation reactions with the anti-Smad3 and Smad4 and isotype control antibodies, along with the indicated primers and KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Wilmington, MA) in a StepOne cycler (Applied Biosystems, Carlsbad, CA). The CT values from triplicate qPCR reactions were extracted from the SteppOne software and were analyzed with the relative quantification method 2−ΔΔCT. The abundance level of a given amplicon per sample/condition was determined relative to its input (non-enriched) chromatin abundance and was additionally corrected relative to isotype control antibody immunoprecipitation sample.

Proximity ligation assay. In vivo differentiated sorted Tgfβr2−/− cells were rested for 5 days in Click’s medium supplemented with 2.5 μg/mL uridine IL-2 (PeproTech 2-121) and were subsequently challenged with 1 μg/mL human TGF-β1 for 30 min. Subsequently, the cells were washed, re-suspended in a minimal volume of PBS supplemented with 10% FBS 0.3% BSA, overlaid onto coverslips and were left to dry for 12 h. The cells were rehydrated with PBS, fixed with 4%PFA and permeabilized with 0.3% PBS-Triton X100. After washing, the specimens were blocked, incubated with caspase-Smad3 and Smad4 antibodies and stained with Duolink® in Situ detection Reagents Red (Sigma). After mounting, the slides were
analysed by confocal microscopy and the acquired images were opened and processed with Fiji software (Image J).

Lymphocyte isolation from human intestine. The human colonic tissue is derived from colon cancer patients admitted into the UKE General Surgery Clinic. The study was approved by the Ethical Physicians Committee of Hamburg (Ethik-Kommission der Aertztekammer Hamburg) and Informed Written Consent was obtained from each one of the sample donors. The general characteristics of the participants cohort were five males and nine females with an average age of 69.5 years (±SD = 11.3). Healthy tissues adjacent to the tumor, were collected by collaborating surgical personnel, immediately after the completion of the surgical procedure. Next, the tissue samples were processed for lymphocyte isolation. Intraepithelial lymphocytes were isolated by digesting the tissue with Dithiothreitol (DTT Sigma-Aldrich) solution at 37 °C for 20 min, followed by incubation with collagenase IV (100 U Sigma-Aldrich) for 30 min at 37 °C. The cells were further separated by a Percoll gradient (GE Healthcare). After this the cells were stained for FACS analysis. Only the healthy colonic tissue data analysis with respect to separated by a Percoll gradient (GE Healthcare) were proceeded.

Sorting of human TH17 cells from Buffy Coat. Enriched human Buffy Coat samples retrieved directly from the UKE Transfusion Medicine, were diluted with PBS to a total volume of 350 mL. The diluted blood samples were overlaid to buffered Biocoll (Hettich Zell & Medizin Technik, Tuttingen, Germany) and with human anti-CD4/FITC, anti-CD45RA/Alexa700, anti-CD127/PE.Cy7 and anti-CD25/BV650 and sorted with an Aria Illusion instrument (BD Biosciences). In all, 5–8 × 10^7 sorted CD4+ IL-17A+ CD45RA+ CD127+ CD25− cells were rested in RPMI medium (supplemented with 10% FBS, 10 mM HEPES, Glutamax, Penicillin/Streptomycin 100 U/mL) plus 2 ng/mL IL-2 for FACS analysis. Only the healthy colonic tissue data analysis with respect to TGF-β-kaptoethanol, Glutamax, Penicillin/Streptomycin 100 U/mL) plus 2 ng/mL IL-2 was proceeded.

CD4+ T cells were selected with magnetic human CD4 microbeads (#130-045-101, Miltenyi, Bergisch-Gladbach, Germany). IL-17A+ cells were stained with the IL17A secretion assay detection kit (#130-094-537, Miltenyi, Bergisch-Gladbach Germany) and with human anti-CD4/FITC, anti-CD45RA/Alexa700, anti-CD25/PE.Cy7 and anti-CD25/BV650 and sorted with an Aria Illusion instrument (BD Bioscience). In all, 5–8 × 10^7 sorted CD4+ IL-17A+ CD45RA+ CD127+ CD25− cells were rested in RPMI medium (supplemented with 10% FBS, 10 mM β-mercaptoethanol, Glutamax, Penicillin/Streptomycin 100 U/mL plus 2 ng/mL IL-2 (PeproTech). After 48 h the cells were challenged with increasing concentrations of TGF-β1 for five days. Subsequently, cells were stimulated with PMA/Ionomycin, stained for CD4, IL-17A, and IL-10 expression and analysed by FACS.

Statistics. FACS data were visualized and analyzed with FlowJo 10.5.3 (BD Bioscience). Statistical analyses were calculated in Prism (GraphPad Software). According to the experimental set-up we used Mann–Whitney U test or Welch's t-test (Data specified in Figs. 1–5), two-way ANOVA (Fig 6b) and χ^2 test (Fig 6d). The p values presented in Fig 7 were calculated via the Mann–Whitney U test. The specific statistical analyses used in each figure are further described in the figure legends. When not otherwise specified, the statistical test used is always two-sided.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. We declare that the data supporting the findings of this study are available within the paper (and its Supplementary Information files). Source data are provided with this paper.

Received: 3 October 2019; Accepted: 11 June 2020; Published online: 03 July 2020.

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Acknowledgements
This work was supported by European Research Council (ERC) (StG 715271 to N.G.) and Deutsche Forschungsgemeinschaft (DFG) (Project number335447717; SFB1328, Project A14 to N.G. and E.T.). This work was supported in part by grants provided by AbbVie (to R.A.F.). The authors thank Elaine Hussey for editing the manuscript, the authors thank Dr. Kristoffer Riecken and Prof. Dr. Boris Fehse in the Center of Oncology, Department of Stem Cell transplantation in UKE, Hamburg for the human HEK293T cells donation, Dr. Markus Heine and Prof. Dr. Joerg Heeren at the Center of Experimental Medicine, Institute of Biochemistry and Molecular cell Biology, UKE Hamburg for their help with the confocal microscopy. Furthermore, the authors wish to thank the UKE, Hamburg Animal Models and Fluorescent Activation Cell sorting core facilities for excellent experimental support.

Author contributions
H.X., T.A., N.G., and R.A.F. designed the study and wrote the paper. H.X. and T.A. designed and performed experiments. R.W., D.P. and J.I. provided and analyzed the human colonic biopsies. W.B. assisted with the sgRNA silencing experiments. V.K. provided the Tif1gFlox/Flox mouse. J.Z., B.S., M.C.A., P.B., R.J., J.G., P.L.L., and E.E. S.H. and discussed and interpreted the results. E.T. provided help with the human T(Th)7 cell culture and protocols. S.H. analyzed histological samples and interpreted the results. All authors edited and approved the paper.

Competing interests
The authors declare no competing interest.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-17097-5.

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Peer review information Nature Communications thanks Toshinori Nakayama, Wenjun Ouyang and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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