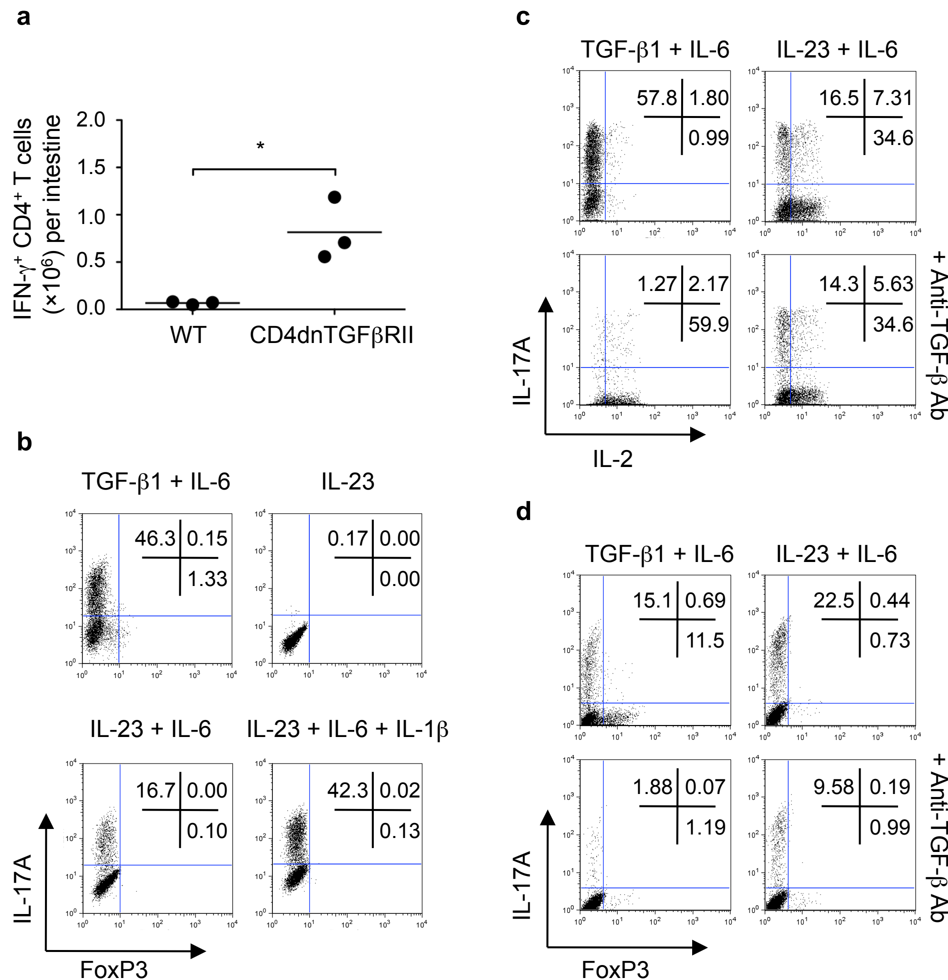
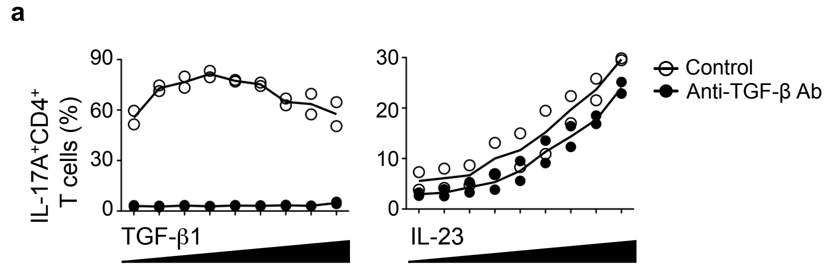
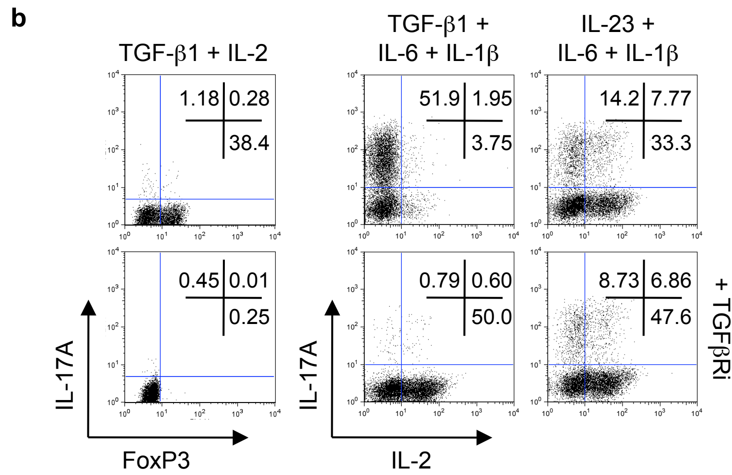
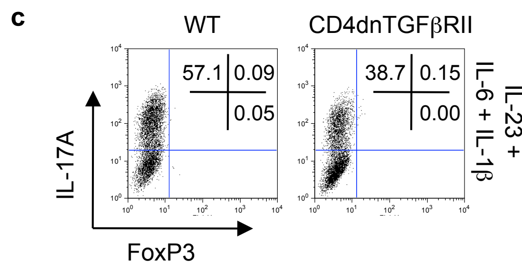


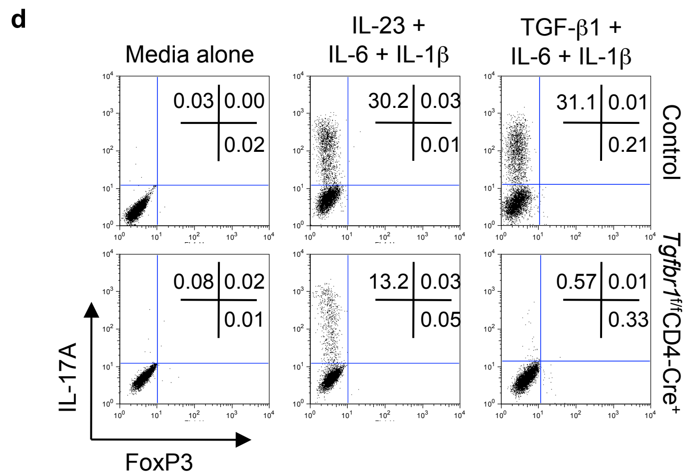
Supplementary Figure 1 Consequences of interrupted TGF-β-signaling on IFN-γ and IL-17 expression in CD4⁺ T cells.

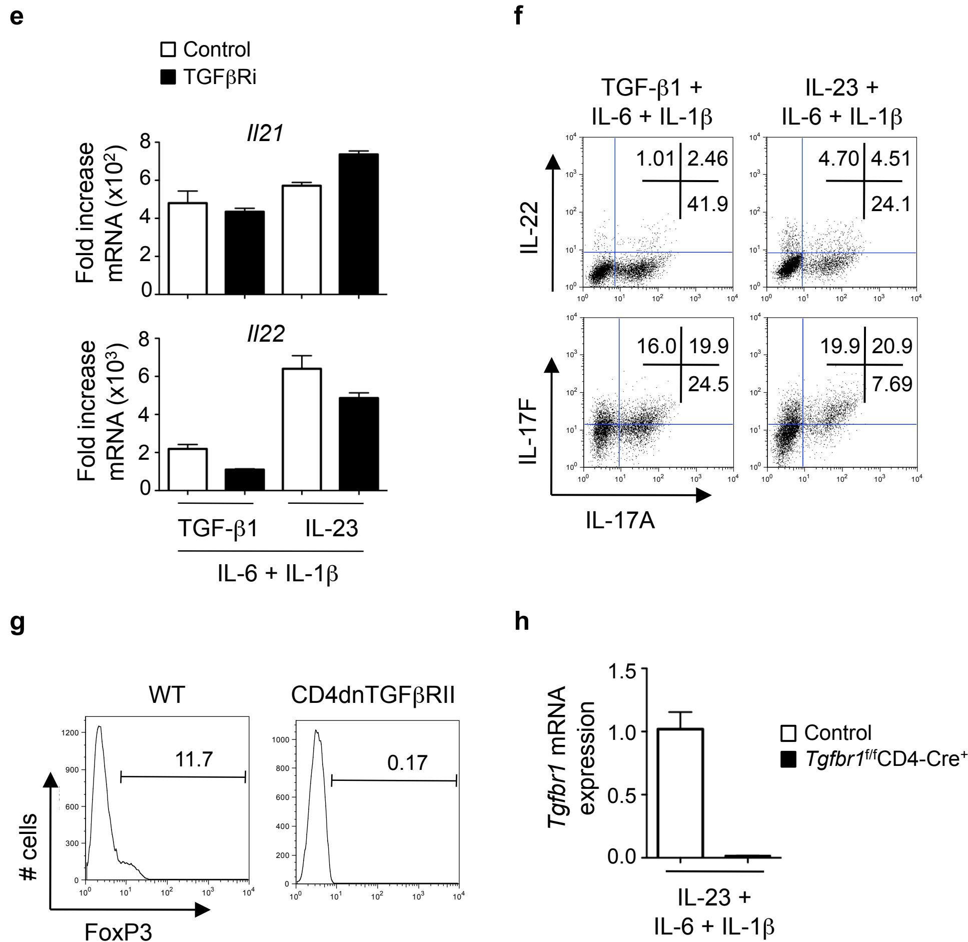
a, The proportions of IFN-γ⁺CD4⁺ T cells in the lamina propria of CD4dnTGFβRII mice and age-matched controls were determined by intracellular cytokine staining and flow cytometry (*P<0.01). **b-d,** Naïve CD4⁺ T cells were isolated by cell sorting and activated in serum-free media (**b, c**) or in media containing 10% FBS (**d**) with plate-bound anti-CD3/anti-CD28 for 4 days together with the indicated cytokines. IL-17A and FoxP3 or IL-2 protein expression were analyzed by intracellular staining. Neutralizing anti-TGF-β antibodies prevented IL-6 and TGF-β1-dependent differentiation of Th17 cells, but not IL-23 and IL-6-induced differentiation (**c, d**).


a
IL-17A+CD4+ T cells (%)
90
60
30
0
TGF-β1
30
20
10
0
IL-23
Control
Anti-TGF-β Ab


b
TGF-β1 + IL-2
TGF-β1 + IL-6 + IL-1β
IL-23 + IL-6 + IL-1β
1.18
0.28
38.4
51.9
1.95
3.75
14.2
7.77
33.3
0.45
0.01
0.25
0.79
0.60
50.0
8.73
6.86
47.6
+ TGFβRi
IL-17A
FoxP3
IL-17A
IL-2

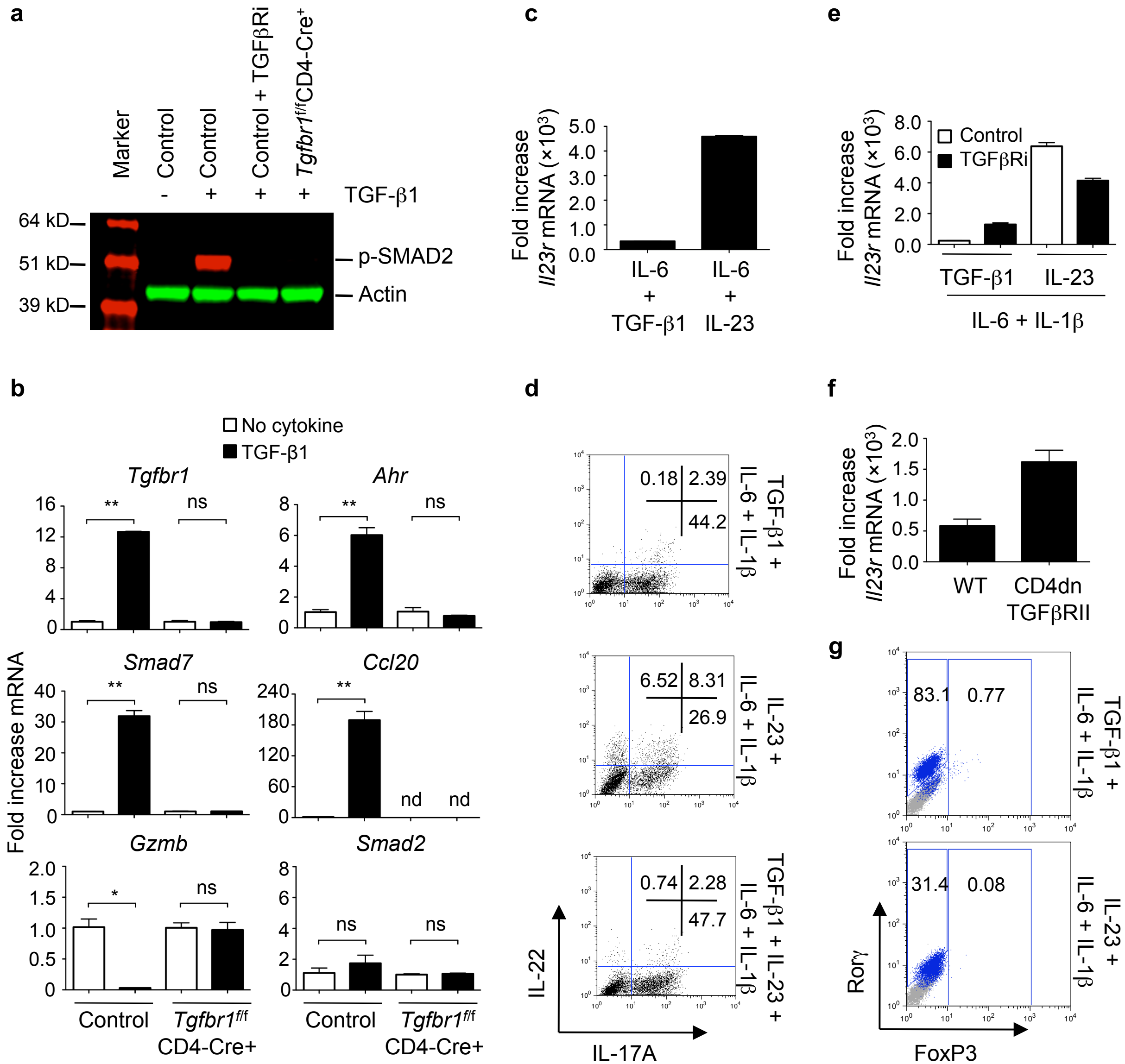

c
WT
CD4dnTGFβRII
57.1
0.09
0.05
38.7
0.15
0.00
IL-23 + IL-6 + IL-1β
IL-17A
FoxP3


d
Media alone
IL-23 + IL-6 + IL-1β
TGF-β1 + IL-6 + IL-1β
0.03
0.00
0.02
30.2
0.03
0.01
31.1
0.01
0.21
Control
0.08
0.02
0.01
13.2
0.03
0.05
0.57
0.01
0.33
Tgfbr1f/fCD4-Cre+
IL-17A
FoxP3



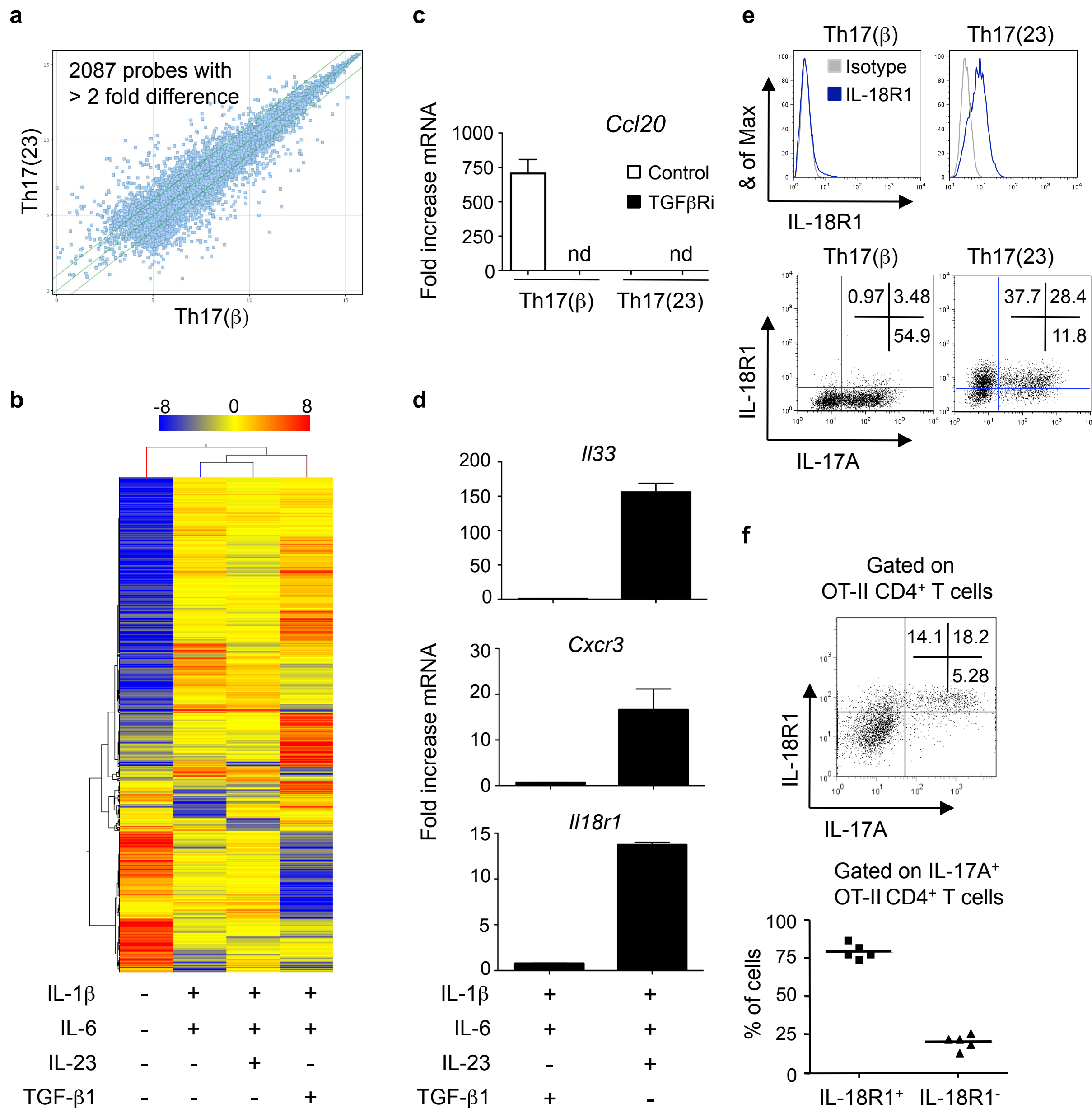
Supplementary Figure 2 Antagonism of TGF-β signaling by multiple modalities permits *in vitro* Th17 differentiation.

a, Sorted naïve CD4$^+$ T cells were activated in serum-free media in the presence of IL-6 and IL-1β with increasing doses of either TGF-β1 (0.03-10 ng ml^{-1}) or IL-23 (0.625-160 ng ml^{-1}). Neutralizing anti-TGF-β antibodies abrogated TGF-β1-induced Th17 differentiation, but did not affect IL-23-mediated Th17 differentiation. Data from two individual experiments (circles) are combined and mean values are shown (solid line). **b,** Naïve CD4$^+$ T cells were differentiated for 4 days in serum-free media alone or in the presence of a TGFβR serine kinase inhibitor (TGFβRi) with the indicated cytokines. Intracellular levels of cytokines and FoxP3 are shown. **c, d,** IL-23 in combination with IL-6 and IL-1β induced substantial IL-17 production in naïve CD4$^+$ cells isolated from mice deficient in TGF-β signaling (CD4dnTGFβRII, **c** and *Tgfbr1*$^{f/f}$CD4-Cre$^+$, **d**). **e,** Th17 cells were generated from naïve T cells as described in **b**. Levels of mRNA for IL-21 and IL-22 are shown as mean (± s.e.m.). **f,** Differentiation of naïve CD4$^+$ T cells with IL-23/IL-6/IL-1β induced higher expression of IL-22, than TGF-β1/IL-6/IL-1β; both cytokine combinations induced equivalent levels of IL-17F. **g,** T cells deficient in TGF-β signaling do not respond to this cytokine as shown by intracellular FoxP3 expression after activation in the presence of TGF-β1 and IL-2 in serum-free media. **h,** *Tgfbr1* expression was determined in IL-23/IL-6/IL-1β/-induced Th17 cells generated from naïve T cells from *Tgfbr1*$^{f/f}$CD4-Cre$^+$ mice or littermate controls.



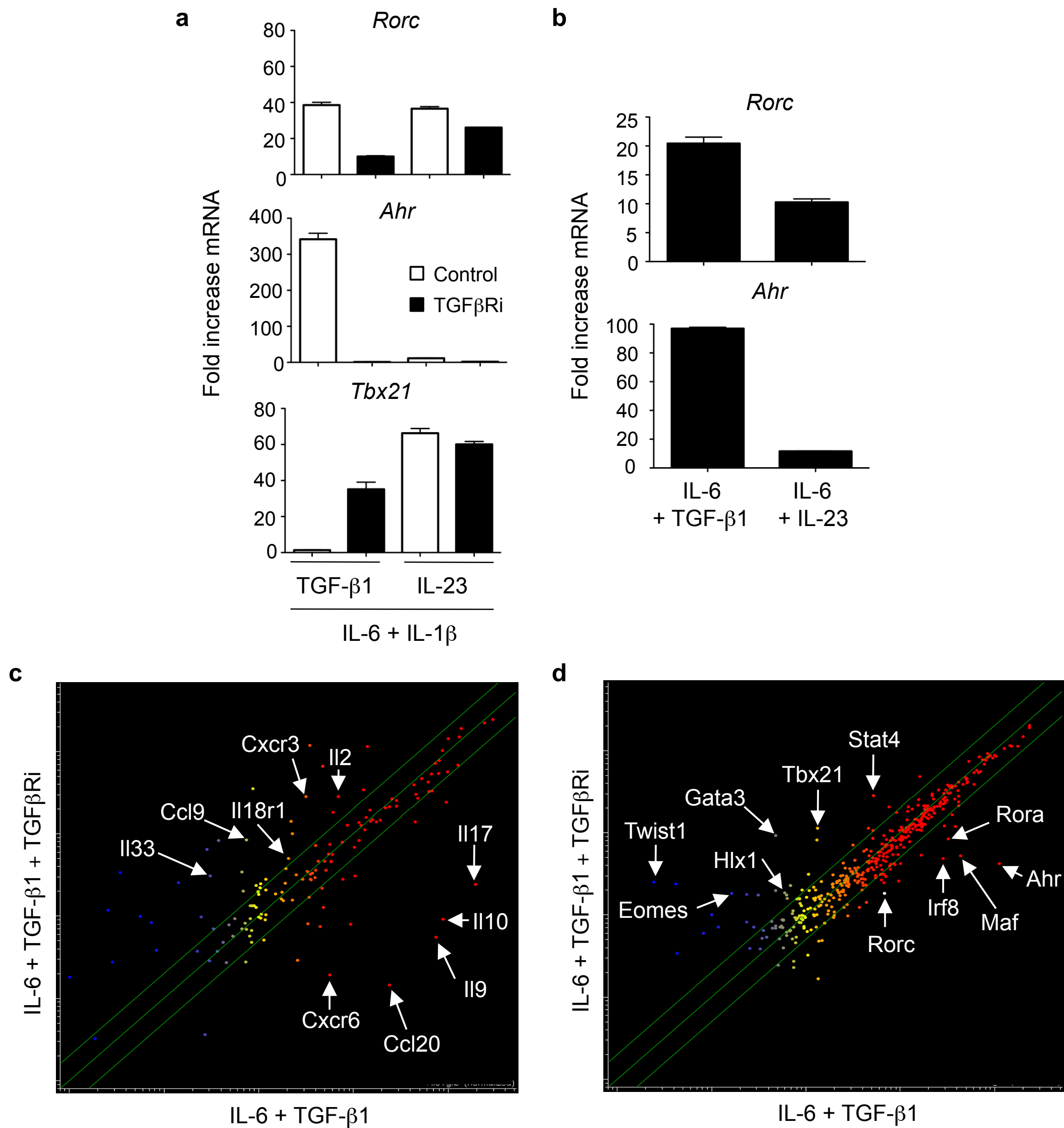
Supplementary Figure 3 IL-23 induces *Il23r* and IL-22 expression in the absence of TGF-β signaling, whereas TGF-β inhibits *Il23r* and IL-22 expression.

a, Sorted CD4^{+} T cells from WT mice (control) or *Tgfbr1*$^{f/f}$CD4-Cre^{+} mice were stimulated for 30 minutes with TGF-β1 (0.5 ng ml^{-1}) in the absence or presence of the TGFβRi as indicated. Whole cell lysates were analyzed for SMAD2 phosphorylation and actin levels by Western blotting. **b,** Sorted CD4^{+} T cells from *Tgfbr1*$^{f/+}$CD4-Cre^{+} (control) and *Tgfbr1*$^{f/f}$CD4-Cre^{+} mice were activated in serum-free media in the absence (control) or presence of TGF-β1. RNA levels (mean ± s.e.m.) of TGF-β1-responsive genes were analyzed after 2 h (Smad7) or 3 days of stimulation with plate-bound anti-CD3/CD28 (*P<0.01, **P<0.001). **c,** IL-23 but not TGF-β1 enhanced IL-23R expression, as shown in media containing 10% FBS. **d,** Naïve CD4^{+} T cells were stimulated in the presence of IL-6 and IL-1β with either TGF-β1, IL-23 or the combination of TGF-β1 and IL-23. IL-17A and IL-22 expression were determined by intracellular staining. **e,f,** IL-23R expression was enhanced by IL-23 in the absence of TGF-β signaling, when using a TGFβRi in serum-free media (**e**), or in naïve T cells from CD4dnTGFβRII mice, activated with IL-6 and TGF-β1 (**f**) (mean ± s.e.m.). **g,** Th17 cells were generated as in **d** with the indicated cytokines. The levels of RORγt and FoxP3 were determined by intracellular staining (control staining-black, transcription factor staining-blue).



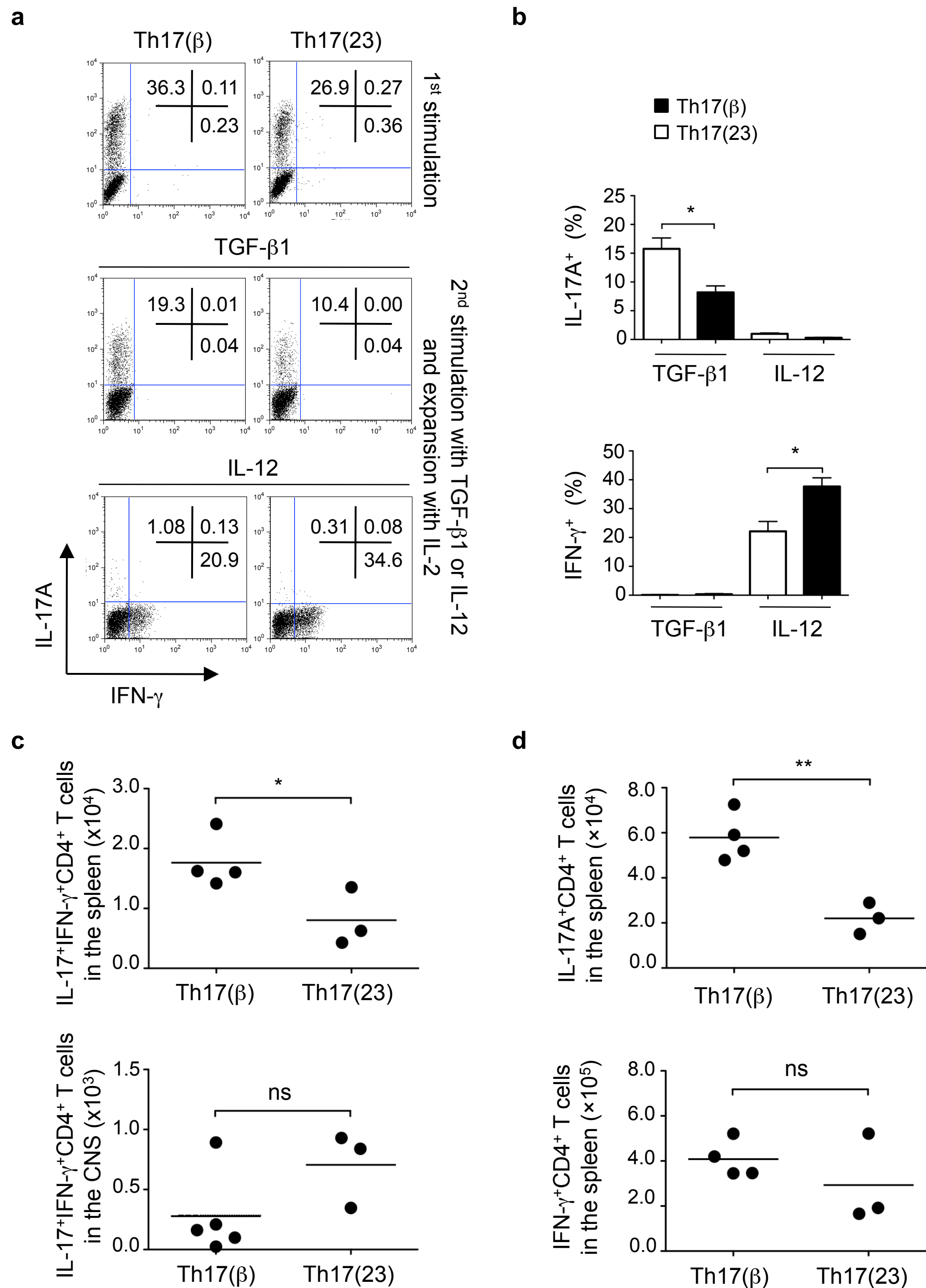
Supplementary Figure 4 Differential global gene expression in IL-23-induced Th17 cells versus TGF-β-induced Th17 cells identifies selective expression of cytokines and receptors.

a, b, Transcriptional profiling of CD4^{+} T cells activated with no cytokines, IL-6 and IL-1β alone or in combination with either IL-23 (Th17(23)) or TGF-β (Th17(β)). Differential expression between Th17(23) cells and Th17(β) cells is depicted as a scatter plot (**a**) and heat map (**b**). **c-e,** Naïve CD4^{+} T cells were stimulated in the presence of IL-6 and IL-1β with either TGF-β1 or IL-23 and plate-bound anti-CD3/CD38 antibodies. TGF-β1 signaling is associated with *Ccl20* transcription (**c**). Th17(23) express *Il33*, *Cxcr3, Il18r1* mRNA and IL-18R1 protein (**d-e**). **f,** Naïve OT-II CD4^{+} T cells were transferred into WT mice that were subsequently immunized with OVA/CFA. IL-18R1 expression of *in vivo* generated Th17 cells was analyzed on day 7 after immunization by flow cytometry. The figure depicts individual mice and the mean of IL-18R1^{+}IL-17A^{+} or IL-18R1^{-}IL-17A^{+} OT-II CD4^{+} T cells.



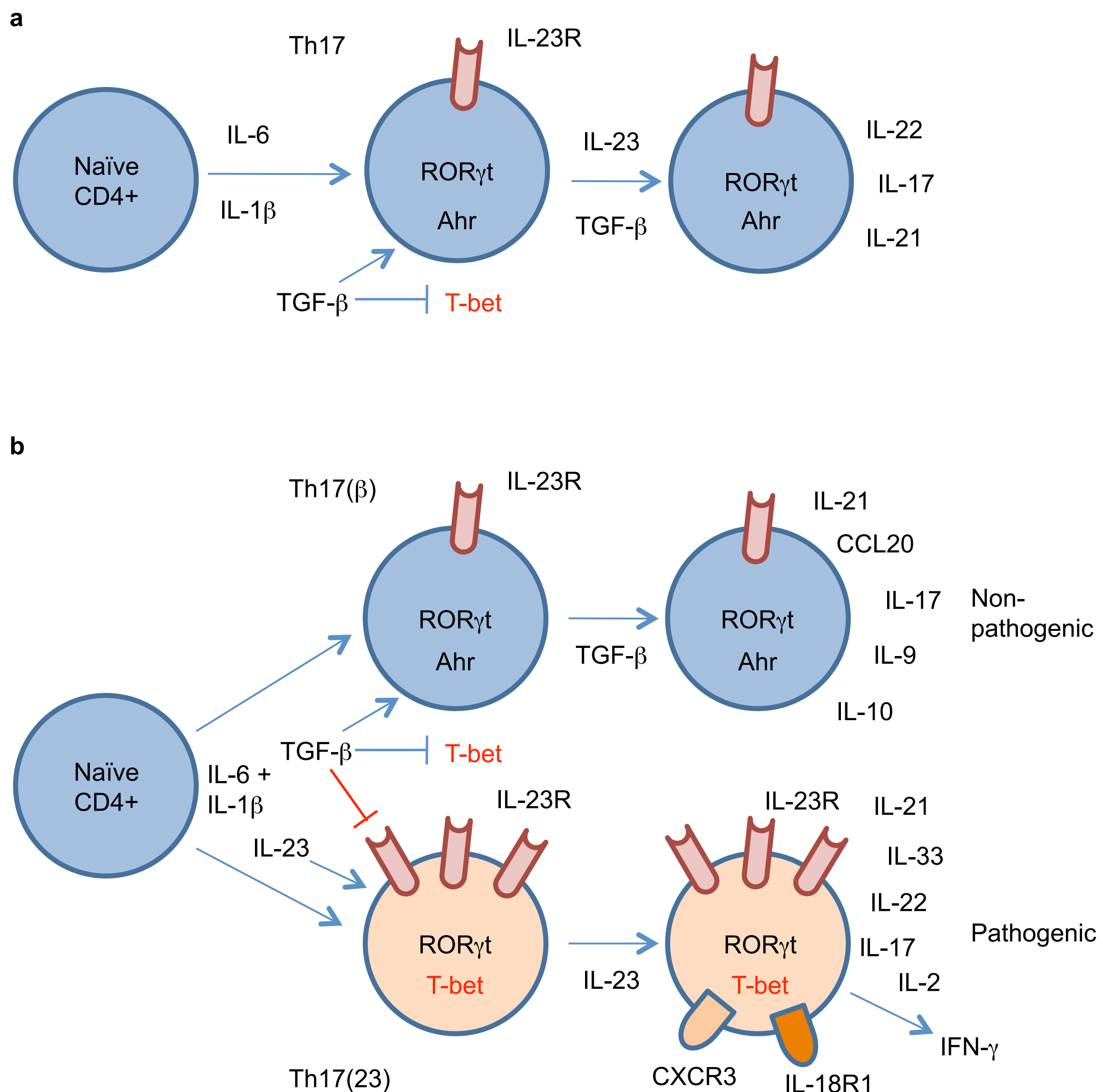
Supplementary Figure 5 Transcription factor profile of IL-23-induced Th17 cells versus TGF-β-induced Th17 cells.

a, b, IL-23 induces *Rorc* mRNA in the absence of TGF-β, but TGF-β signaling is required for *Ahr* transcription. In contrast, *Tbx21* is differentially regulated during Th17 differentiation: Th17 cells express T-bet only in the absence of TGF-β signaling. Similar results have been observed in serum-free media (**a**) as well as in media containing 10% FBS (**b**). RNA levels (mean ± s.e.m.) have been analyzed in Th17 cells differentiated from sorted naïve T cells with the indicated cytokines. **c**, **d,** Microarray analysis shows the differential expression of cytokines and receptors (**c**) or transcription factors (**d**) in Th17(β) cells stimulated in the absence or presence of a TGFβR serine kinase inhibitor (TGFβRi).



Supplementary Figure 6 Th17 cells generated in the absence of TGF-β produce more IFN-γ upon re-activation *in vitro* and *in vivo*.

a, b, Polarized Th17(β) or Th17(23) were analyzed for IFN-γ and IL-17 expression (**a**, top panels). After polarization for three days the cells were expanded with IL-2, restimulated with anti-CD3/anti-CD28 (1 μg ml^{-1}) and either TGF-β1 or IL-12. The cells were then further expanded with IL-2 and analyzed for IFN-γ and IL-17 production by flow cytometry (**a**, middle and lower panels; **b,** mean percentage of $CD4^{+}IFN\text{-}\gamma^{+}$ and $CD4^{+}IL\text{-}17^{+}$ cells in T cell cultures ± s.e.m.; *P<0.05). **c, d,** Th17(β) or Th17(23) polarized TCR(2D2)-transgenic $CD4^{+}$ T cells were adoptively transferred into naïve $Rag2^{-/-}$ mice. Cytokine production of adoptively transferred $CD4^{+}$ T cells isolated from the spleen and CNS of recipient mice was analyzed by intracellular staining. Data shows individual numbers and mean of the two different groups (*P<0.05, **P<0.01).



Supplementary Figure 7 Sequential and alternative models of Th17 differentiation.
a, Sequential model: Naïve Th cells can differentiate into Th17 cells in the presence of IL-6, IL-1β, and IL-23. TGF-β and IL-23 help to maintain the phenotype of Th17 cells during further maturation and proliferation in a sequential process. TGF-β inhibits T-bet expression, making the cells less pathogenic. **b,** Alternative model: RORγt^{+} Th17 cells can be generated by IL-6 and IL-1β in combination with TGF-β or alternatively, together with IL-23 in the absence of TGF-β signaling. While TGF-β-induced Th17 cells express Ahr, Ccl20, IL-9 and IL-10, IL-23-induced Th17 cells express T-bet, Cxcr3, IL-18R1 and can turn into IL-17/IFN-γ double producing cells. The former are not pathogenic, whereas the latter are.