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The alarmins IL-1 and IL-33 differentially regulate the functional specialisation of Foxp3$^+$ regulatory T cells during mucosal inflammation

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CD4$^+$Foxp3$^+$ regulatory T (T_{REG}) cells are critical mediators of peripheral tolerance and modulators of immune responses. Functional adaptation of T_{REG} cells, through acquisition of secondary transcription factors is critical for their effector differentiation towards local inflammatory stimuli including infections. The drivers and consequences of this adaptation of T_{REG} cell function remain largely unknown. Using an unbiased screen, we identified receptors of the IL-1 family controlling the adaptation of T_{REG} cells. Through respiratory infection models, we show that the IL-33 receptor (ST2) and the IL-1 receptor (IL1R1) selectively identify stable and unstable T_{REG} cells at mucosal surfaces, respectively. IL-33, not IL-1, is specifically required for maintaining the suppressive function of T_{REG} cells. In the absence of ST2, T_{REG} cells are prone to lose Foxp3 expression and acquire RORγT and IL1R1, while, in the absence of IL-1R1, they maintain Foxp3 expression and resist the acquisition of a Th17 phenotype. Finally, lack of IL-1 signalling enhances the accumulation of ST2$^+$ T_{REG} over pro-inflammatory T_{REG} cells in a *Cryptococcus neoformans* infection. These observations show that IL-1 and IL-33 exert opposing functions in controlling the functional adaptation of T_{REG} cells, ultimately dictating the dynamics of adaptive immunity to pathogens.

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INTRODUCTION

The exposure to environmental stimuli causes mucosal surfaces to develop local mechanisms to maintain homeostasis at steady-state conditions, all-the-while mounting strong immune responses when required. CD4$^+$Foxp3$^+$ regulatory T (T_{REG}) cells are critical mediators of peripheral immune self-tolerance and modulators of immune responses directed towards a spectrum of self and non-self antigens.[1,2] Foxp3 is a lineage-specifying transcription factor essential for the development and function of T_{REG} cells. Induction and sustained expression of Foxp3 defines the core transcriptional programme of T_{REG} cells, establishing their phenotype and suppressive function, as well as regulating key cell-intrinsic, homeostatic processes, including cytokine signalling pathways.[3] Inheritable mutations of the *foxp3* gene result in the onset of a severe autoimmune disease in scurfy mice and the immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) in humans.[4,5]

In addition to secondary lymphoid organs, Foxp3$^+$ T_{REG} cells are also abundant in non-lymphoid tissues, particularly mucosal surfaces, where they contribute to maintain immune homeostasis.[6,7] Local inflammatory signals can force CD4$^+$ T cells to undergo significant functional plasticity in order to acquire specialised effector functions in situ, and adapt to the evolving nature of immune responses, particularly during infections.[8] Growing evidence indicates that Foxp3$^+$ T_{REG} cells can also acquire tissue-specific adaptations that promote their local homeostasis and function.[9,10] We and others have previously shown that the stability of Foxp3 expression and T_{REG} cell function is a dynamic process dictated by the inflammatory environment. Settings of lymphopenia, chronic inflammation, or infections favour the lineage and functional plasticity of Foxp3$^+$ T_{REG} cells by downregulating Foxp3 expression, compromising suppressive function, and reprogramming them into IFNγ- or IL17-producing-like *ex*Foxp3$^+$ T cells which, in turn, promote potent effector immune responses in the host.[11] Recently, studies have shown that Foxp3$^+$ T_{REG} cells can also co-express lineage-defining transcription factors of their target T_{EFF} cells such as T-bet, RORγT or GATA3, allowing T_{REG} cells to localise and suppress the associated CD4$^+$ T-helper (Th) cell immune responses.[12–14] For instance, T_{REG}-mediated suppression of Th17 responses is dependent on CCR6, a characteristic chemokine receptor of Th17 cells, and STAT3, a signal transducer and activation of transcription downstream of IL-6 and IL-23.[15] Moreover, T_{REG} cells from the lamina propria adapt to the local microbial environment

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of the gut by expressing the Th17-associated transcription factor RORγT, a feature that confers suppressive function during gut-specific immune responses.[16] Similarly, CXCR3$^+$T-bet$^+$ T_{REG} cells suppress Th1 cell responses,[17,18] while GATA3$^+$ T_{REG} cells were identified in the lung and gut of healthy mice[19] and accumulated more readily during Th2-mediated inflammation.[20] Thus, inflammatory cues can modulate the epigenetic and transcriptional landscape of Foxp3$^+$ T_{REG} cells, forcing them to specialise their functions for a context-dependent adaptation to evolving immune responses.

By applying an unbiased screen to uncover the mechanistic events leading to a downregulation of Foxp3 expression in T_{REG} cells, we identify a unique role of the IL-1 family of cytokines in modulating the reprogramming potential of T_{REG} cells during inflammation. We show that cell surface expression of the IL-33R (ST2, *Il1rl1*) identifies a subset of functionally stable Helios$^+$Foxp3$^+$ T_{REG} cells that are resistant to plasticity and loss of suppressive function, whereas IL1R1 (*Il1r1*) expression identifies RORγT$^+$ Helios$^-$ T_{REG} cells that acquire a Th17 cell phenotype and fail to suppress. While a Th17 polarizing milieu induces IL-1R1 expression on T_{REG} cells, IL-33 favours the stability of the Helios$^+$ST2$^+$ T_{REG} cell phenotype in similar conditions. Importantly, ST2$^+$ T_{REG} cells accumulate early in the lungs upon infection with influenza virus or the fungal pathogen *Cryptococcus neoformans*, and segregates suppressive from inflammatory T_{REG} cells throughout the course of infection. Abrogation of IL-33, but not IL-1 signalling, in T_{REG} cells compromises their suppressive function and fuels inflammatory responses in vivo. Importantly, lack of IL-1 signalling alters the dynamics of ST2$^+$ and RORγT$^+$ Foxp3$^+$ T_{REG} populations in the lung, and augments susceptibility to fungal infection. Overall, we show that IL-1 and IL-33 exert opposite roles in controlling the adaptation and functional specialisation of Foxp3$^+$ T_{REG} cells at mucosal surfaces.

RESULTS

Differential IL-33R (ST2) and IL1R1 expression distinguishes functionally stable from unstable T_{REG} cells

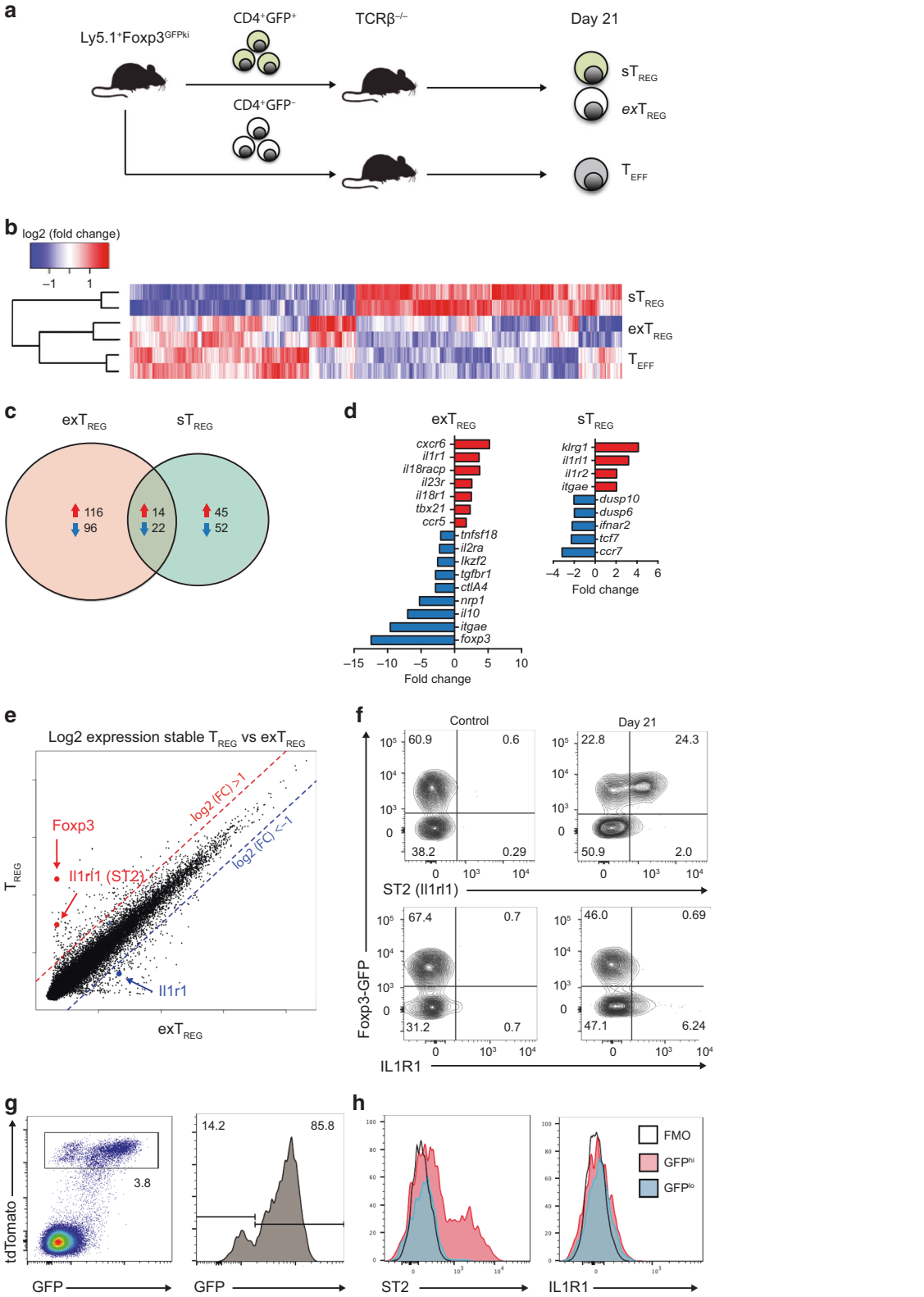
To delineate the mechanistic basis of functional plasticity of T_{REG} cells in vivo, we exploited a Foxp3$^+$ T_{REG} cell transfer model in lymphopenic hosts that we previously described.[11] Specifically, Foxp3$^+$ T_{REG} (GFP$^+$) cells from Ly5.1$^+$ Foxp3^{GFPki} reporter mice were FACS-purified and transferred into TCRβ$^{-/-}$ Ly5.2$^+$ recipient mice. After 21 days, T_{REG} cells that preserved Foxp3 expression (GFP$^+$, stable T_{REG} [sT_{REG}]) and T_{REG} cells that lost Foxp3 expression (GFP$^-$, unstable T_{REG}, termed *ex*T_{REG} cells) were isolated based on GFP expression from donor Ly5.1$^+$ CD4$^+$ splenocytes, and subjected to transcriptional microarray analysis. Donor Ly5.1$^+$CD4$^+$GFP$^-$ T_{EFF} cells were also FACS-isolated at day 21 from mice that received only T_{EFF} (Ly5.1$^+$CD4$^+$GFP$^-$) cells, thus devoid of any Foxp3-expressing cells at day 0 (Fig. 1a). Freshly sorted CD4$^+$ GFP$^-$ and GFP$^+$ T cells from Foxp3^{GFPki} reporter mice were also included in the microarray analysis as control. Hierarchical cluster analysis of the top 654 genes ($p < 0.05$) identified between T_{REG}, *ex*T_{REG} and activated T_{EFF} cells reveals a closer relationship between *ex*T_{REG} cells and activated T_{EFF} cells compared to stable T_{REG} cells (Fig. 1b). We observed that the majority of genes that are significantly modulated upon transfer (>1 log2 fold change) are not shared between sT_{REG} and *ex*T_{REG} cells (Fig. 1c). We observed that *ex*T_{REG} cells down-regulated some of the major T_{REG} signature genes (Ctl4, IL-10 and Nrp1) and up-regulated genes associated with Th1 and Th17 T cells (Tbx21 and *Il23r*; Fig. 1d). Interestingly, the mRNA levels of IL-33 receptor (*Il1rl1*, IL-33R, ST2) were significantly higher in T_{REG} cells that maintained Foxp3 (sT_{REG}) compared to *ex*T_{REG} and T_{EFF} cells. In contrast, mRNA expression of IL-1 receptor (*Il1r1*) was significantly reduced in stable T_{REG} cells when comparing with *ex*T_{REG} (Fig. 1e). Analysis of cell surface-bound ST2 expression by flow cytometry confirmed findings of increased mRNA levels, demonstrating that a subset of sT_{REG} cells readily expressed ST2 after transfer (Fig. 1f). In order to further confirm these results, we transferred total CD4$^+$ T cells from the fate-mapping Foxp3$^{GFP\text{-}Cre}$ X Rosa26-lox-stop-lox tdTomato mice[21] into TCRβ Ly5.1$^+$ mice (Fig. 1g, h; S1). Again, we observed a difference in the surface expression of ST2 between stable (tdTomato$^+$GFP$^+$) vs. *ex*T_{REG} cells (tdTomato$^+$ GFP$^-$) in the spleen (Fig. 1g, h; S1). We also observed a tendency towards higher expression of the IL1R1 in the *ex*T_{REG} when compared to sT_{REG} cells (S1). These results illustrate that differential expression of ST2 and IL1R1 segregates distinct subsets of T_{REG} cells with divergent effector outcomes.

ST2 expression delineates stable T_{REG} cells from IFNγ- and IL-17A-producing T_{REG} cells during viral infection

We hypothesised that ST2$^+$ T_{REG} cells increase in an inflammatory microenvironment that harbours elevated levels of IL-33, an alarmin induced by infectious pathogens particularly at mucosal surfaces.[22] To test this hypothesis, we examined the functional dynamics of ST2$^+$ T_{REG} cells in the context of a type-1 immune-driven respiratory viral infection in vivo. Infection with Influenza A H1N1/PR8 induces pulmonary expression and release of IL-33 early during the course of disease.[23,24] Infected mice demonstrated an increase in pulmonary CD4$^+$ T cells that coincided with the peak of weight loss (Fig. 2a). ST2$^+$ T_{REG} cell numbers in the lungs were found to increase starting by day 4 and to peak at day 10 post-infection (Fig. 2b–d). Interestingly, the increase in ST2$^+$ T_{REG} cells during the course of infection was seen only in lungs of infected mice (Fig. 2e), suggesting that IL-33 acts locally in the lung mucosal environment. Since Foxp3$^+$ T_{REG} cells homing to inflammatory sites have the potential to adopt an inflammatory phenotype by co-expressing RORγT as well as IL-17A or IFNγ,[11,25] we determined whether ST2 expression by T_{REG} cells correlated with expression of these inflammatory phenotypes. We observed that at peak infection the production of IFNγ and IL-17A originated exclusively from pulmonary ST2$^-$ T_{REG} and ST2$^-$ T_{EFF} cells (Fig. 2f–h). Similarly, the proportions and absolute cell numbers of IFNγ and IL-17A secreting Foxp3$^+$ T_{REG} cells increased during peak infection and were restricted to the ST2$^-$ cell subset (Fig. 2g, h). These data show that IL-33-responsive (ST2$^+$) T_{REG} cells do not secrete inflammatory cytokines and represent a stable population of T_{REG} cells during influenza virus infection.

IL-1 inhibits ST2 expression on Helios$^+$ T_{REG} cells

To further detail the nature and origin of ST2$^+$ T_{REG} cells, we assessed their phenotype in secondary lymphoid organs (pLN) as well as mucosal tissues (colon) of naïve C57BL/6 mice. We observed a significant frequency of ST2$^+$ T_{REG} cells in the colonic lamina propria that is not observed in pLN (Fig. 3a). At steady state, ST2$^+$ T_{REG} cells possess a memory profile (S2), a result consistent with a study by Schiering et al.[19] Spleen-derived ST2$^+$ T_{REG} cells consistently expressed high levels of Nrp1, Helios and TIGIT, a phenotype reflecting thymic origin (Fig. 3b). Furthermore, thymic-derived CD4^{SP} Foxp3-GFP$^+$ T cells upregulate the ST2 receptor,[26] and also maintain high levels of Helios expression (S3A-B). We confirmed that IL-33 was sufficient to induce the expression of ST2 and the transcription factor GATA3 in T_{REG} cells (S3C).[19,26] Moreover, this induction in ST2 expression correlated with the accumulation of Helios$^+$ T_{REG} cells in IL-33 treated cultures (Fig. 3c, d), and an increase in CD25 and Foxp3 expression in ST2$^+$ T_{REG} cells (Fig. 3e, f). As IL-33 enhanced the proliferative capacity of T_{REG} cells,[27] but did not affect the suppressive ability of T_{REG} cells in vitro (S4), we then hypothesised that the specific accumulation of Helios$^+$ T_{REG} cells could lead to an overall increase in a suppressive environment at the mucosa, as Helios was recently associated with increased suppressive function in T_{REG} cells.[28] Interestingly, upon transfer of GFP$^+$ (Foxp3$^+$) T cells in TCRβ$^{-/-}$ C57BL/6 recipient mice,



stable Helios^{+} T_{REG} cells expressed ST2 (Fig. 3g, green panel), whereas Helios^{-} T_{REG} cells showed increased IL1R1 expression (Fig. 3g, orange panel). Analysis of ST2 expression on de novo generated GFP^{+} (pT_{REG}) cells from mice that received only GFP^{-} T cells revealed that pT_{REG} cells, in contrast to T_{REG} cells, fail to upregulate the ST2 receptor (Fig. 3h). This was further confirmed in vitro, as TGFβ generated iT_{REG} cells did not upregulate ST2 in the presence of IL-33 (S5).

As IL1R1 was preferentially expressed in unstable T_{REG} cells with reprogramming potential, we further assessed ST2 expression in T cells from mice lacking IL1R1 (IL1R1$^{-/-}$; Fig. 3j–l). Importantly, we observed an increase in the frequencies of ST2^{+} Helios^{+} T_{REG}

Fig. 1 Identification of ST2 as a marker of functionally stable T_{REG} cells. **a** $CD4^+GFP^+Ly5.1^+$ T cells from $Foxp3^{GFPki}$ C57BL/6 mice were FACS-sorted and adoptively transferred into $TCR\beta^{-/-}$ mice. The cells were harvested 21 days post-transfer based on GFP expression and microarray analysis (see Methods) was performed (splenocytes of $TCR\beta^{-/-}$ mice were isolated and pooled before sorting the distinct populations; Two separate T cell transfers were performed, the cells isolated and processed individually for microarray analysis). The top 654 genes that varied ($p < 0.05$ cut-off) between the groups are shown. **b** Transcriptional signatures of stable T_{REG} (sT_{REG}), exT_{REG} and T_{EFF} cells isolated at day 21 post transfer. **c** Venn diagram showing the distribution of differentially expressed genes (cut-off of >1 log2 fold change) between exT_{REG} and sT_{REG} relative to T_{REG} ($CD4^+GFP^+$) from day 0. **d** Highest ranking (>2 fold change) genes in exT_{REG} and sT_{REG} at day 21 relative to T_{REG} cells from day 0. **e** Pearson correlation of log2 fold change gene expression directly comparing sT_{REG} cells and exT_{REG} cells identifying the differential expression of *Il1r1* (blue) and *Il1rl1* (red). Dotted lines show ±1 log2 fold change. **f** Representative flow cytometry profile of surface expression of IL1RL1 (IL-33R, ST2) (top) and IL1R1 (bottom) in $CD4^+CD45.1^+$ T cells in the spleen at day 21 after transfer ($N = 4$); Controls are fluorescence minus one (FMO). **g**, **h** $CD4^+$ T cells from $Foxp3^{GFP\text{-}CRE}$ $Rosa26^{RFP}$ fate-tracking mice were sorted and adoptively transferred (i.p.) into a TCRβ $Ly5.1^+$ mouse, and examined 10 days post-transfer ($N = 4$). **g** (Left) Representative plots showing frequencies of GFP^+RFP^+ (Foxp3+; sT_{REG}) and GFP^-RFP^+ (exT_{REG}) cells in $CD4^+CD45.2^+$ (right panel). Cut-off population of GFP^{hi} and GFP^{lo} T cells. **h** Representative histograms of expression levels of ST2 (left panel) and IL1R1 (right panel) between GFP^{hi} (red) and GFP^{lo} (blue) $CD4^+CD45.2^+$ RFP^+ T cells

cells at the steady-state in the lungs of $IL1R1^{-/-}$ mice when compared to WT mice (Fig. 3j, k). Moreover, we show that $ST2^+$ T_{REG} cells from $IL1R1^{-/-}$ mice expressed higher levels (MFI) of ST2 than their counterparts from WT mice (Fig. 3l) suggesting that IL-1 inhibits the expression of ST2 in T_{REG} cells. Finally, we show that ST2 and IL1R1 are differentially expressed among T_{REG} cells at the resting state in the lung of BALB/c mice (Fig. 3m, n). Overall, these results illustrate that subsets of T_{REG} cells possess the ability to respond to either IL-1 or IL-33 at mucosal sites, and that IL-1 signalling regulates ST2 expression in T_{REG} cells.

Absence of IL-33-, but not IL-1-, signalling converts $Helios^+T_{REG}$ cells to a pro-inflammatory phenotype that fuels intestinal inflammation

To determine whether IL-1 or IL-33 signalling affected the stability and suppressive function of T_{REG} cells in vivo, we exploited the $CD4^+$ T_{EFF} cell-induced model of intestinal inflammation (colitis). Specifically, we transferred T_{EFF} cells alone ($Thy1.1^+$, $CD90.1^+$) or in the presence of WT, $ST2^{-/-}$ or $IL1R1^{-/-}$ T_{REG} cells ($Thy1.2^+$, $CD90.2^+$) into SCID/beige mice and monitored the dynamics of T_{REG}/T_{EFF} responses relative to colitis development (Fig. 4a). In contrast to mice receiving T_{EFF} cells alone ($Thy1.1^+$), all mice receiving T_{REG} cells did not display significant weight loss by day 28 post-transfer (Fig. 4b). SCID/beige mice that received $ST2^{-/-}$ T_{REG} cells displayed a higher colitis score than the ones with WT T_{REG}, which confirmed the results from Schiering and colleagues.[19] Strikingly, we show that mice that received $IL1R1^{-/-}$ T_{REG} effectively prevented colitis onset by day 28 (Fig. 4c). Concomitantly, mice co-transferred with $ST2^{-/-}$ T_{REG} cells showed significantly increased numbers of $CD11b^+Ly6G^+$ neutrophils in the colon compared to mice receiving WT or $IL1R1^{-/-}$ T_{REG} cells (Fig. 4d). In addition, $IL1R1^{-/-}$, but not $ST2^{-/-}$, T_{REG} fully inhibited the accumulation of IL17A-producing T_{EFF} cells in the colon highlighting the lack of suppressive ability of T_{REG} cells in the colon when IL-33, but not IL-1, signalling is abrogated in T_{REG} cells (Fig. 4e). Moreover, mice co-transferred with $IL1R1^{-/-}$ T_{REG} cells show enhanced frequencies of $Foxp3^+$ T_{REG} cells in the colon and mesLN, but not in the spleen, compared to mice that received WT or $ST2^{-/-}$ $Foxp3^+$ T_{REG} cells (Fig. 4f; S6). $IL1R1^{-/-}$ T_{REG} cells expressed higher levels of ST2, and this coincided with increased Helios expression in the colon (Fig. 4g, h). Consistently, mice that received $IL1R1^{-/-}$ T_{REG} cells also showed increased numbers of $ST2^+$ T_{REG} cells and reduced numbers of $ROR\gamma T^+IL\text{-}17A^+T_{REG}$ cells, compared to mice that received WT or $ST2^{-/-}$ T_{REG} cells (Fig. 4i, j). Strikingly, a greater number of IL-17A producing T_{REG} cells that lost Foxp3 expression (exT_{REG}) were found in mice that received $ST2^{-/-}$ T_{REG} cells in contrast of recipients of WT or $IL1R1^{-/-}$ T_{REG} cells (Fig. 4k). Importantly, the increased numbers of colonic neutrophils positively correlated with the increased frequencies of $ROR\gamma T^+IL17A^+$ T_{REG} cells in all groups (Fig. 4l). Finally, we assessed if we could prevent T_{REG} cell reprogramming in vivo, as determined by the production of IL17A and expression of RORγT in T_{REG} cells by IL-33 supplementation. To this end, as described in Fig. 1, we adoptively transferred 2×10^5 $Ly5.1^+CD4^+GFP^+$ ($Foxp3^+$) T_{REG} cells i.v. into $TCR\beta^{-/-}$ mice, and injected rIL-33 i.p. (100 ng/mouse) every 48 h. 14 days after the T_{REG} transfer, we observed a significant ($p < 0.05$) reduction in the frequencies of $IL17A^+$ T cells and an increase in the accumulation of $IL17A^+$ exT_{REG} cells (S7A-B). Concomitantly, we observed a trend towards reduced expression of the transcription factor RORγT in $Foxp3^+$ T cells and a significant increase in ST2 expression (S7C-D). These results highlight that IL-1 and IL-33 exert opposite functions in controlling adaptation and functional specialisation of T_{REG} cells during intestinal inflammation.

Th17 polarizing conditions induce IL-1R1 expression on T_{REG} cells and promote their proliferation

Our results show that lack of IL-33 signalling in T_{REG} cells compromises their function, while IL-1R1 deficiency favours T_{REG} cell responses highlighting the inhibitory role of IL-1 for the stability and function of T_{REG} cells. We hypothesised that IL-1R1 expression could promote RORγT expression in T_{REG} cells. We then examined the effects of IL-1β on the phenotype and function of T_{REG} cells in vitro by establishing a mixed culture of $Ly5.2^+$ T_{REG} and $Ly5.1^+$ T_{EFF} cells under neutral or Th17 differentiated conditions (Fig. 5a). Indeed, under Th17 polarizing conditions, $Foxp3^+$ T_{REG} cells readily express RORγT, which is undetectable when cultured in control medium (Fig. 5b). Moreover, $ROR\gamma T^+$-$Foxp3^+$ T cells expressed higher levels of IL-1R1 than medium-treated T_{REG} and $ROR\gamma T^+$ T_{EFF} cells (Fig. 5c, d). Inclusion of IL-1β to the Th17 polarizing conditions was critical for enhanced proliferation and survival of T_{REG} cells in vitro (Fig. 5e, f). Consistently, Anakinra, a potent IL-1R antagonist (Kineret®), significantly blocked the proliferation of T_{REG} cells in Th17 polarizing conditions, suggesting a specific role for IL-1β in the expansion and/or survival of $ROR\gamma T^+$ T_{REG} cells (Fig. 5e, f; S8). This feature was not due to an indirect effect of IL-1β on T_{EFF} cells, as replacing WT with $IL1\text{-}R1^{-/-}$ T_{REG} in this assay impaired the proliferation of these cells upon IL-1β stimulation (S8C-D). Furthermore, we observed that RORγT expression was highest in $Helios^-$ T_{REG} cells, which coincided with the expression of IL-1R1 (S8B). Finally, $IL\text{-}1R1^+$ T_{REG} cells displayed a diminished suppressive ability on in vitro differentiated Th17 cells in the presence of IL-1β (Fig. 5g), an observation not due to a reduction in the frequency of T_{REG} in this culture (S9). Thus, Th17 polarizing conditions skew the functional adaptation of T_{REG} cells and modulate their responsiveness to IL-1β.

IL-33 favours the stability of $Helios^+ST2^+$ T_{REG} cells in Th17 polarizing conditions

We showed that $ST2^+$ T_{REG} cells are robust suppressors and refractory to plasticity. We then determined whether $ST2^+$ T_{REG} cells maintain this resistance and their phenotype even in Th17 polarizing conditions. To this end, we TCR-activated $ST2^+$ and

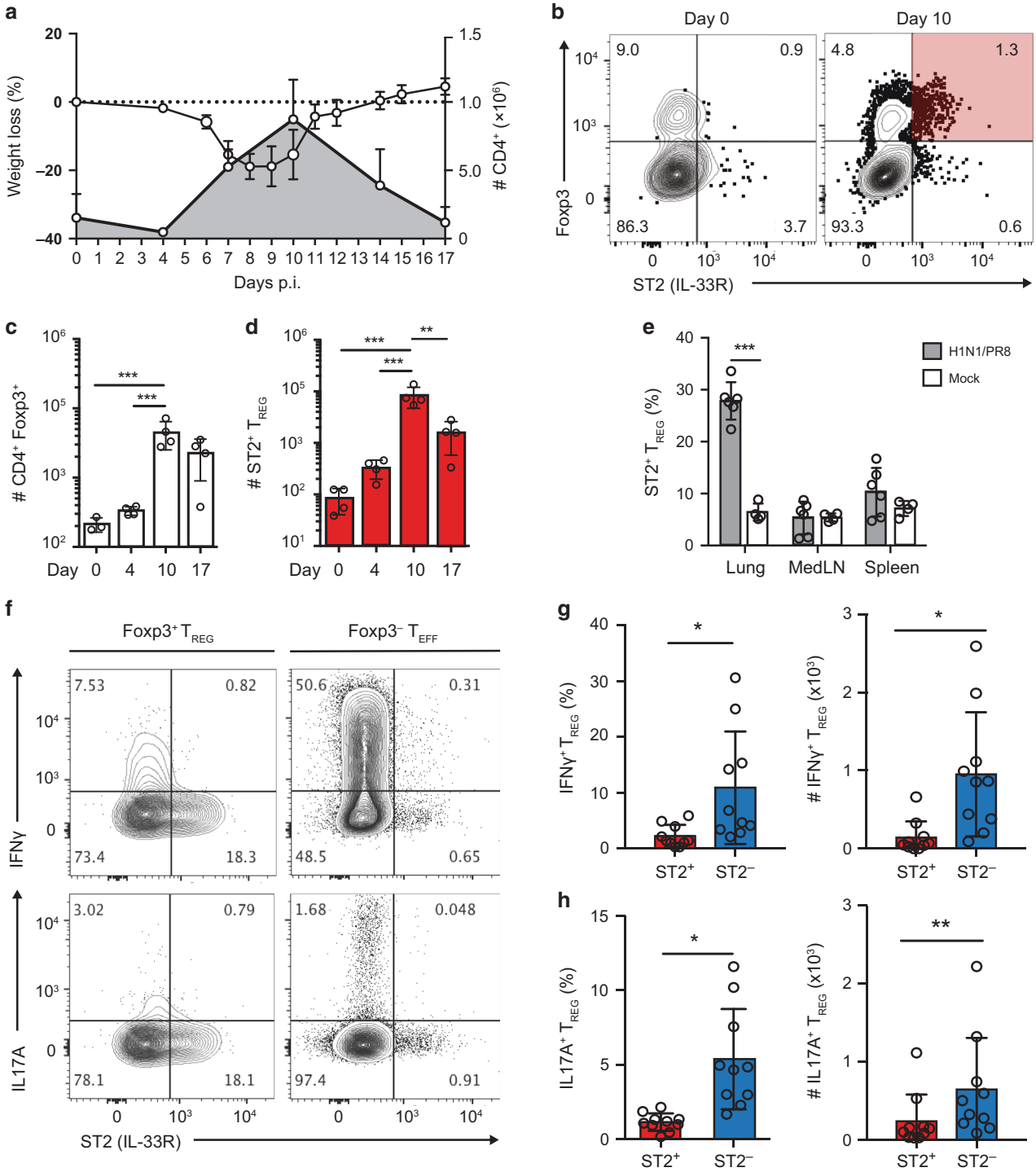
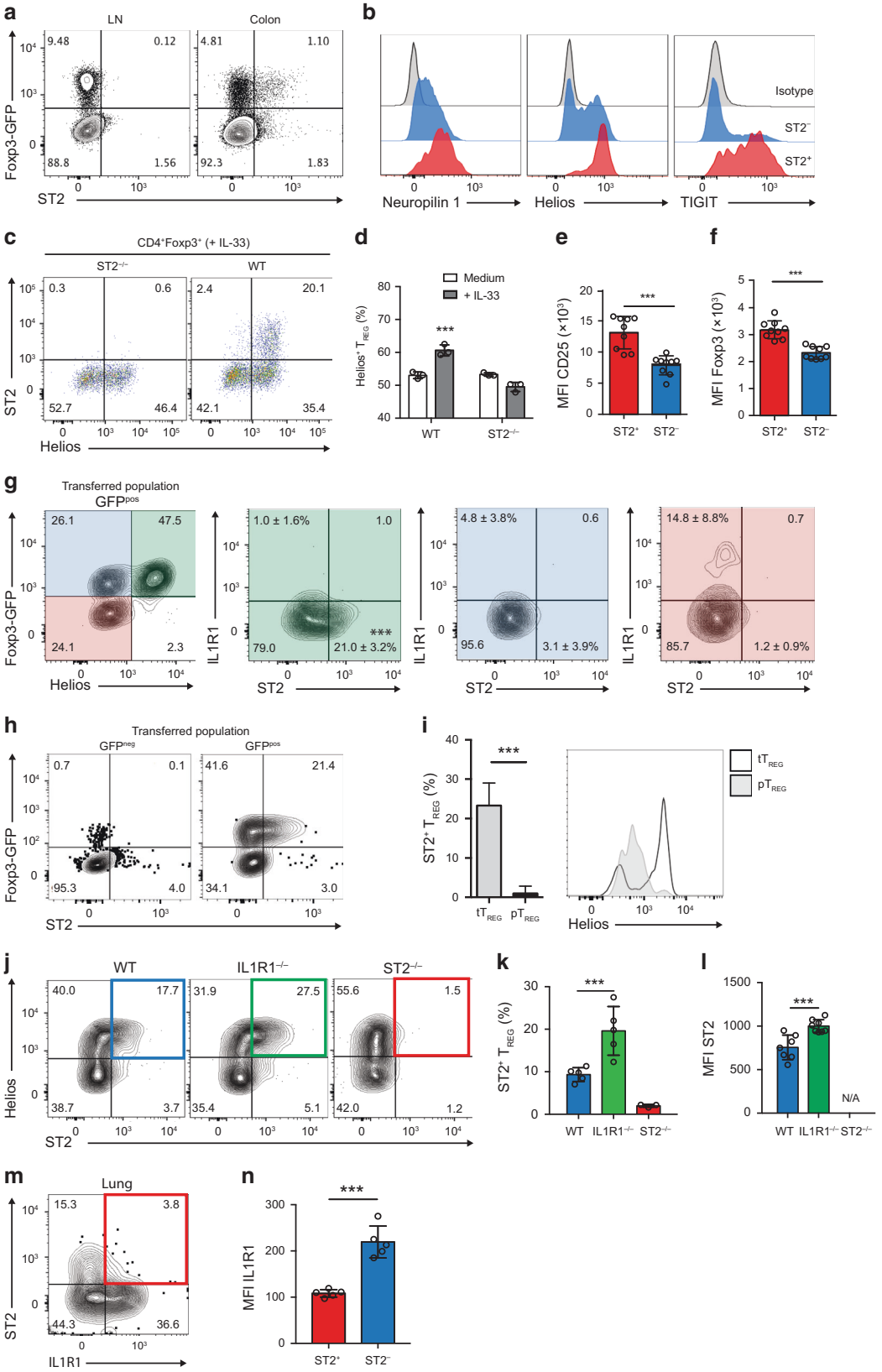


Fig. 2 The ST2 receptor segregates stable T_{REG} cells from pro-inflammatory IFNγ and IL-17A-producing T_{REG} cells in vivo. **a** $CD4^+$ T cells peak in the lung by day 4 post-infection with Influenza A PR8/H1N1. C57BL/6 mice were infected with 20 pfu of PR8/H1N1 intra-nasally and necropsy was performed at days 0, 4, 10 and 17 post-infection. Representative of 4 distinct experiments. **b** ST2 expression (red quadrant) is selectively increased in $CD4^+Foxp3^+$ T cells during the course of an Influenza A infection. ($N = 5$). **c**, **d** T_{REG} cells and $ST2^+$ T_{REG} cells accumulate in the lung and peak at day 10 after infection. (One way ANOVA. ***$p < 0.001$; **$p < 0.01$). **e** The increase in $ST2^+$ T_{REG} cells at day 10 post-infection is conscribed to the lung. **f** Representative flow cytometry plots of $Foxp3^+$ (left) and $Foxp3^-$ (right) $CD4^+$ T cells in the lungs of infected mice at day 10 post-infection. **g**, **h** $ST2^-$ (blue), but not $ST2^+$ (red), T_{REG} cells produce IFNγ or IL17-A at day 10 post-infection. Student *t*-test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

$ST2^-$ T_{REG} cell subsets in the presence or absence of Th17 differentiation conditions (IL-6, TGFβ and IL-1β), and assessed their ability to lose Foxp3 and Helios expression and acquire RORγT and IL-1R1 expression. We confirmed that IL-33 contributed to the maintenance of Helios and ST2 expression by $ST2^+$ T_{REG} cells (Fig. 6a). Interestingly, even when deprived of IL-33, only a small portion of $ST2^+$ T_{REG} cells downregulates ST2 and Helios expression in the span of 72 h and up-regulated RORγT and IL-1R1



(Fig. 6b). This portion of T_{REG} cells was significantly lower than what we observed in ST2$^-$ T_{REG} cells. Since ST2$^+$ T_{REG} could resist the inflammatory signals that lead to T_{REG} instability, we wanted to assess the effect of IL-33 in the total pool of T_{REG} cells in Th17 polarizing conditions. When we added increasing amounts of IL-33 to these conditions, we observed an increase in RORγT$^-$ IL1R1$^-$ ST2$^+$ T_{REG} cells in culture (Fig. 6c). In fact, the IL-33-induced expression of ST2 was not affected by Th17 conditions (Fig. 6d).

Fig. 3 IL-1 inhibits ST2 expression on Helios$^+$ T_{REG} cells. **a** Representative flow cytometry profile of ST2 expression in T_{REG} cells from pLN and colon of naïve C57BL/6 mice ($N = 5$ mice). **b** Expression levels of Neuropilin 1, Helios and TIGIT by ST2$^+$ (red) and ST2$^-$ (blue) T_{REG} cells from the spleen of naïve C57BL/6 mice were analysed by flow cytometry. Light grey histograms represent staining with isotype control. Representative of 3 mice, repeated in 3 separate experiments. **c**, **d** CD4$^+$ T cells from spleens of WT and ST2$^{-/-}$BALB/c mice were FACS-isolated and activated by plate-bound α-CD3 and α-CD28 in the presence of IL-2 (50 U/ml) and/or IL-33 (10 ng/ml) for 72 h. Expression levels of Foxp3, ST2 and Helios were analysed by flow cytometry, and **d** frequencies of Foxp3$^+$Helios$^+$ T_{REG} cells were determined. One-way ANOVA; *$p < 0.05$. Triplicates. Representative of three distinct experiments. **e**, **f** Mean fluorescence intensity of CD25 and Foxp3 expression of ST2$^+$ (red) and ST2$^-$ (blue) T_{REG} cells in (**c**). Student *t*-test. ***$p < 0.001$. (triplicates; $N = 5$). **g** ST2$^+$ T_{REG} cells segregate with Helios expression in the T_{REG} adoptive transfer model. CD4$^+$GFP$^+$ T cells were transferred as described in Fig. 1a and analysed at day 21. Expression levels of ST2 and IL1R1 in Helios$^+$Foxp3$^+$ (green), Helios$^-$Foxp3$^+$ (blue) and Helios$^-$Foxp3$^-$ (red). ($N = 4$ per group; Representative of 3 experiments). **h** Representative flow cytometry plots of ST2 and GFP (Foxp3) expression by the transferred GFP^{neg} and GFP^{pos} (Ly5.1$^+$ T cells) in the spleen at day 21. **i** Peripherally -induced T_{REG} cells (pT_{REG}) do not induce ST2 expression after transfer. Student *t*-test. ***$p < 0.001$. ($N = 4$ per group; Representative of 3 experiments). **j–l** IL1R1$^{-/-}$ mice show increased frequencies of ST2$^+$ T_{REG} cells in the lung at the steady state. **j** Representative flow cytometric analysis of Helios vs. ST2 expression in the lungs of naïve (WT), IL1R1$^{-/-}$ or ST2$^{-/-}$ BALB/c mice. **k** Frequencies of ST2$^+$ T_{REG} cells in the lung. **l** Mean fluorescence intensities (MFI) of ST2 expression among ST2$^+$ T_{REG} cells. One-way ANOVA; Tukey correction. ***$p < 0.001$. ($N = 3$–6 per group; Representative of 3 separate experiments). **m** Representative flow cytometry plots of the expression of ST2 and IL1R1 on CD4$^+$Foxp3$^+$ T_{REG} cells isolated from the lungs of BALB/c mice, and (*N*) Mean Fluorescence intensity (MFI) of IL1R1 on ST2$^+$ (red) and ST2$^-$ (blue) T_{REG} cells. Paired Student's *t*-test; ***$p < 0.01$. Representative of three distinct experiments ($N = 5$ mice per experiment)

We observed that the ST2$^+$ T_{REG} cells in the culture resisted IL-1R1 and RORγT expression (Fig. 6e, f). Finally, in order to understand the effect of IL-2 on the differentiation of IL-1R1$^+$ T_{REG} cells, we added IL-2 to Th17 polarizing conditions in the presence of saturating amounts of IL-1β and IL-33 (S10). We observed that IL-2 was sufficient to skew the differentiation of Foxp3$^+$ T cells towards stable ST2$^+$ T_{REG} cells even in increasing amounts of IL-6 (S10 A-B). Interestingly, although ST2$^+$ T_{REG} cells expand in the presence of IL-2 and IL-33, IL1R1$^+$ T_{REG} show an increased competitive expansion as they accumulate faster in the presence of IL-6 (S10C). In fact, the expression of IL-1R1 on T_{REG} cells increased in the presence of IL-6 in a dose-dependent manner (S10D). These conditions reveal the dichotomy between ST2$^+$ and IL1R1$^+$ T_{REG} cells and illustrate how distinct cytokine signals in situ promote the accumulation of stable vs. unstable T_{REG} cells.

Lack of IL-1 signalling alters the dynamics of ST2$^+$ and RORγT$^+$ Foxp3$^+$ T_{REG} subsets in fungal infection

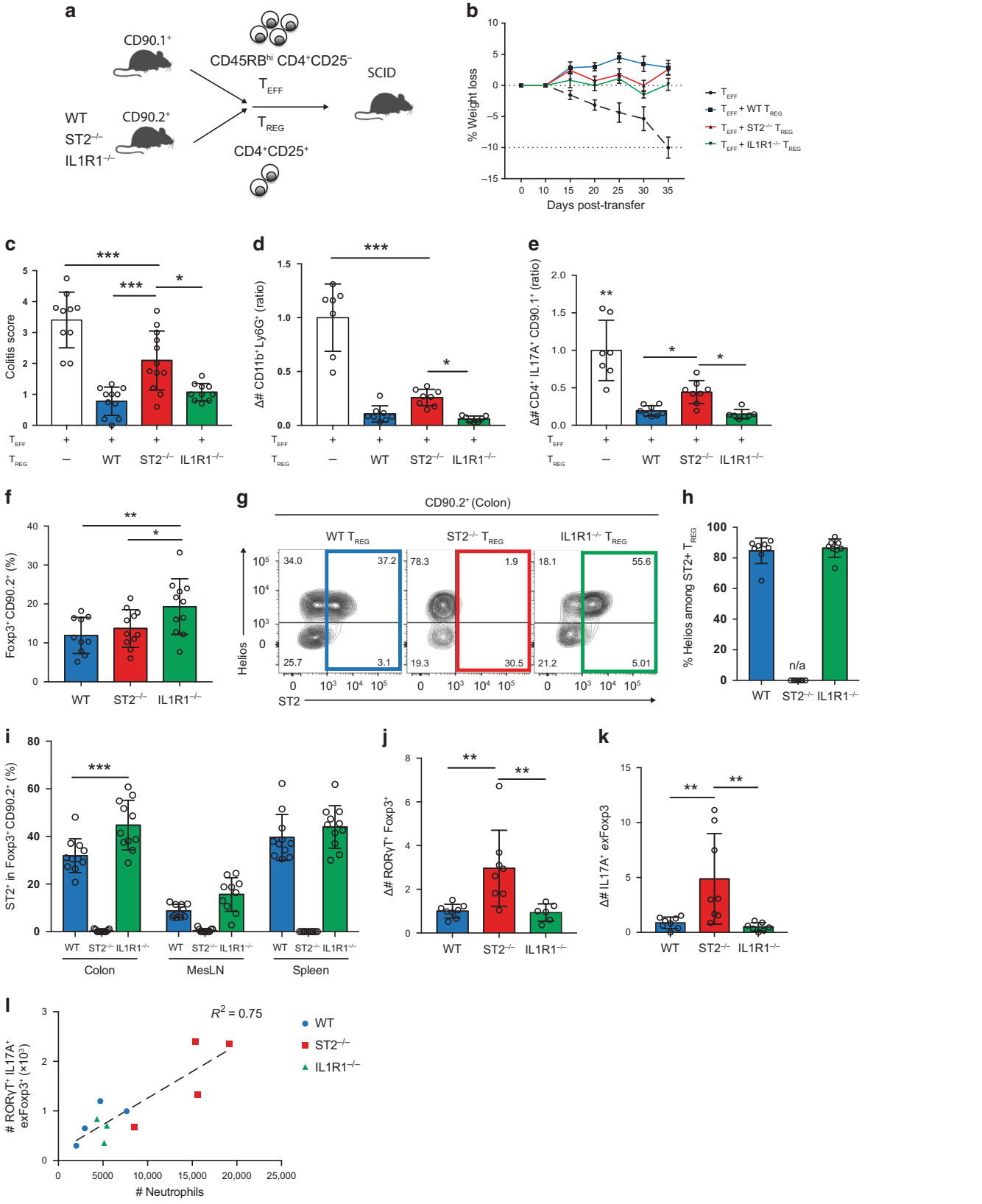
It is well established that IL-1 and IL-33 mediate distinct cellular responses of various immune cell types, including T cells.[29] Notably, IL-1 signalling plays a fundamental role in host defence against fungal diseases,[30] whereas IL-33 expression is exploited by the pathogen to evade clearance.[31] After observing that IL1R1$^{-/-}$ mice exhibited increased ST2-expressing T_{REG} cells at the steady state (Fig. 3j–l), we hypothesised that absence of IL-1R1 signalling would further favour IL-33 activity in vivo to enhance ST2$^+$ T_{REG} cell responses during infection. To understand the roles of ST2 and IL-1R1 on the dynamics of the T_{REG} cell responses in disease, we infected mice intratracheally with the fungal pathogen *Cryptococcus neoformans* which induces the production of IL-33[31] and IL-1α/β.[32,33] When compared to WT mice, IL-1R1$^{-/-}$ mice exhibited reduced fungal clearance in lung and brain (Fig. 7a). Further characterisation of CD4$^+$ T cell populations revealed that lack of IL-1R1 signalling leads to significantly reduced Th17 responses during the course of infection, as evidenced by the expression of the Th17 associated transcription factor RORγT in both Foxp3$^+$ and Foxp3$^-$ T cells (S11A). Interestingly, the lack of IL-1R1 signalling significantly increased the proportion and production of ST2 in Foxp3$^+$ T_{REG} cells in the lungs at day 14 of infection (Fig. 7b, c). These differences correlated with a significant increase in the frequencies of CD4$^+$Foxp3$^+$ T cells in the lungs of IL-1R1$^{-/-}$ mice (Fig. 7d) and an increased ratio of T_{REG} (# CD4$^+$Foxp3$^+$) to T_{EFF} (# CD4$^+$Foxp3$^-$) cells in the lungs (Figure S11B–D) underlining how the lack of IL-1R1 signalling affects the T_{REG}/T_{EFF} cell balance in the lungs. Importantly, the frequencies of pulmonary ST2$^+$ T_{REG} cells initially increased in both WT and IL-1R1$^{-/-}$ mice but remained high throughout the course of infection in the lungs of IL-1R1$^{-/-}$ mice when compared to WT mice (Fig. 7e). Moreover, IL-1R1$^{-/-}$ mice showed significantly decreased frequencies of RORγT$^+$ T_{REG} cells in the lungs during the course of disease (Fig. 7f). Concomitantly, we observed a decrease in IL-17A-producing T_{REG} cells at day 14 (Fig. 7g). As we previously observed in vitro, RORγT$^+$ T_{REG} cells were largely ST2$^-$ and Helios$^-$ (Fig. 7h; S11E). In fact, ST2$^+$ T_{REG} cells expressed the transcription factor GATA3 (Fig. 7i). Moreover, ST2$^+$ contrary to RORγT$^+$ T_{REG} cells maintained high levels of Helios expression (S11F). As such, GATA3$^+$ and RORγT$^+$ expressing Foxp3$^+$ T cells represent two distinct subpopulations of pulmonary T_{REG} cells during infection (Fig. 7j), and the absence of IL-1R1 significantly shift the populations in the lungs at day 14 of infection in favour of GATA3$^+$Foxp3$^+$ T_{REG} cells (Fig. 7k; S11G). These results reveal the antagonistic roles of IL-33 and IL-1 in modulating the functional specialisation of Foxp3$^+$ T_{REG} cell during lung infection.

DISCUSSION

CD4$^+$Foxp3$^+$ T_{REG} cells, key regulators of innate and adaptive immunity, accumulate locally during the course of various acute and chronic infections, inhibit immune responses and limit pathology.[34] In viral infections, T_{REG} cells reduce the efficacy of CD4$^+$ and CD8$^+$ T cell responses in the early stages of viral infections.[35] However, Foxp3$^+$ T_{REG} cells also have a protective role after viral clearance by controlling immune-mediated and microbe-associated pathology.[36,37] These divergent roles suggest a delicate control in the functional dynamics of Foxp3$^+$ T_{REG} cells for the regulation of immune responses to infections.

An increasing body of evidence reveals that Foxp3$^+$ T_{REG} cells do not have a fixed lineage identity but rather display considerable functional adaptability by altering their epigenetic and transcriptional landscapes to adapt to inflammatory conditions, in turn acquiring specialised roles for efficient modulation of immune responses.[38] This functional specialisation was characterized by the loss of Foxp3 expression in T_{REG} cells, the consequential loss of their suppressive phenotype, and reprogramming into inflammatory Th1/Th17-like *ex*Foxp3 T_{EFF} cells in lymphopenia, infections, organ-specific autoimmunity, and in tumour microenvironments.[9,11,14,21] Moreover, Foxp3$^+$ T_{REG} cells can also undergo a more subtle form of functional adaptability by transiently co-expressing lineage-specifying transcription factors for a more efficient control of T_{EFF} cells expressing the same transcription factors in inflammatory sites.[12–14] These cellular outcomes suggest a delicate control in the stability and fate of Foxp3$^+$ T_{REG} cells, allowing them to adjust their suppressive potential, and concurrently differentiate into inflammatory T_{EFF} cells.

By applying an unbiased screen to delineate the mechanistic events leading to the loss of Foxp3 in T_{REG} cells, we identified the



differential expression of the cell surface receptor for IL-33 (IL-33R, ST2) or IL-1 (IL-1R1) between stable (Foxp3^{hi}) and *ex*T_{REG} cells, respectively. We make the novel finding that IL-33 and IL-1 play opposing roles in dictating the dynamics and functional specialisation of T_{REG} cells at inflamed sites during viral and fungal infection as well as chronic inflammation.

Distinct types of T_{EFF} cells express the receptors for IL-1 (IL-1R1), IL-18 (IL-18RA) and/or IL-33 (T1/ST2, IL-33 receptor) in order to

Fig. 4 Absence of IL-33-, but not IL-1-, signalling converts Helios$^+$ T_{REG} cells to a pro-inflammatory phenotype that fuels intestinal inflammation. **a** SCID/beige mice received CD4$^+$CD45RB^{hi}CD25$^-$Thy1.1$^+$ effector T cells (T_{EFF}, 4×10^5) either alone or with CD4$^+$CD25^{hi} T_{REG} cells (2×10^5) from WT, ST2$^{-/-}$, or IL1R1$^{-/-}$ BALB/C mice. Necropsy was done at day 28 post-transfer. Pooled results are shown from three separate experiments. Where applicable, the results were compiled using a common denominator in each experiment (average of the WT). **b** Mice that received T_{EFF} alone show weight loss by day 28 post-transfer. **c** Mean colitis score of each mouse (see Methods). Five blinded observers. One-way ANOVA. $^{**}p < 0.01$. **d** Relative number of cells (to the average of WT) of CD11b$^+$Ly6G$^+$ cells in the colon of mice, assessed by flow cytometry. **e** Relative number of cells (to the average of WT) of Th17 cells (CD4$^+$IL-17A$^+$ amongst Thy1.1$^+$ cells) assessed by flow cytometry. One-way ANOVA. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$. **f** IL1R1$^{-/-}$ T_{REG} cell show less Foxp3 loss in the colon after transfer. Two-way ANOVA. (Tukey) $^{**}p < 0.01$. **g** IL1R1$^{-/-}$ T_{REG} cells co-express Helios and ST2. **h** ST2-expressing CD90.2$^+$ T_{REG} cells are Helios$^+$. **i** A higher frequency of IL1R1$^{-/-}$ T_{REG} cells express ST2 compared to WT T_{REG}. Two-way ANOVA. Tukey correction. $^{***}p < 0.001$. **j** CD90.2$^+$ (Thy1.2$^+$) CD4$^+$Foxp3$^+$ T_{REG} cells from ST2$^{-/-}$ donors accumulate as RORγT$^+$ in the colon of the mice (relative to the average number of WT). One-way ANOVA. $^{**}p < 0.01$. **k** exT_{REG} cells originating from ST2$^{-/-}$ T_{REG} cells accumulate as IL17A$^+$ T cells in the colon of mice (relative to the average number of WT). **l** The numbers of Th17-like exT_{REG} cells correlate with increased neutrophil accumulation in the colon of mice. Pearson correlation analysis. Representative of three separate experiments

enhance their proliferation and cytokine production.[39] Recent studies reveal the presence of an ST2$^+$ T_{REG} cell subset in mucosal and lymphoid tissues.[19,26,40] IL-33 and IL-1α/β are released locally by many non-hematopoietic and hematopoietic cells at external and internal barrier surfaces upon cell stress where they function as endogenous alarmins.[22] Interestingly, IL-33 was recently shown to favour the expansion of T_{REG} cells in the gut as ST2$^{-/-}$ T_{REG} cells show diminished functions in the same tissue environment.[19] However, the role of IL-1 for T_{REG} cell function was largely unknown. This was probably due to the requirement for IL-1R1$^+$ T_{REG} cells to differentiate into RORγT$^+$Foxp3$^+$ dual expressers prior to IL-1R1 signalling.

We confirm here that IL-33-mediated effects on T_{REG} cells occur in the presence of IL-2[19], whereas expression of IL-1R1 is induced in the presence of IL-6 and TGFβ, essential polarizing cytokines for Th17 differentiation.[41] Interestingly, IL-33-stimulated dendritic cells[42] and mast cells[43] were shown to produce IL-2 upon IL-33 stimulation, highlighting a positive regulatory loop to support ST2$^+$ T_{REG} cell stimulation. On the other hand, production of IL-1β by dendritic cells is enhanced by IL-6, IL-21 and IL-23, and promotes Th17 differentiation.[44] Interestingly, both IL-1β and IL-33 enhanced survival and proliferation of polarized T_{REG} cells within their respective inflammatory milieu in vitro. Previous studies have shown that thymic-derived T_{REG} cells were able to respond to IL-33 and IL-2 in order to upregulate the ST2 receptor.[26,45] We also confirm the strong link between ST2 and the expression of the transcription factor Helios and the surface receptors Neuropilin 1 and TIGIT, whose collective expression reflects T_{REG} cells of thymic origin and enhanced T_{REG} stability.[28,46–48] In contrast, RORγT$^+$ T_{REG} cells show reduced Helios expression both in vivo and in vitro. We did not observe ST2 expression in in vitro or peripherally -induced T_{REG} cell subsets (iT_{REG} and pT_{REG}, respectively), in *ex*T_{REG} cells, nor was ST2 expression prominently seen in Helios$^-$ T_{REG} cells, in contrast to a recent report,[19] although further characterization of ST2$^+$ T_{REG} cells in the gut environment, relative to the lung, is required. Interestingly, Helios expression by tT_{REG} cells was necessary to maintain immune homeostasis in later stages of life.[28] Future studies are required to confirm whether Helios plays an important role in stabilising tT_{REG} cell function, and if inflammatory conditions can modulate Helios expression or function.

To characterise the dynamics of ST2$^+$ T_{REG} cells in the context of an immune challenge, we studied their fate in two mouse models of lung infections: an acute model of murine-adapted Influenza A virus H1N1/PR8, and a chronic *Cryptococcus neoformans* fungal infection. In both models, we observed that ST2$^+$ T_{REG} cells peak early during the course of infection and at timepoints that precede the onset of peak anti-pathogen immune responses. Recently, it was suggested that ST2$^+$ T_{REG} cells play a particularly important role in tissue protection.[40] As such, we observed that ST2$^+$ T_{REG} cells resisted production of pro-inflammatory cytokines throughout the course of disease, in contrast to their ST2$^-$ counterparts, which display significant plasticity by co-expressing RORγT and develop the potential to secrete inflammatory cytokines like IL-17A. The early accumulation of ST2$^+$ T_{REG} cells during lung injury may reflect an attempt by local mucosal T_{REG} cells to maintain homeostasis in the early events. However, as inflammation progresses, the adaptive immune response switches to an effective inflammatory response, where newly adapted ST2$^-$ T_{REG} cells appear. This mechanism is particularly evident during chronic infection with *Cryptococcus neoformans*, where the pathogen favours IL-33,[31] IL-1α and IL-1β[32,33] release in the early phase of disease to enhance pathogenicity. In fact, we observed that marked elevation of ST2$^+$ T_{REG} cells lead to an overall increase of the T_{REG} to T_{EFF} ratio and increased virulence and pathogenicity. Thus, IL-33 release observed with pathogenic *Cryptococcus neoformans* strains may promote the stability of immunosuppressive ST2$^+$ T_{REG} cell pool, thereby counteracting anti-microbial immune responses. On the other hand, recent evidence reveals that excessive IL-33 levels, as evidenced by lung treatment with IL-33, could alter the suppressive function of ST2$^+$ T_{REG} cells,[49] a phenomenon we did not observe in our Th1 and Th17-driven infectious models. Thus, other signalling pathways are probably involved in the loss of function of ST2$^+$ T_{REG} cells and will be the focus of further investigation.

In this study, we assessed the effect of IL-1β on the suppressive response of T_{REG} on Th17 cells. We show that IL-1β stimulation almost completely abrogated the suppressive ability of T_{REG} cells. The inability of IL-1R1$^+$ T_{REG} cells to suppress Th17 cells is further substantiated by their increased survival and proliferation in the presence of IL-1, consistent with a study by Ben-Sasson et al. showing that IL-1 enhances Th17 cell proliferation.[50] In accordance with these results, during infection with *Cryptococcus neoformans*, no elevation of RORγT and IL-17 expression in ST2$^-$ T_{REG} cells was observed in IL-1R1-deficient mice, unlike their wild-type counterparts. In contrast, we found that IL-1R1-deficiency led to marked elevation of GATA3$^+$ ST2$^+$ T_{REG} cells with an overall increase of the T_{REG} to T_{EFF} ratio and increased virulence and pathogenicity. Moreover, we observed that lack of IL-1R1 on T_{REG} cells caused enhanced stability and suppressive ability of the Foxp3$^+$ T_{REG} cell population during the course of T-cell-induced colitis. In agreement with a previous report,[19] we observed that ST2$^{-/-}$ T_{REG} cells were inefficient in suppressing T cell-induced colitis. Strikingly, we show that the lack of IL-33R signalling in T_{REG} cells leads to the acquisition of pro-inflammatory characteristics, and promotion of the colitogenic process. Conversely, we also show that IL-1R1$^{-/-}$ T_{REG} cells have increased maintenance of Foxp3 expression in donor T_{REG} cells, and an up-regulation of the IL-33R in this inflammatory setting. In addition, we observed that IL-1R1 is required for the proliferation of co-expressing RORγT$^+$-Foxp3$^+$ cells, further highlighting the role of IL-1R1 for the functional adaptability of T_{REG} cell responses. In their 2010 report, Li et al. showed that IL-1β enhanced IL17A production by T_{REG} cells in undifferentiated co-cultures with T_{EFF} cells in vitro,[41] an

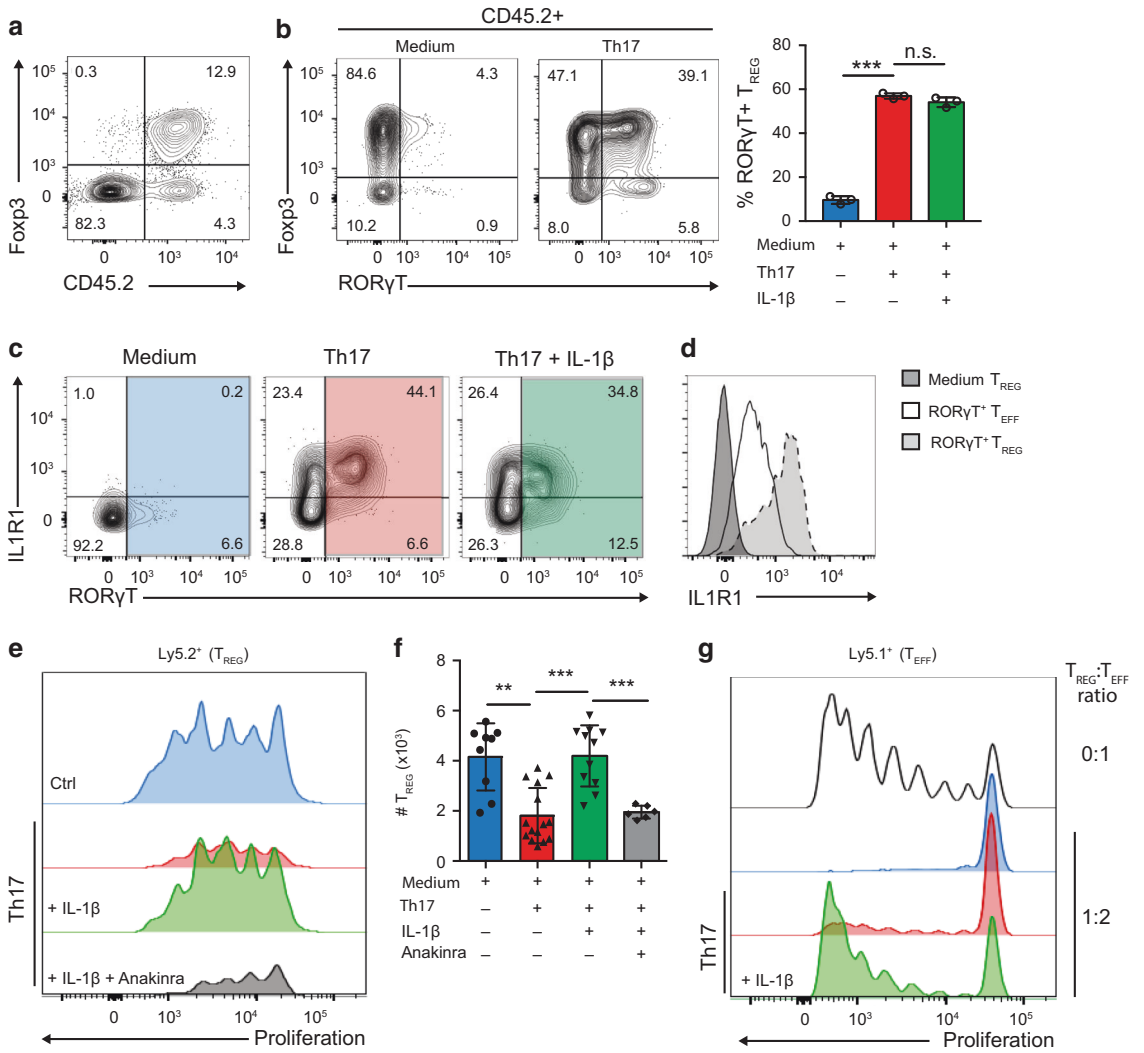


Fig. 5 Th17 polarizing conditions induce IL1-R1 expression on T_{REG} cells, favour their proliferation but hinder their suppressive ability. Ly5.2^{+}CD4^{+}GFP^{+} T_{REG} cells were FACS-sorted from C57BL/6 Foxp3^{GFPki} mice and co-cultured (1:2 ratio) with Ly5.1^{+}CD4^{+}GFP^{-} (T_{EFF}) sorted from similar mice, α-CD3 (1 μg/ml) and irradiated APCs (CD4^{neg} fraction from the Ly5.2^{+}) for 72 h. Representative of five experiments in triplicates. **a** Representative flow cytometry plot of the gating strategy. **b** Th17 polarizing conditions [IL-6 (10 ng/ml) + TGFβ (1 ng/ml)], not IL-1β (25 ng/ml), upregulate RORγT in CD4^{+}Foxp3^{+} T cells in vitro. One way ANOVA. Tukey correction. ***$p < 0.001$. **c** RORγT is co-expressed with IL-1R1 on T_{REG} cells in Th17 polarizing conditions. **d** RORγT^{+} Foxp3^{+} T cells express the IL-1R1 receptor upon Th17 polarization to a greater extent than RORγT^{+} T_{EFF} cells. **e** IL-1β enhances the proliferation of T_{REG} cells in Th17 polarizing conditions. Ly5.2^{+}CD4^{+}GFP^{+} T cells were stained with V450 proliferation dye prior to culture. ANAKINRA (Kineret®). T_{REG} in medium (blue), in Th17 polarizing conditions (red), in Th17 + IL-1β (green) or in Th17 + IL-1β + Anakinra (100 μg/ml) (grey). **f** CD4^{+}Foxp3^{+}Ly5.2^{+} T cell counts after 72 h. One way ANOVA, Tukey correction. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. **g** IL-1β signalling enhanced proliferation of T_{EFF} (Ly5.2^{+}) cells even in the presence of T_{REG} cells at a 1:2 ratio. CD4^{+}Foxp3^{-}Ly5.1^{+} T_{EFF} cells alone (white), in the presence of T_{REG} at 1:2 ratio (blue), in Th17 polarizing conditions (red) or in the presence of IL-1β (green)

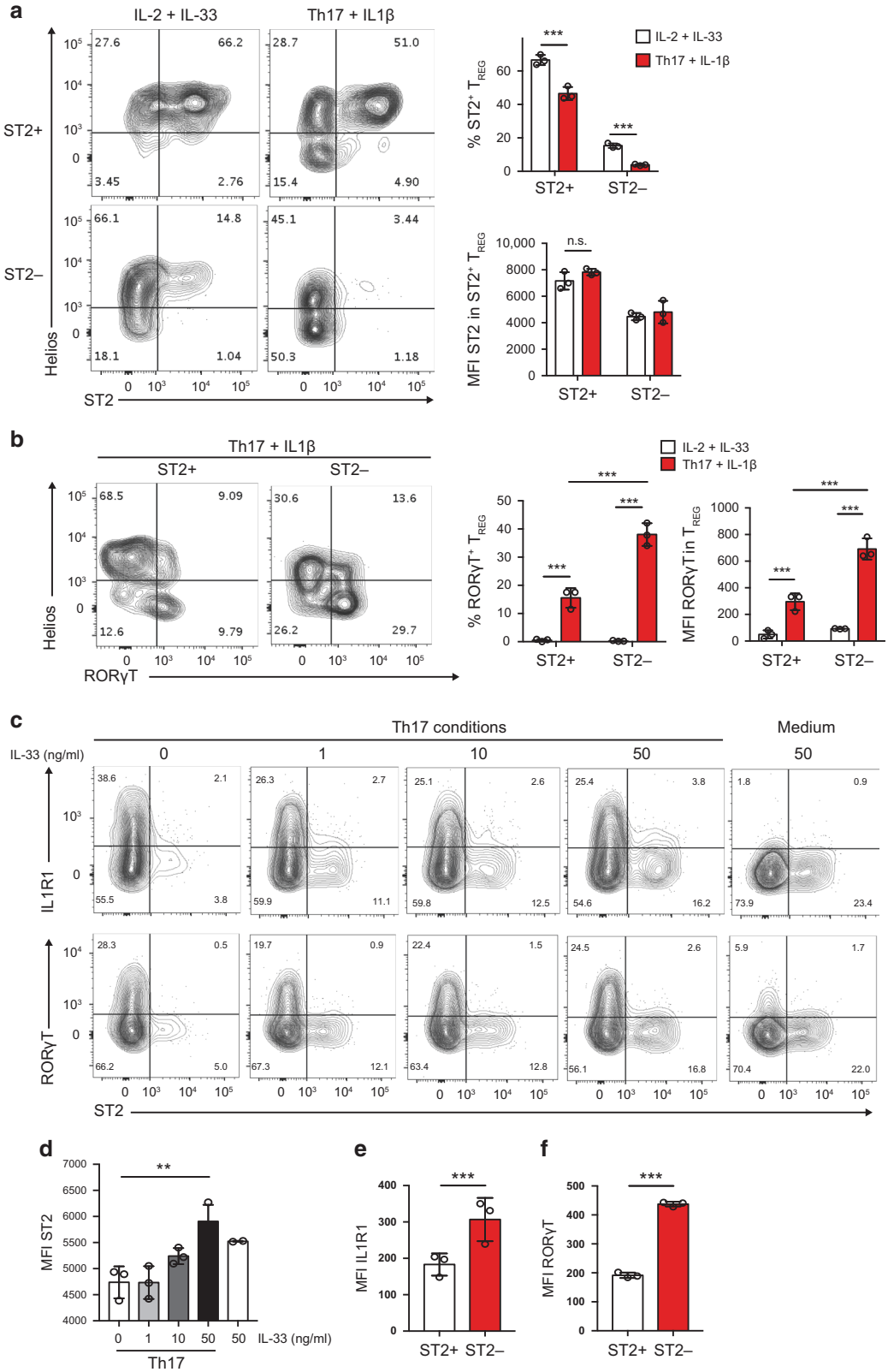
observation not aligned with our conclusions. The reasons for this are unclear but may relate to the reduced T_{REG} cell-mediated regulation of APC function in the absence of IL-1R1 signalling, and consequential induction of IL-17 secretion by T_{EFF} cells in culture supernatants. Nonetheless, these results illustrate that distinct polarizing signals on T_{REG} cells are required for them to acquire the ability to respond to the distinct members of the IL-1 family induced during immune challenge such as IL-1 and IL-33.

Overall, we show that two distinct members of the IL-1 family of cytokines, IL-33 and IL-1, have differential effects on the functional adaptation of Foxp3^{+} T_{REG} cells at mucosal surfaces during immune challenge. We show that IL-1, but not IL-33, impedes the suppression of T_{EFF} cells by T_{REG} cells in highly inflammatory conditions. In contrast, IL-33 stabilises the T_{REG} cell phenotype and function, all-the-while restricting the potential of Foxp3^{+} T_{REG} cells to adopt inflammatory features in challenged mucosal sites. These results highlight a mechanism by T_{REG} cells to adapt to the inflammatory conditions throughout the evolving nature of an infection to fine-tune anti-pathogen adaptive immunity and protect the host from pathology. They provide an additional dimension to the role and fate of T_{REG} cells in disease. Further characterisation of the processes that lead to the adaptation of T_{REG} cells will pave the way towards the development of therapies that aim to modulate their response during the course of disease.

MATERIALS AND METHODS

Mice

WT (Ly5.2^{+}) and congenic (Ly5.1^{+})TCR-β$^{-/-}$ C57BL/6 mice were obtained from Taconic Laboratories while C57BL/6.Foxp3^{GFP} reporter knock-in (Foxp3^{GFP}) mice[11] were provided by Alexander Rudensky and bred into the congenic background (Ly5.1^{+}) for



more than 10 generations. Inbred BALB/c were purchased from Charles River. Thy1.1+ BALB/c (JAX® CBy.PL(B6)-Thy1^{a}/ScrJ #005443), T1/ST2$^{-/-}$ BALB/c mice[51] and IL-1R1$^{-/-}$ BALB/c mice (10-generation backcross from JAX #003018 B6 IL-1R1^{Tm1Rom1} strain on the BALB/c background) were bred on site. IL-1R1$^{-/-}$ mice were kept as homozygous for breeding purposes. Foxp3$^{GFP\text{-}CRE}$ X Rosa26 lox-stop-lox tdTomato fate-tracking mice on C57Bl/6 background were obtained from Jeffrey Bluestone (University of California).[21] All mice were used at 10–12 weeks of age. All mice were housed and bred under specific pathogen-free

Fig. 6 IL-33 favours the stability of Helios^{+}ST2^{+} T_{REG} cells in Th17 polarizing conditions. **a**, **b** ST2^{+} and ST2^{-} CD4^{+}GFP^{+} (T_{REG}) were isolated from the spleen of Foxp3^{GFPki} mice and plated in the presence of Ly5.1^{+}CD4^{+}GFP^{-} (T_{EFF}) sorted from Ly5.1^{+}Foxp3^{GFPki} mice, soluble α-CD3 (1 μg/ml) and irradiated APCs (CD4^{neg} fraction from the Ly5.2^{+}) at a 1:2 ratio for 72 h. Representative of four distinct experiments in triplicates. **a** T_{REG} cells resist the loss of ST2 and Helios expression under pro-inflammatory conditions in vitro. ST2^{+} and ST2^{-} CD4^{+}GFP^{+} T cells from the spleen of Foxp3^{GFPki} were activated in the presence of IL-6 (10 ng/ml), TGFβ (1 ng/ml) and IL-1β (25 ng/ml) for 72 h. **b** Loss of Helios expression is associated with an increase in RORγT expression in Foxp3^{+} T_{REG} cells in vitro. One-way-ANOVA; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. **c–f** CD4^{+}GFP^{+} T_{REG} cells were isolated from the spleen of Foxp3^{GFPki} mice and co-cultured in the presence of CTV-labelled CD4^{+}GFP^{-} T_{EFF} cells (1:2 ratio), α-CD3 (1 μg/ml) and irradiated APCs (CD4^{neg} fraction from the Ly5.2^{+}) for 72 h. **c**, **d** IL-33 facilitates the up-regulation of the ST2 receptor on T_{REG} cells in Th17 polarizing conditions. One way ANOVA. Tukey correction. **$p < 0.01$. **e**, **f** ST2^{+} T_{REG} cells resist the expression of RORγT and IL-1R1. Paired Student's *t*-test; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

conditions and used according to institutional guidelines at McGill University.

Lymphocyte isolation

Isolation of T cells was performed, as previously described.[24] In brief, after CO_2 euthanasia, the lungs were perfused with cold PBS through the right ventricle. The collected lungs were then digested in RPMI 1640 with 5% FBS (Wisent) containing collagenase A (0,5 mg/ml) and collagenase D (0,5 mg/ml) in the presence of DNAse I (0.005 μM) (Sigma-Aldrich) for 45 min at 37 °C and then mechanically processed in the same manner as the mediastinal LN, the spleen and the inguinal lymph nodes. The lamina propria T cells (colon) were obtained through the processing of the colon as previously described.[11] The cells were then filtered through a cell strainer and kept in complete RPMI medium.

Purification of T cell subsets

For in vivo adoptive transfers, T_{REG} cells from splenocytes of Ly5.1^{+} Foxp3^{GFPki} C57BL/6 were isolated the following; first CD4^{+} T cells were enriched using an autoMACS (Miltenyi Biotec), and then CD4^{+}GFP^{+} T cells (purity >99%) were sorted using a FACSAria™ (BD Biosciences). ST2^{+} and ST2^{-} Foxp3^{+} T_{REG} cells were isolated by FACSAria™ as CD4^{+}GFP^{+} ST2^{+} or ST2^{-} T cells. When working on the BALB/c background, CD4^{+}CD25^{hi} (top 50% of CD25^{+} cells) and CD4^{+}CD45RB^{hi}CD25^{-} cells were sorted using a FACSAria™ the day of the transfer.

In vitro assays

FACS-sorted CD4^{+}Foxp3^{+} T_{REG} cells (GFP^{+}, 50×10^{3}) expressing ST2 (ST2^{+}) or not (ST2^{-}) were activated in 96-well flat-bottomed (0.2 ml) plates previously coated with α-CD3 (5 μg/ml) and α-CD28 (2 μg/ml), and in the presence of IL-2 (100 U/ml - or otherwise indicated) in RPMI (Wisent) supplemented with 10% FBS at 37 °C for 72 h. For in vitro stimulation assays, IL-33 (10 ng/ml - or otherwise indicated), IL-6 (10 ng/ml - or otherwise indicated), mouse recombinant TGFβ1 (1 ng/ml) and/or IL-1β (25 ng/ml) (R&D biosystems) were added at the start of the culture unless otherwise stated. In some studies, T cell cultures were performed in the presence of Anakinra (Kineret™; Swedish Orphan Biovitrum, Stockholm, Sweden). For suppression and polarisation assays, 5.0×10^{4} FACS-sorted CD45.2^{+}CD4^{+}GFP^{-} were plated in 96-well flat-bottomed plates together with 2.0×10^{5} irradiated feeder cells (CD4^{neg} fraction) and activated with soluble α-CD3 (1 μg/mL) in the absence of IL-2. CD45.1^{+}CD4^{+}GFP^{+} T_{REG} cells were added at a 1:2 or 1:4 ratio (2.5×10^{4} or 1.25×10^{4}). The cells were labelled with either Violet proliferation dye V450 (BD Bioscience) or CellTrace™ Violet (Thermofisher), depending on the experiment.

Adoptive T cell transfer

For the microarray, FACSAria™ sorted CD4^{+}GFP^{+} or GFP^{-} cells from Ly5.1^{+} Foxp3^{GFPki} C57BL/6 mice were suspended in PBS and transferred intravenously into Ly5.2^{+} TCRβ$^{-/-}$ animals (1.0×10^{5} cells/mouse). Mice were monitored for dehydration and weight loss. Necropsy was performed at day 21 post-injection.

For the recombinant IL-33 injections, the Ly5.2^{+} TCRβ$^{-/-}$ mice received 2×10^{5}/mouse of FACSAria™ sorted CD4^{+}GFP^{+} T_{REG} cells (Ly5.1 + Foxp3^{GFPki} C57BL/6) intravenously and were injected with 100 μg/mice of recombinant IL-33 (R&D biosystems) intraperitoneum (I.P.) starting from day of injection (Day 0) every 48 h until the day of necropsy (Day 14).

In the colitis experiments, FACSAria™ sorted Thy1.1^{+} CD4^{+}CD45RB^{hi}CD25^{-} T cells (naïve T_{EFF}) were kept separate from CD4^{+}CD25^{hi} cells (T_{REG}) originating from WT, T1/ST2$^{-/-}$ or IL-1R1$^{-/-}$ BALB/c mice. At the time of injection, 4.0×10^{5} naïve T_{EFF} and 2.0×10^{5} T_{REG} cells were mixed and injected in the lower right quadrant of the peritoneum (I.P.). Weight of mice was monitored daily. CD4^{+} T cells from Foxp3$^{GFP\text{-}CRE}$ X Rosa26 lox-stop-lox tdTomato fate-tracking mice were sorted and adoptively transferred (I.P) into a TCRβ Ly5.1^{+} mouse and left for homeostatic proliferation for 10 days.

Microarray analysis

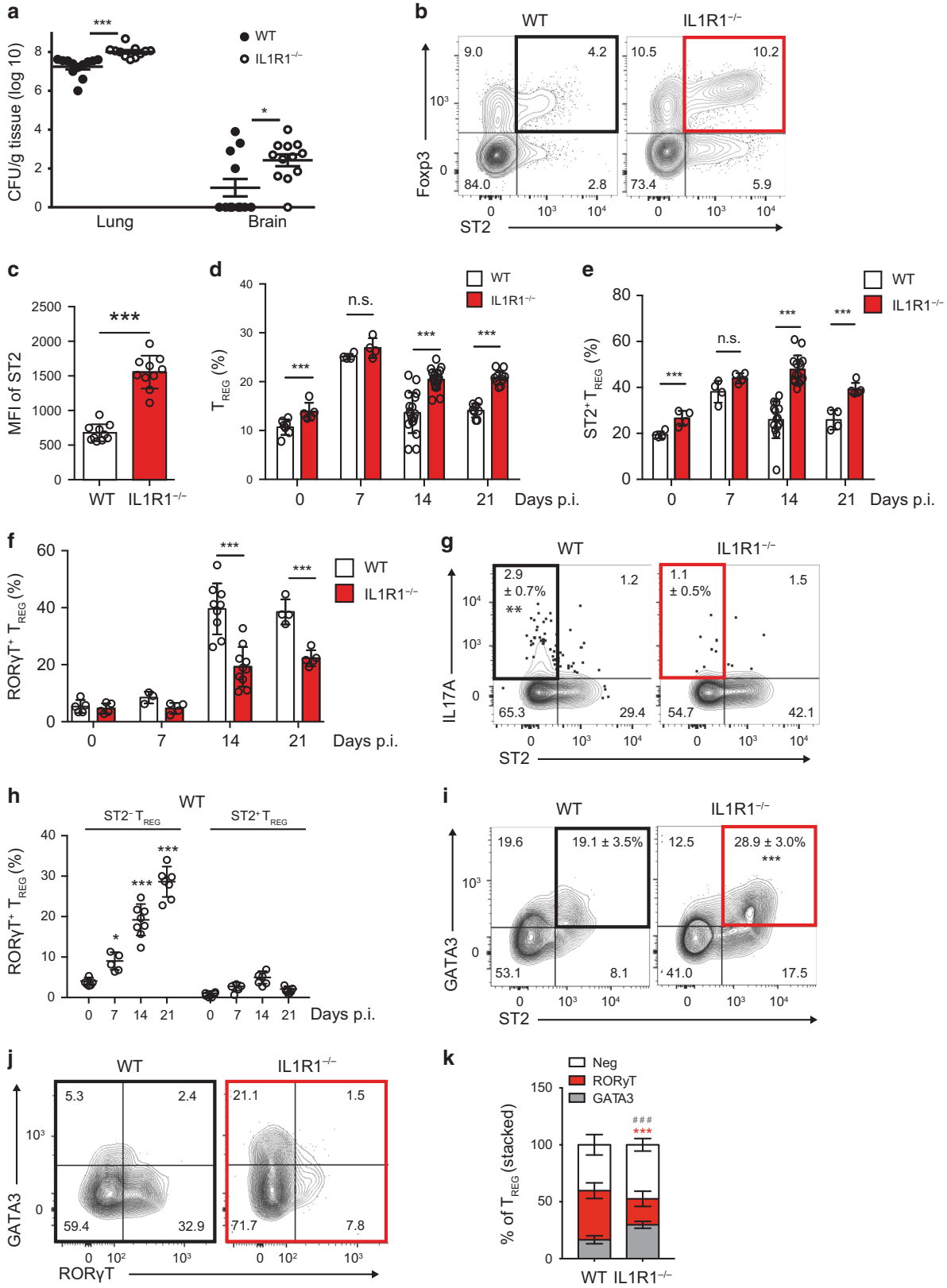
Freshly isolated (Day 0) CD4^{+} GFP^{+} (fresh T_{REG}) and GFP^{-} (fresh T_{EFF}) were isolated from Foxp3^{GFPki} Ly5.1^{+} mice and transferred into TCRβ−/− Ly5.1^{+} (see Fig. 1a). At day 21 post-transfer, Ly5.2^{+}CD4^{+} T cells were isolated and further separated based on GFP signal: (1) GFP^{hi} (T_{REG}), (2) GFP^{neg} (*ex*T_{REG}) or (3) GFP^{neg} (T_{EFF} from GFP^{-} transferred mice). Two additional groups of cells were analysed: (4) freshly-sorted T_{REG} (fresh T_{REG}) and (5) freshly sorted T_{EFF} cells (fresh T_{EFF}) from day 0. RNA was isolated using the RNeasy Mini Kit from Qiagen as per the manufacturer's instructions. Samples were run on a Illumina® MouseWG-6 v2.0 chip which contains 45281 mouse probes and 974 control probes (Illumina® – Genome Québec), and the resulting raw expression data were extracted, annotated, robust spline normalised and background-adjusted using illuminaMousev2.db and bead array packages in R. The top 654 genes that varied ($p<0.05$ cut-off) were selected and the expression patterns were analysed between the groups. A modified ANOVA for microarray analysis (eBayes function from the Limma package) was used to compare across all conditions for each gene.

Intranasal infection with Influenza A

The mouse-adapted Influenza A virus (IAV) H1N1 strain A/Puerto Rico/8/34 was propagated and titrated by plaque assay on Madin-Darby canine kidney cells, as described.[52] Mice were anaesthetised by intramuscular injection with a mixture of 10 mg/kg ketamine (Ayerst Veterinary Laboratories) and 125 mg/kg xylazine (Bayer). For a sublethal infection, 20 plaque-forming units (PFU) of IAV per 20 g body weight were administered intranasally. The mice were monitored daily for clinical score and weight loss.

Intratracheal infection with C. *neoformans*

C.neoformans 52D (ATCC no. 24067) was grown and prepared as previously described.[31] In brief, a single colony of *C. neoformans* from a Sabouraud dextrose agar (Becton Dickinson) was resuspended in Sabouraud dextrose broth (Becton Dickson) and then grown further in a rotating culture until stationary phase (48 h) at room temperature for in vivo infections. Subsequently, the culture was spun, washed twice with PBS, and resuspended to a



desired concentration in PBS. Concentrations of *C. neoformans* were verified by plating on Sabouraud dextrose agar at 37 °C for 72 h followed by determination of CFU. The mice were anaesthetised with 10 mg/kg ketamine (Ayerst Veterinary Laboratories) and 125 mg/kg xylazine (Bayer) intra-peritoneally. To access the trachea, a vertical 1-cm incision of the skin was made below the jaw. A 22-gauge catheter (Becton Dickson) was inserted into the trachea. In a volume of 50 µl sterile PBS, 2×10^5

Fig. 7 Absence of IL-1R1 signalling *in vivo* skews the T_{REG} cell response in favour of $ST2^+$ T_{REG} cells during the course of disease. **a** $IL\text{-}1R1^{-/-}$ mice show increased fungal burden in the lungs (left) and brains (right) upon intra-tracheal exposure to 10^4 CFU/mice of *Cryptococcus neoformans* 52D. ($N = 4$–5 per group; Representative of 3 distinct experiments). **b** Representative flow cytometry profiles of ST2 relative to Foxp3 expression in $CD4^+$ T cells at day 14 post-infection (lung). **c** Higher frequencies of $Foxp3^+$ T_{REG} cells in the $IL\text{-}1R1^{-/-}$ (red) compared to WT mice (white). **d** ST2-expressing T_{REG} cells represent a major population of $Foxp3^+$ T cells in $IL\text{-}1R1^{-/-}$ mice (red) throughout the course of infection in opposition to WT mice (white). **e** $ST2^+$ T_{REG} cells accumulate early in the lungs during infection but decrease in WT (white) compared to $IL\text{-}1R1^{-/-}$ mice (red). **f** $IL\text{-}1R1^{-/-}$ mice (red) show decreased frequencies of $ROR\gamma T^+$ $CD4^+Foxp3^+$ T_{REG} cells in the lungs at the peak of adaptive immunity. **g** IL-17A production by $Foxp3^+$ T cells originates from $ST2^-$ T_{REG} cells in the lungs at day 14 post-infection. **h** $ST2^-$ T_{REG} expressing RORγT represent a significant fraction of T_{REG} cells in the lungs during the course of infection. Two-way ANOVA. Tukey correction. ***$p < 0.001$. **$p < 0.01$. *$p < 0.05$. **i** ST2 expression on T_{REG} cells correlates with increased GATA3 expression in the lungs at day 14 in both WT (white) and $IL\text{-}1R1^{-/-}$ (red). **j, k** Absence of IL-1R1 influences the balance between $GATA3^+$ and $ROR\gamma T^+$ T_{REG} cells in the lungs (day 14) in WT (white) when compared to $IL\text{-}1R1^{-/-}$ (red). One-way ANOVA. ***$p < 0.001$. **$p < 0.01$. *$p < 0.05$

CFU/ml *C. neoformans* was instilled, followed quickly by 50 µl volume of air. The incision was closed using a 9 mm EZ Clip Wound Closing Kit (Stoelting). Mice were monitored daily following surgery.

Colitis score

Mean colitis score of the colon of each mouse was assessed by 5 distinct double-blinded observers following the guidelines from Erben et al.[53] (4–5 mice per group; 3 distinct experiments).

Flow cytometry

After lymphocyte isolation, single-cell suspensions were stained with the following fluorescence-conjugated mAbs, purchased from Thermofisher (eBioscience) unless otherwise stated: α-CD4–Alexa700 (GK1.5), α-CD8-V500 (53-6.7) (BD Biosciences), α-ST2-PerCP710 (RMST2-2), α-CD25-PECy7 (PC61) (BD Biosciences), α-Foxp3-FITC or PE (FJK-16s), α-IL-17A-APC (eBio17B7), α-IFNγ-PECy7 (XMG1.2), α- RORγT-PE (AFKJS-9) or α-RORγT BV786 (Q31-378) (BD Bioscience), α-GATA3-Alexa647 (BD Biosciences), α-CD45.1 PE (A20) (PharMingen), α-Helios-Pacific Blue or PE (22F6) (Biolegend), α-CD121a/IL-1R1 (35F5) (BD Biosciences) and CD90.2 Alexa 780 (53-2.1). Non-viable cells were excluded using fixable viability dye eFluor780 or 506 reagent (Thermofisher). Data were acquired using a FACS Fortessa X-20 flow cytometer (BD Biosciences) and analysed using FlowJo version 9 software (TreeStar).

Statistical analysis

For all experiments, the mean and standard deviation are shown, unless otherwise stated. Multiple comparisons were tested using a two-way ANOVA with a Tukey post-test for comparison of all individual means within a figure or One-way ANOVA when required. For single comparisons, *N* unpaired Student's *t*-test was used with the p-value expressed in the figure legend. All statistical analysis was performed with GraphPad Prism version 5 software (GraphPad Software).

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AUTHOR CONTRIBUTIONS

F.A., J.H.F. and C.A.P. designed the conceptual framework of the study, designed experiments and wrote the paper. F.A. designed and performed experiments and analysed data. R.I. contributed to the in vivo Influenza experiments. M.S. and S.Q. contributed on the design and experiment involving *Cryptococcus neoformans*. N. P. and T.A. contributed to the Influenza and Colitis experiments. All authors provided valuable input throughout the study and the writing of the manuscript.

ADDITIONAL INFORMATION

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