Supplemental Figure Legends

Fig. 1. Foxp3 and RORγt expression in cytokine-stimulated and lamina propria CD4$^+$ T cells. a, Naïve CD4$^+$ T cells were stimulated with anti-CD3/CD28 in the presence of the indicated cytokines for 48 hours, and were then stained with anti-RORγ (red) and anti-Foxp3 (green) mAbs. **b,** CD4$^+$GFP^{int} and CD4$^+$GFP$^-$ lamina propria T cells were isolated from the gut of *RORc(γt)$^{+/gfp}$* mice as in Fig. 1b. Sorted cells were stained with anti-RORγ (red) and anti-Foxp3 (green) mAbs as well as DAPI (blue nuclear staining, omitted in the corner panels). In the GFP$^-$ T cell fraction, rare RORγ$^+$ cells are present that do not express Foxp3. In the GFP^{int} fraction, Foxp3$^+$RORγ$^+$ cells (white arrowheads) can be detected. Representative data from two experiments are shown.

Fig. 2. Th17 cells express Foxp3 during development in vivo. Total small intestinal lamina propria cells were isolated from *Rosa26$^{stop\text{-}YFP/+}$; Foxp3$^{cre/+}$* female mice. Cells were stimulated with PMA/ionomycin in the presence of GolgiStop, and labelled with antibodies specific for cell surface markers. These were then divided into 2 fractions, and fixed/permeabilized with BD fix/perm for intracelluar cytokine staining (**a** and **c**), or eBioscience fix/perm for intranuclear Foxp3 staining (**b**). Intranuclear staining for Foxp3 was performed in a separate tube because this procedure results in loss of YFP protein. The numbers in red indicate the percentage of TCRβ$^+$CD4$^+$ cells that are YFP, Foxp3 or IL-17 positive. **a,** YFP expression in both TCRβ$^+$CD4$^+$ and TCRβ$^+$CD8$^+$ cells. **b,** TCRβ$^+$Foxp3$^+$ cells in the lamina propria are CD4$^+$ and not CD8$^+$. **c,** IL-17-expressing TCRβ$^+$CD4$^+$, but not TCRβ$^+$CD4$^-$ cells, have switched on YFP. Littermate male control

mice (*Rosa26*$^{stop\text{-}YFP/+}$*; Foxp3*$^{+/Y}$) have no YFP expression (data not shown). Representative data from two experiments are shown.

Fig. 3. An shRNA against Foxp3 alleviates the inhibition by TGF-β of RORγt-directed IL-17 production. IL-17 production was measured by intracellular staining in retrovirally-transduced naïve CD4$^+$ T cells cultured in the absence or presence of TGF-β. Cells co-transduced with control MCD2 or RORγt (hCD2 reporter) and shRNA vectors LMP or LMP1066 (GFP reporter) were gated for GFP expression level and IL-17 expression levels were examined within each population. Representative data from three experiments are shown.

Fig. 4. Interaction of Foxp3 and RORγt in co-transfected cells. HeLa cells were transfected with the indicated constructs and localization of ectopically expressed proteins was examined by confocal microscopy. RORγt facilitates nuclear translocation of FKH-deficient Foxp3 only if the Foxp3 exon 2 sequence is intact. The scale bar is 20 μm.

Fig. 5. Human FOXP3 inhibits RORγt-directed IL-17 expression. Naïve CD4$^+$ T cells were co-transduced with retroviruses encoding murine RORγt (MIT vector, Thy1.1 reporter) and various human 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 three experiments are shown.

Fig. 6. Foxp3ΔEx2 suppresses expression of IL-2 and IFNγ in mouse CD4$^+$ T cells. Naïve CD4$^+$ T cells were transduced with retroviral constructs encoding MSCV-IRES-GFP (MIG), WT Foxp3-IRES-GFP (Foxp3 WT), or Foxp3Δexon2 -IRES-GFP (Foxp3ΔEx2). IL-2 and IFNγ expression were measured by intracellular staining 48 hours after transduction. Representative data from at least three experiments are shown.

Fig 7. IL-23 enhances the level of IL-17 expression at low concentrations of TGF-β. **a,** IL-23 enhancement of Th17 cell differentiation at low concentrations of TGF-β. IL-17 expression was examined by intracellular staining in naïve CD4$^+$ T cells after 96 hours of stimulation with the indicated cytokines. The numbers above and below bracket lines in each histogram indicate percent IL-17$^+$ cells in the presence or absence of IL-23, respectively (blue or red). **b,** Naïve CD4$^+$ T cells were stimulated with the indicated cytokines for 96 hours, and mean fluorescence of intensity (MFI) of IL-17$^+$ cells in the indicated cytokine conditions was examined by flow cytometry.

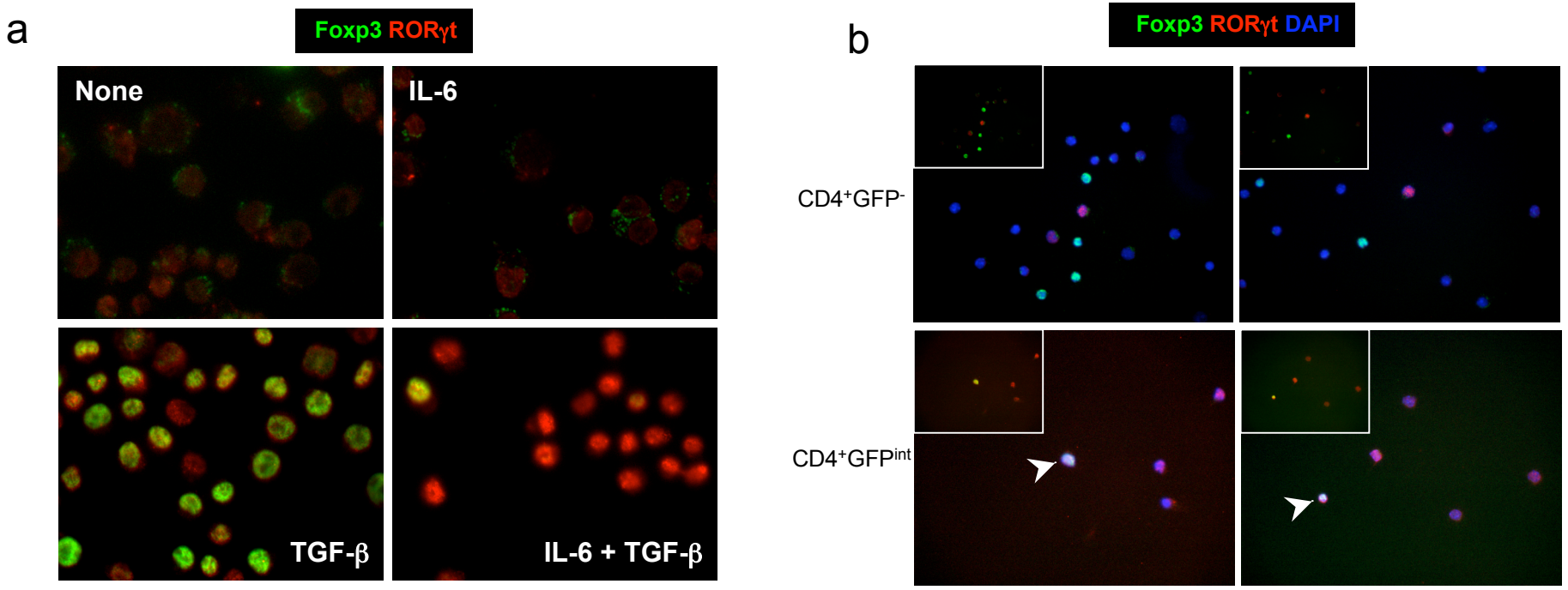
Fig. 8. IL-23/IL-23R signaling inhibits TGF-β-induced Foxp3 expression. a, Naïve CD4$^+$ T cells were transduced with MIG or IL-23R viruses and treated with different cytokines. On day 5 (96 h after adding the indicated cytokines), Foxp3 expression was measured by intracellular staining. **b,** Levels of Foxp3 mRNA after treatment with IL-6 or IL-23. Viral transduction was done exactly as in **(a)**. On day 3 (48 h after adding the indicated cytokines), RNA from transduced GFP$^+$ cells was isolated. Foxp3 expression was measured by real-time RT-PCR and was normalized to the actin expression level.

Error bars represent standard deviation obtained using the standard curve method. Representative data from at least three experiments are shown.

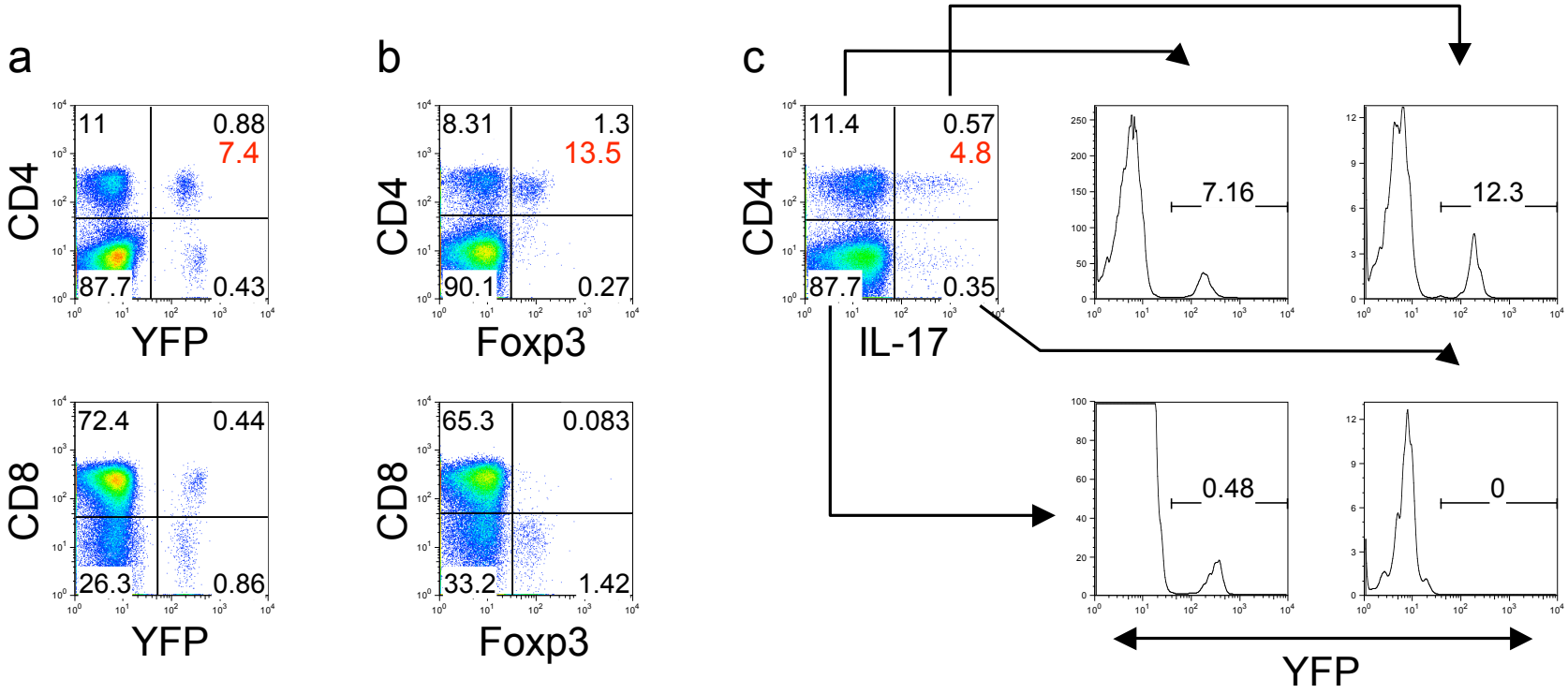
Fig. 9. Foxp3 and IL-17 co-expression in $CD4^+$ T cells stimulated with IL-6 + TGF-β correlates with inhibition of Foxp3 suppressive activity by IL-6 or IL-21. a, Naïve CFSE-labeled $CD4^+$ T cells were stimulated with anti-CD3/CD28 and exogenous hIL-2 in the presence of IL-6 + TGF-β for the indicated times. CFSE dilution is shown and cells were gated based on IL-17 or Foxp3 expression. **b,** Naïve $CD4^+$ T cells were co-transduced as in Fig.3d. After transduction, cells were stimulated with or without IL-6 or IL-21. IL-17 expression was assessed on day 4 in cells gated for expression of both Thy1.1 and GFP. The level of retrovirally transduced-Foxp3 protein and proportion of $Foxp3^+$ cells were similar in the presence or absence of IL-6 or IL-21, as determined by intracellular staining (data not shown). Representative data from three experiments are shown.

Fig. 10. TGF-β concentration governs the Th17:Treg choice. TGF-β induces the expression of both Foxp3 and RORγt in naïve $CD4^+$ T cells, but the function of RORγt is inhibited by Foxp3. Maximal expression of IL-23R is induced synergistically by low concentrations of TGF-β, together with proinflammatory cytokines (IL-6/IL-21/IL-23), which inhibit the expression and activity of Foxp3 and therefore favor Th17 cell differentiation. Maximal expression of Foxp3 is induced by high concentrations of TGF-β, which inhibits IL-23R, IL-22, and possibly IL-17 expression and therefore promotes Treg cell differentiation.

Supplementary Figure 1

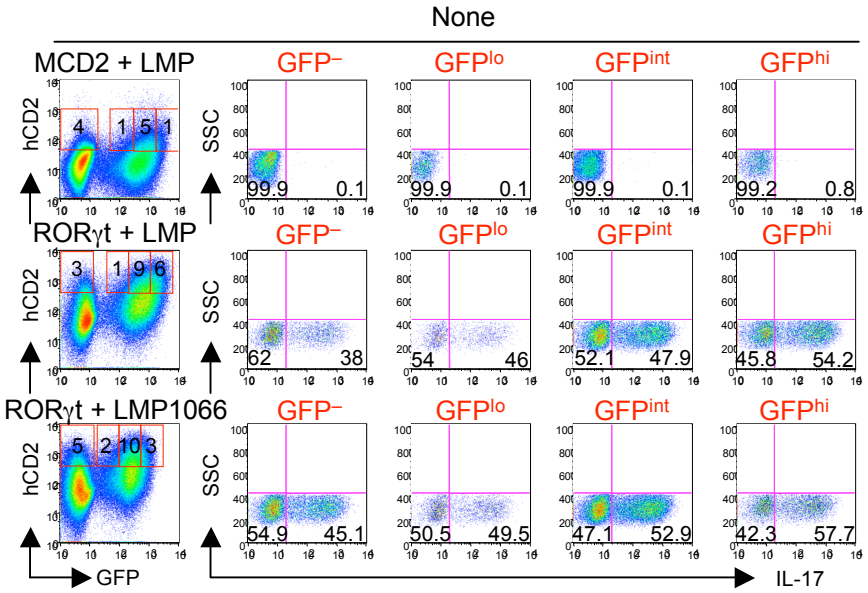


Supplementary Figure 2



Supplementary Figure 3

a



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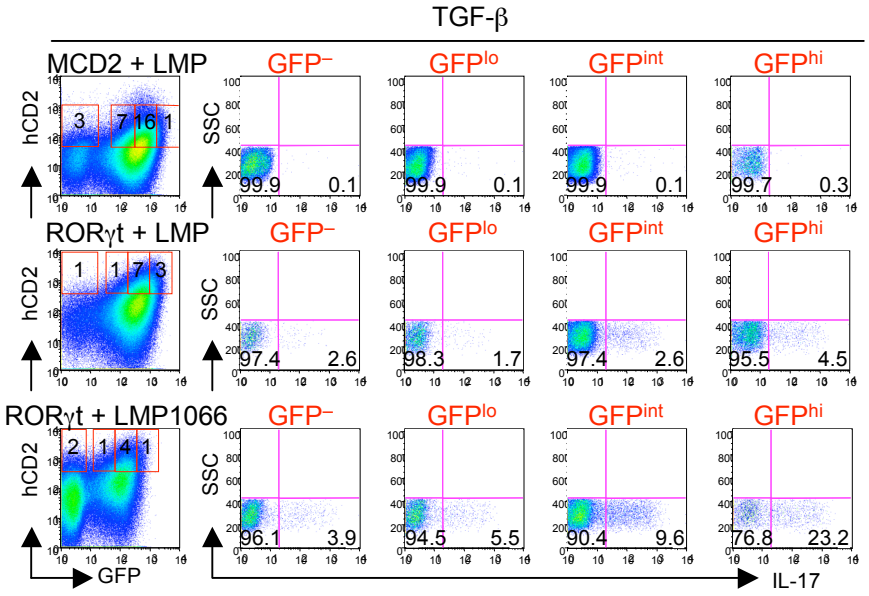

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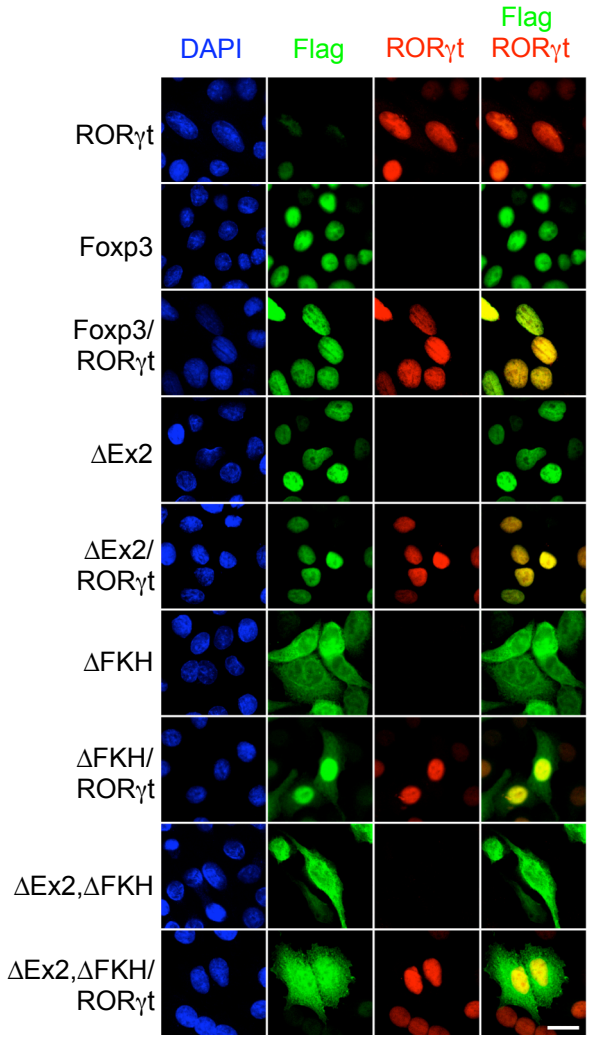

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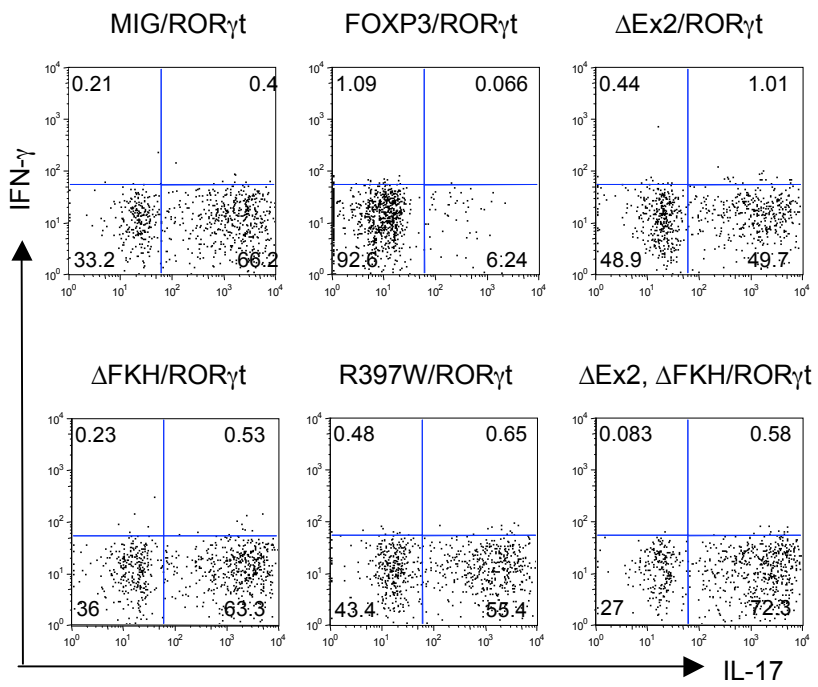
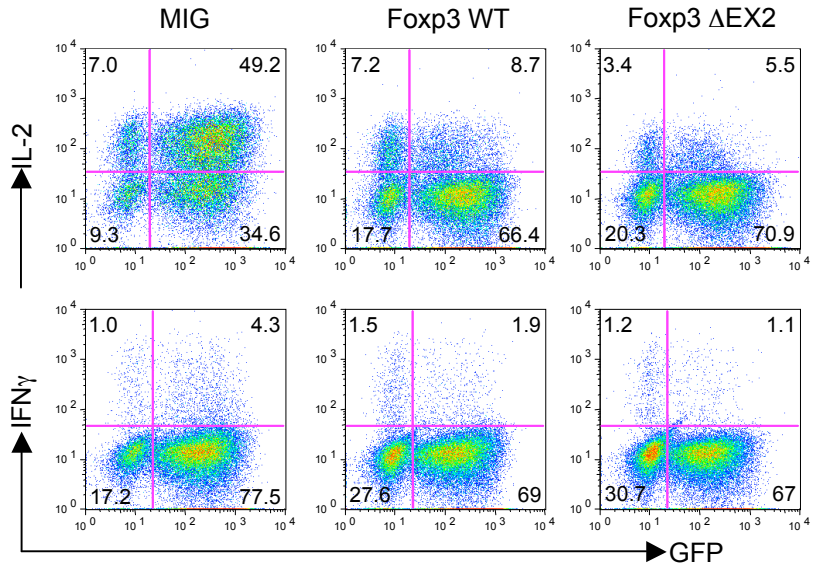
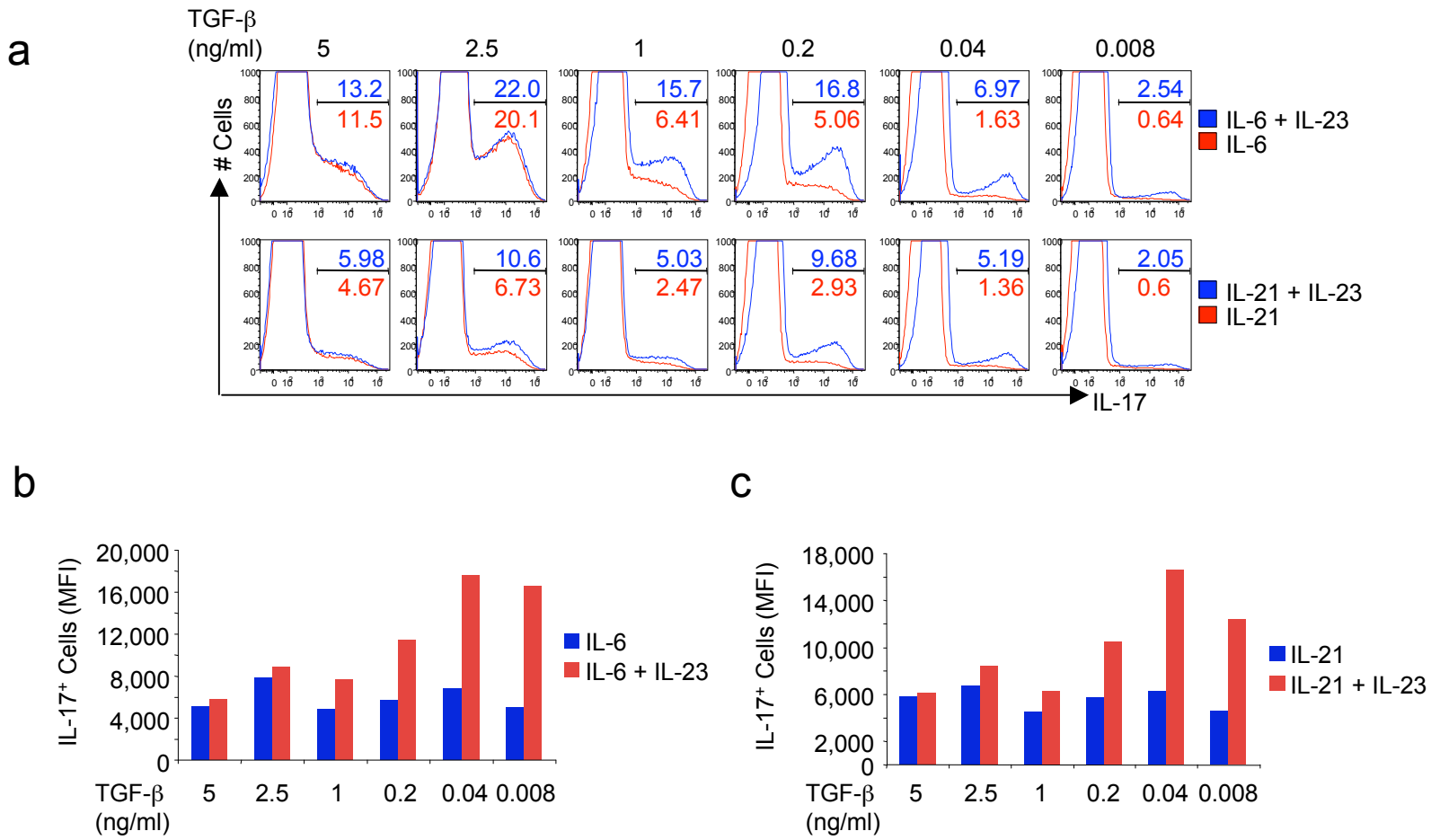


Supplementary Figure 6

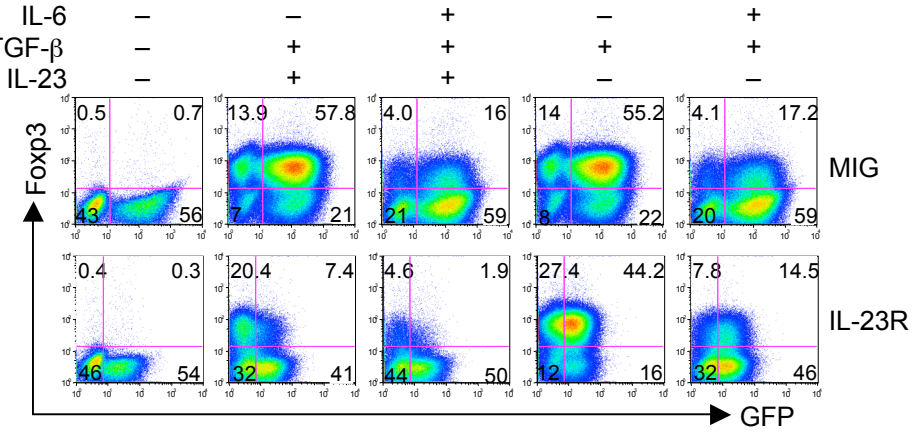


Supplementary Figure 7



Supplementary Figure 8

a



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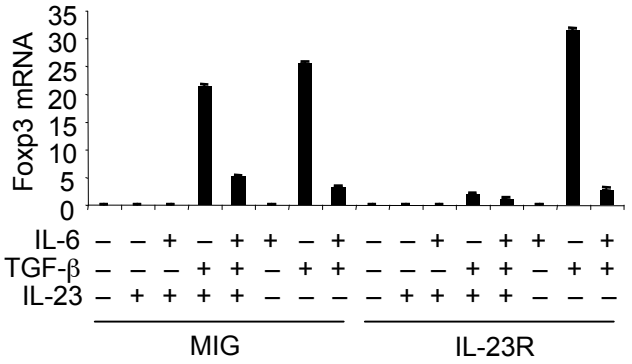
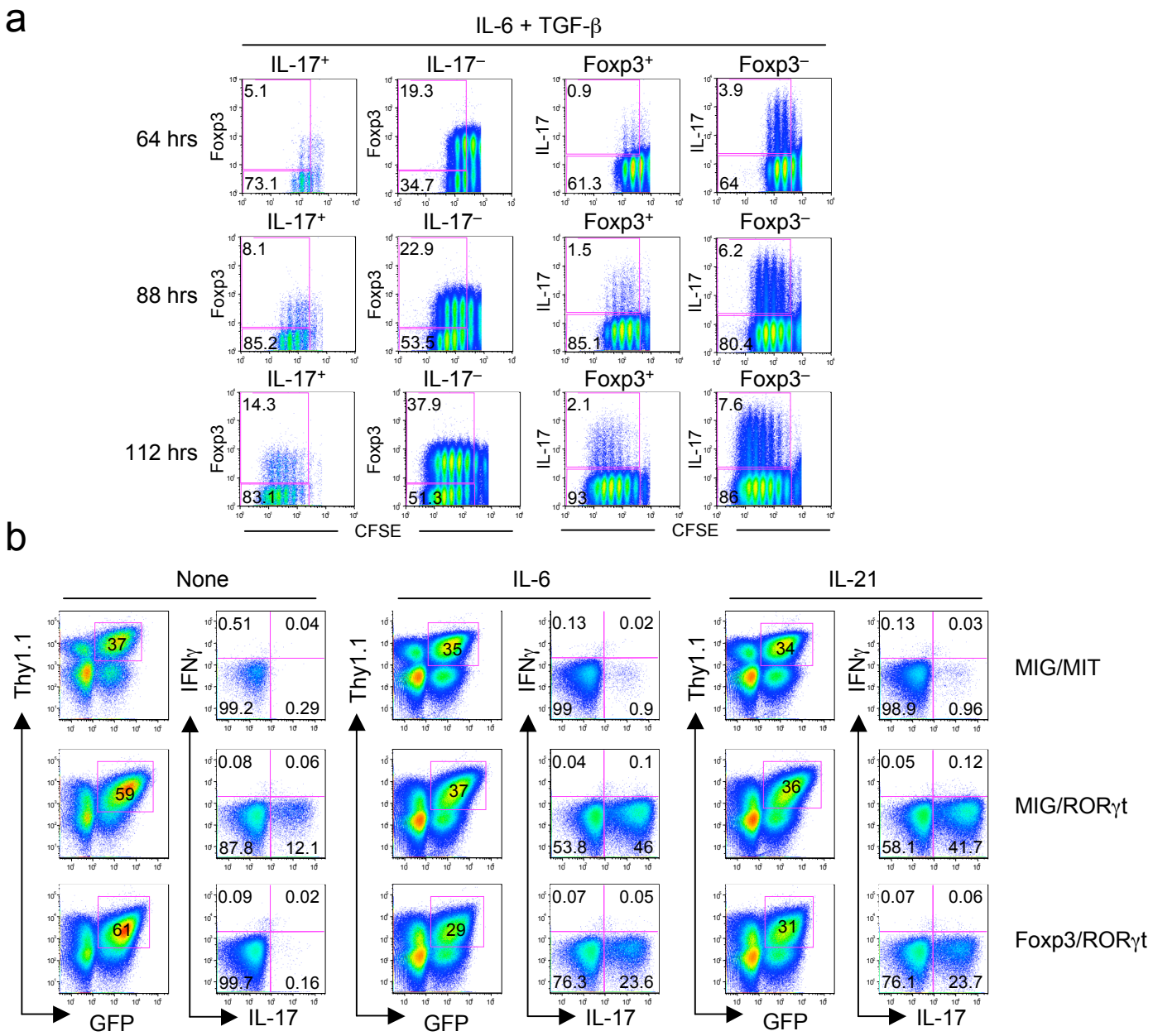


Supplementary Figure 9



Supplementary Figure 10