IL-1 signaling modulates activation of STAT transcription factors to antagonize retinoic acid signaling and control the $T_{\text{H}17}$ cell–iT$_{\text{reg}}$ cell balance

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Interleukin 17 (IL-17)-producing helper $T$ cells ($T_{\text{H}17}$ cells) and CD4$^+$ inducible regulatory $T$ cells (iT$_{\text{reg}}$ cells) emerge from an overlapping developmental program. In the intestines, the vitamin A metabolite retinoic acid (RA) is produced at steady state and acts as an important cofactor to induce iT$_{\text{reg}}$ cell development while potently inhibiting $T_{\text{H}17}$ cell development. Here we found that IL-1 was needed to fully override RA-mediated expression of the transcription factor Foxp3 and induce protective $T_{\text{H}17}$ cell responses. By repressing expression of the negative regulator SOCS3 dependent on the transcription factor NF-$\kappa$B, IL-1 increased the amplitude and duration of phosphorylation of the transcription factor STAT3 induced by $T_{\text{H}17}$-polarizing cytokines, which led to an altered balance in the binding of STAT3 and STAT5 to shared consensus sequences in developing $T$ cells. Thus, IL-1 signaling modulated STAT activation downstream of cytokine receptors differently to control the $T_{\text{H}17}$ cell–iT$_{\text{reg}}$ cell developmental fate.

Interleukin 17 (IL-17)-producing helper $T$ cells ($T_{\text{H}17}$ cells) and CD4$^+$ induced regulatory $T$ cells (iT$_{\text{reg}}$ cells) emerge from a shared developmental axis$^1$. While transforming growth factor-$\beta$ (TGF-$\beta$) contributes to the developmental programming of both subsets, the proinflammatory cytokine IL-6 favors $T_{\text{H}17}$ cell development at the expense of iT$_{\text{reg}}$ cell development$^2$–$^6$. Conversely, retinoic acid (RA), a metabolite of vitamin A produced by intestinal stromal cells and dendritic cells (DCs) that express retinaldehyde dehydrogenases$^7$, acts in concert with TGF-$\beta$ to promote expression of the transcription factor Foxp3 and iT$_{\text{reg}}$ cell development while potently inhibiting $T_{\text{H}17}$ cell development$^8$–$^{12}$. A substantial percentage of $T_{\text{H}17}$ cells resident in the intestinal lamina propria have expressed Foxp3 at some point during their development, which indicates a dynamic relationship between ROR$\gamma^+$ $T_{\text{H}17}$ cells and Foxp3$^+$ iT$_{\text{reg}}$ cells developing in the intestines$^5$. Whereas IL-6 signaling induces phosphorylation of the transcription factor STAT3 that is required for expression of the transcription factor ROR$\gamma$ and $T_{\text{H}17}$ cell development, the actions of RA are at least partially dependent on IL-2, which induces phosphorylation of STAT5 that is required for Foxp3 expression and iT$_{\text{reg}}$ cell development, and which suppresses $T_{\text{H}17}$ cell development$^9$,$^{13,14}$. Many DNA-binding sites targeted by STAT3 in gene loci associated with the $T_{\text{H}17}$ lineage can also bind STAT5, which provides a mechanism for competitive antagonism of these trans-acting factors downstream of signaling via the IL-6 and IL-2 receptors$^{15}$. While IL-6 is important for reciprocally promoting $T_{\text{H}17}$ cell development and repressing iT$_{\text{reg}}$ cell development, its effects in countering RA-mediated inhibition of $T_{\text{H}17}$ cell development are incomplete$^9$,$^{14}$. Thus, even at saturating doses of IL-6, $T_{\text{H}17}$ cell development is suppressed by RA, which suggests that additional signals might be needed to override the dominant RA signaling in the gut. IL-1 is another proinflammatory cytokine that promotes $T_{\text{H}17}$ cell development while subverting iT$_{\text{reg}}$ cell development$^6$,$^{16}$, and it has been shown to be important in the development of $T_{\text{H}17}$ cells in the gut, at least at steady state$^{17}$. However, other reports have suggested that signaling via IL-1$\beta$ and its receptor IL-1R is dispensable in the differentiation of intestinal $T_{\text{H}17}$ cells at steady state, on the basis of studies of mice deficient in the adaptor MyD88, which have impaired signaling via IL-1R and Toll-like receptors$^{18,19}$. The role of IL-1 signaling during pathogen-induced intestinal inflammation has not been well studied. Therefore, both the mechanism by which IL-1 promotes intestinal $T_{\text{H}17}$ cell development and the role of IL-1 in $T_{\text{H}17}$ cell development during acute intestinal inflammation, particularly in disease models in which $T_{\text{H}17}$ cells are protective, remain largely undefined$^{20,21}$.

In this study, we sought to define the role of IL-1 in regulating the developmental balance of $T_{\text{H}17}$ cells and iT$_{\text{reg}}$ cells, with emphasis on its effects on the intestine during challenge with the enteropathogenic bacterium Citrobacter rodentium, which elicits a $T_{\text{H}17}$ pathway response that is required for host protection$^{5,21,22}$. Our results indicated that IL-1 signaling was needed to enhance IL-6 signaling.

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to fully override RA-mediated Foxp3 expression and induce protective Th17 cell responses. Through a mechanism dependent on the transcription factor NF-κB, IL-1 signaling repressed the expression of SOCS3, a feedback inhibitor of tyrosine phosphorylation of STAT3 induced by Jak kinase. Inhibition of SOCS3 expression by IL-1 signaling resulted in an increased amplitude and duration of the tyrosine-phosphorylation of STAT3 downstream of signaling by Th17-polarizing cytokines without altering the IL-2-induced tyrosine-phosphorylation of STAT5. IL-1 signaling therefore altered the balance of the binding of STAT3 versus that of STAT5 to shared consensus sequences in developing T cells, including the conserved noncoding sequence 2 (CNS2) element in the Foxp3 locus that regulates stability of Foxp3 expression, as well as target sequences in the Il17a–Il17f locus. Thus, IL-1 signaling modulated STAT activation downstream of cytokine receptors differently to control the Th17 cell–iTreg cell developmental fate.

RESULTS

IL-1β reverses RA-induced inhibition of Th17 differentiation

IL-6 counteracts the effects of the RA-mediated suppression of Th17 cell development, albeit incompletely\(^3\). In the course of investigating the role of IL-1β in promoting Th17 cell development, we found that in contrast to IL-6, IL-1β completely reversed the impairment of Th17 differentiation observed when DCs from mesenteric lymph nodes (MLNs) were used to activate naive CD4+ T cells (Fig. 1a, b). Moreover, IL-1β was comparable to the retinoic acid receptor (RAR) inhibitor LE450 in blocking the effects of RA (Fig. 1a, b). Accordingly, the addition of IL-1β overrode the inhibition of Th17 differentiation by RA, regardless of RA concentration (Fig. 1c, d). This result was not due to downregulation of the receptor subunits RAR or RXR, as all members of this family were either unchanged or modestly increased by IL-1 signaling, and this occurred despite partial RA-mediated down-modulation of IL-1R1, which had higher expression by developing Th17 cells than by Th0 cells (Supplementary Fig. 1).

To extend the studies reported above, we investigated the effects of IL-1 on reversing the expression of Foxp3 supported by RA signaling in developing Th17 cells (Fig. 1e, f). Using Th17 cells derived from naive precursor cells from Il17f/−/−Foxp3/−/− dual-reporter mice (homozygous for sequence encoding the alloantigen Thy-1.1 knocked into the Il17f locus and for sequence encoding green fluorescent protein (GFP) knocked into the Foxp3 locus), we sorted Foxp3−/−IL-17F− cells and restimulated them under Th17-polarizing conditions in the presence or absence of added RA. In the absence of added RA, a significant fraction of sorted Foxp3−/− cells lost Foxp3 expression and expressed IL-17F. This transition was inhibited

Figure 1 IL-1β counteracts the RA-dependent inhibition of Th17 cell development. (a) Flow cytometry of Il17f/−/− naive CD4+ T cells (CD4+CD25−CD62Lhi CD44lo) activated for 4 d with soluble anti-CD3 on IL-1β-deficient (Il17f/−/−) MLN DCs under Th17-polarizing conditions alone (left margin) in the presence or absence of increasing concentrations of RA (above plots). (d) Frequency of cells as in b among CD4+ T cells activated for 4 d with soluble anti-CD3 and Il17f/−/− DCs under Th17-polarizing conditions with or without IL-1β (left margin) in the presence or absence of increasing concentrations of RA (above plots). (d) Frequency of cells as in b among CD4+ T cells activated for 4 d with soluble anti-CD3 and Il17f/−/− DCs under Th17-polarizing conditions with or without IL-1β (left margin) in the presence or absence of increasing concentrations of RA (above plots). (d) Frequency of cells as in b among CD4+ T cells activated for 4 d with soluble anti-CD3 and Il17f/−/− DCs under Th17-polarizing conditions with or without IL-1β (left margin) in the presence or absence of increasing concentrations of RA (above plots). (d) Frequency of cells as in b among CD4+ T cells activated for 4 d with soluble anti-CD3 and Il17f/−/− DCs under Th17-polarizing conditions with or without IL-1β (left margin) in the presence or absence of increasing concentrations of RA (above plots). (d) Frequency of cells as in b among CD4+ T cells activated for 4 d with soluble anti-CD3 and Il17f/−/− DCs under Th17-polarizing conditions with or without IL-1β (left margin) in the presence or absence of increasing concentrations of RA (above plots).
IL-1R signaling is required for the host-protective T\textsubscript{H}17 and T\textsubscript{H}17 cell-iT\textsubscript{reg} cell balance in vivo. (a) Serial whole-body imaging of IL17f\textsubscript{–/–}, Il1r1\textsuperscript{–/–}, and Il17f\textsubscript{–/–} Il1r1\textsuperscript{–/–} mice inoculated with a luminescent strain of C. rodentium and left untreated or treated with an IL-17F+ cell–depleting mAb to Thy-1.1 (α-Thy-1.1), imaged 5, 9 or 13 d after infection (above images). (b) Kinetics of the colonization of mice as in a at 5–13 d after infection, presented as counts per second. (c,d) Quantitative enzyme-linked immunosorbent assay (ELISA) of IL-17A (pg/ml) in supernatants of cultured homogenates of colonic tissue collected from IL17f\textsubscript{–/–} and Il1r1\textsuperscript{–/–} mice left uninfected (UI) or 1–13 d after inoculation with C. rodentium. (e) Flow cytometry (right) of IL17f\textsubscript{–/–} (CD45.1\textsuperscript{–}) and IL17f\textsubscript{–/–} (CD45.1\textsuperscript{–}) splenic lymphocytes (SPL) and colonic lamina propria lymphocytes (LPL) from recipient Tcrb\textsuperscript{–/–} mice reconstituted with a mixture of 3 × 10\textsuperscript{6} IL17f\textsubscript{+}/IL17f\textsubscript{–/–} (CD45.2\textsuperscript{+}) donor CD4\textsuperscript{+} T cells, followed by infection of recipients with C. rodentium 2 weeks after reconstitution (protocol, Supplementary Fig. 2d) and analysis, 7 d later, of the expression of Thy-1.1 (IL-17F) and intracellular Foxp3 with gating on activated CD4\textsuperscript{+} T cells. Far left, numbers above outlined areas indicate percent CD45.1\textsuperscript{+} (IL17f\textsubscript{–/–}) (right) or CD45.1\textsuperscript{–} (IL17f\textsubscript{–/–}) (left) TCR\textsuperscript{β} donor cells in the recipient mice. (f) Frequency of IL-17F\textsuperscript{+} (Thy-1.1\textsuperscript{+}) cells among IL1r1\textsuperscript{+/+} and IL1r1\textsuperscript{–/–} populations from the spleen, MLNs and lamina propria of the large intestine (LP) of recipient Tcrb\textsuperscript{–/–} mice reconstituted as in e and left uninfected (UI) or infected (Inf) with C. rodentium 2 weeks after reconstitution. NS, not significant; *P < 0.05 and **P < 0.01 (two-tailed unpaired t-test). Data are from one experiment representative of two (anti-Thy-1.1 treated IL-17F+ mice) or three (untreated IL17f\textsubscript{–/–} II17f\textsubscript{–/–} mice) independent experiments with similar results (a), pooled from two or three independent experiments with nine to eleven mice per group (b, mean and s.e.m.) or two independent experiments with six mice per group (f, mean and s.e.m.), are from two independent experiments with six mice per group (c,d, mean and s.e.m.) or are representative of one of two similar independent experiments (e).

IL-1R1 deficiency impairs the development of T\textsubscript{H}17 cells

To explore the role of IL-1 signaling in modulating the T\textsubscript{H}17 cell–iT\textsubscript{reg} cell balance in vivo, we assessed infection by the intestinal pathogen C. rodentium, protection against which is mediated by the T\textsubscript{H}17 pathway\textsuperscript{21,22}. It has been suggested on the basis of studies of steady-state intestinal T\textsubscript{H}17 cell development that conflicting reports on the role of IL-1 have arisen due to use of the stimulation of recovered T cells ex vivo with the phorbol ester PMA and ionomycin\textsuperscript{23}. We therefore used IL-1R1-deficient and IL-1R1-deficient IL17f\textsubscript{–/–} Foxp3\textsuperscript{GFP} mice to directly examine gene expression ex vivo without requirement for an in vitro recall response induced by stimulation with PMA plus ionomycin or with antibody to the invariant signaling protein CD3 (anti-CD3)\textsuperscript{24}. Because IL17f expression occurs early in T\textsubscript{H}17 cell development, at which time it is dominant over IL17a expression\textsuperscript{24}, the IL17f\textsubscript{–/–} reporter model provides a sensitive ‘readout’ of early gene expression in T\textsubscript{H}17 cells. Moreover, the IL17f\textsubscript{–/–} reporter model enables specific depletion of IL-17F–producing cells in vivo by administration of monoclonal antibody (mAb) to Thy-1.1 (ref. 25).

Depletion of IL-17F–producing cells from C. rodentium–inoculated IL17f\textsubscript{–/–} single- reporter mice through the use of mAb to Thy-1.1 during the peak of infection (3–7 d after infection)\textsuperscript{23} resulted in impaired clearance of bacteria and heightened injury of the intestinal mucosa (Fig. 2a,b and Supplementary Fig. 2a,b). Infection of mice deficient in IL-1R1 (Il1r1\textsuperscript{–/–}) mice showed similarly impaired host protection with a concomitant decrease in colonic production of IL-17A but not of interferon-γ (IFN-γ) (Fig. 2c,d). Those results were similar to published findings obtained with mice deficient in the IL-17 receptor component IL-17RA and were consistent with the possibility of a role for IL-1 in promoting T\textsubscript{H}17 pathway–dependent host protection\textsuperscript{21}. Depletion of IL-17–producing cells from Il1r1\textsubscript{–/–} Il17f\textsubscript{–/–} mice did not significantly alter this result (Fig. 2a,b), which suggested that a major effect of IL-1R1 deficiency during this period of the infection was mediated by its actions on these cells. Production of IL-22 in the infected colon was reduced in IL-1R1-deficient mice, but only at the peak time point (day 7 after infection; Supplementary Fig. 2c).

We observed a transient increase in Foxp3\textsuperscript{+} CD4\textsuperscript{+} T cells in the large intestine and draining lymph nodes of IL-1R1–deficient mice during infection (days 3–5; Supplementary Fig. 3a,b), as well as a shift in the balance of IL-17\textsuperscript{+} cells versus Foxp3\textsuperscript{+} cells recovered from the large intestine of infected IL-1R1–deficient mice (Supplementary Fig. 3c,d). Therefore, we posited that infection-induced IL-1 signaling in CD4\textsuperscript{+} T cells might be needed to overcome the T\textsubscript{reg} cell...
programming that is favored in intestinal tissues by the production of RA, as was observed \textit{ex vivo}. To test this hypothesis, we obtained congenically marked CD4+ T cells from IL-1R1-sufficient (CD45.1+) C. rodentium Il17f−/− mice or IL-1R1-deficient (CD45.1−) C. rodentium Il17f− CD4+ T cells isolated from infected IL-1R1-sufficient and IL-1R1-deficient Il17f−/− Foxp3+ GFP mice at day 10 after infection with C. rodentium. (f) Histopathology scores of colons from mice as in e, assessing the epithelium (Epi) and inflammation (Infl), as well as the aggregate histopathology score (Total). *P < 0.05 and **P < 0.01 (two-tailed unpaired t-test). Data are from one experiment representative of three (a) or two (c,e) independent experiments with similar results (a,c,e) or are pooled from three (b) or two (d,f) independent experiments with ten (b), six (d) or four (f) mice per group (b,d,f; mean and s.e.m.).

\textbf{In vivo blockade of RA compensates for IL-1 signaling deficiency}

To further investigate the possible transition of Foxp3+ precursor cells into IL-17-producing effector cells, we tracked the fate of Foxp3+ IL-17F− CD4+ T cells isolated from infected IL-1R1-sufficient and IL-1R1-deficient Il17f−/− Foxp3+ GFP mice after their transfer into infected TCRβ−/− mice. In accord with the possibility of a role for IL-1 signaling in promoting the fruiting of Foxp3 and induction of IL-17, we recovered a significantly greater frequency of Foxp3+ T cells from the recipients of IL-1R1-deficient Foxp3+ T cells than from the recipients of IL-1R1-sufficient Foxp3+ T cells, with a deficit in IL-17+ T cells (Fig. 3a,b and Supplementary Fig. 3e). We then determined whether the impaired Treg cell development in the IL-1R1-deficient mice was due to a failure to overcome RA-mediated repression. We treated C. rodentium–infected IL-1R1-deficient Il17f−/− Foxp3+ GFP mice with an antagonist of RAR or vehicle following inoculation with C. rodentium (Fig. 3c,d). Blockade of RAR signaling in infected IL-1R1-deficient mice significantly reversed the deficit in IL-17-producing T cells in both the lamina propria of the large intestine and MLNs but not in the spleen; this blockade in the IL-1R1-deficient mice resulted in decreased bacterial loads associated with RA.

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\caption{In the absence of IL-1β, \textit{in vivo} blockade of RA facilitates the iTreg cell to T H17 cell transition during enteropathogenic bacterial infection. (a) Expression of Thy-1.1 (IL-17F) and Foxp3-GFP on splenic and MLN cells and LPLs isolated from Tcrb−/− recipients left uninfected or infected with C. rodentium 2 d before transfer of 2.5 × 10⁶ Foxp3+ IL-17F− donor CD4+ T cells (collected from C. rodentium–infected Il1r1−/− mice (WT) or Il1r1−/− Il17f−/− Foxp3+ GFP mice (IL1R1-deficient)) and sorted on day 2 after infection; protocol, Supplementary Fig. 3e), analyzed by flow cytometry on day 8 after infection, without restimulation and MLN cells and LPLs isolated from infected (infected), then assessed the frequency of Foxp3+ cells and IL-17F+ (infected), and Foxp3+ cells as prepared in a. (c) Expression of Thy-1.1 (IL-17F) and Foxp3 (GFP) on CD4+CD25+ T cells from the spleen, MLN and lamina propria of Il1r1−/− Il17f−/− Foxp3+ GFP mice inoculated with 2 × 10⁹ colony-forming units of C. rodentium and given vehicle alone (Veh) or the RA inhibitor LE135 by gavage on days 3–7 after infection, analyzed by flow cytometry on day 8 after infection, without restimulation \textit{ex vivo}. (d) Frequency of IL-17F+ (Thy-1.1+) and Foxp3-GFP+ cells among the Foxp3+ (GFP+) or activated (CD4+CD25+) fraction of splenic and MLN cells and LPLs of C. rodentium–infected Il1r1−/− Il17f−/− Foxp3+ GFP mice treated with vehicle or LE135. Each symbol represents an individual mouse; small horizontal lines indicate the mean. (e) Histopathology of hematoxylin- and eosin-stained colonic tissue sections derived from vehicle- or LE135-treated Il1r1−/− Il17f−/− Foxp3+ GFP mice at day 10 after infection with C. rodentium. (f) Histopathology scores of colons from mice as in e, assessing the epithelium (Epi) and inflammation (Infl), as well as the aggregate histopathology score (Total). *P < 0.05 and **P < 0.01 (two-tailed unpaired t-test). Data are from one experiment representative of three (a) or two (e) independent experiments with similar results (a,e) or are pooled from three (b) or two (d,f) independent experiments with ten (b), six (d) or four (f) mice per group (b,d,f; mean and s.e.m.).}
\end{figure}
with protection from mucosal injury and less weight loss, but such blockade in their IL-1R1-sufficient counterparts did not (Fig. 3e,f and Supplementary Fig. 4). Collectively, these findings identified a role for pathogen-induced IL-1 signaling in overriding the iT_{reg} cell programming favored by RA-producing intestinal DCs to induce protective T_{H}17 cell responses to microbial antigens.

**IL-1β counters RA-driven repression of T_{H}17 cells**

RA suppresses T_{H}17 cell development in favor of iT_{reg} cell development at least in part via an IL-2-dependent mechanism. To determine whether IL-1 signaling might also modulate IL-2-dependent suppression of T_{H}17 differentiation, we assessed the effect of adding increasing concentrations of IL-2 to cultures of naive CD4^{+} T cells activated by antigen under T_{H}17-polarizing conditions, with or without the addition of IL-1β. In the absence of added IL-1β, increasing concentrations of IL-2 suppressed the frequency of IL-17^{+} cells, with a reciprocal increase in the frequency of Foxp3^{+} cells (Fig. 4a,b), as reported. IL-1β abrogated the suppressive effects of IL-2, regardless of the concentration of IL-2 (Fig. 4a,b). We obtained comparable effects of IL-1β on IL-17 expression and Foxp3 repression under DC-free conditions of T_{H}17 polarization, wherein the induction of higher concentrations of endogenously produced IL-2 favored less-robust T_{H}17 cell development and higher expression of Foxp3 (Supplementary Fig. 5 and data not shown). Notably, IL-23 signaling had no comparable effect on the T_{H}17 cell–iT_{reg} cell balance during the early stages of differentiation (3–4 d), when IL-1 signaling induced an enhanced T_{H}17 response, and the addition of both IL-23 and IL-1β did not augment the effect of IL-1β alone (Supplementary Fig. 5b,c and data not shown). So, in line with its effects on overriding the RA-mediated repression of T_{H}17 differentiation, IL-1β antagonized the effects of IL-2, and this effect was independent of a requirement for co-signaling via IL-23.

The similar effects of IL-1 signaling on the reversal of RA- and IL-2-mediated inhibition of T_{H}17 differentiation were consistent with our finding that the production of IL-2 by T cells was enhanced about threefold by the addition of RA, at all concentrations tested (Fig. 4c). This result indicated that the RA-mediated repression of T_{H}17 differentiation was due at least in part to enhanced IL-2 production. Consistent with the actions of IL-2 in inducing higher expression of the inducible components of IL-2R, the addition of RA also upregulated the expression of IL-2Rα (CD25) and IL-2Rβ (CD122) (Fig. 4d). Notably, neither the enhanced IL-2 production nor the upregulation...
of the expression of components of the IL-2 receptor by RA was significantly affected by IL-1β (Fig. 4c,d), which suggested that the principal effect of IL-1β in blocking the effects of RA was probably due to interference with IL-2 signaling downstream of receptor binding of IL-2. Accordingly, blockade of IL-2 signaling reversed the reciprocal effects of RA on the Th17 cell–iTreg cell developmental balance, similar to the effects induced by IL-1β (Fig. 4e,f). Furthermore, in Cd4+ T cells deficient in STAT5 expression and therefore RA-dependent signaling, the effects of RA in repressing IL-17 expression in favor of Foxp3 expression were similarly overridden and were not further augmented by IL-1β signaling (Fig. 4e,f).

Although RA acted to enhance IL-2 production and expression of the inducible components of the IL-2 receptor by T cells, the reversal of RA-mediated effects by IL-1β signaling appeared to be mediated mainly through interference of IL-2 signaling and not through altered expression of IL-2 or its receptor.

IL-1β enhances phosphorylated STAT3 via repression of SOCS3

To explore mechanisms by which IL-1 might interfere with the RA-mediated modulation of IL-2 signaling, we assessed the tyrosine-phosphorylation of STAT3 following stimulation of T cells under Th17-polarizing conditions with or without the addition
of either RA or RA plus IL-1β. Consistent with its effects on enhancing the expression of IL-2 and its receptor, RA enhanced the tyrosine-phosphorylation of STAT5 in TH17 cells, a result that was not altered substantially by concurrent IL-1 signaling (Fig. 5a). In contrast, RA had no effect on IL-6-induced tyrosine-phosphorylation of STAT3, whereas the addition of IL-1 resulted in a substantial increase in tyrosine-phosphorylated STAT3 (Fig. 5a). The effect of IL-1 signaling on differentiating TH17 cells resulted in both increased and more-sustained tyrosine-phosphorylation of STAT3 (Fig. 5b) and was not limited to IL-6-induced tyrosine-phosphorylation of STAT3, as we obtained similar results when STAT3 was activated downstream of signaling by IL-21 (Supplementary Fig. 6) or IL-23 (Fig. 5c). Therefore, while IL-1 signaling failed to directly repress the RA-mediated enhancement of IL-2-induced tyrosine-phosphorylation of STAT5, it did alter the ratio of tyrosine-phosphorylated STAT5 to tyrosine-phosphorylated STAT3 through its enhancement of IL-6-, IL-21- and IL-23-induced tyrosine-phosphorylation of STAT3, which established an indirect mechanism by which IL-1 might act in concert with each of the STAT3-activating components (relA and NF-κB). In addition to its effects on the tyrosine-phosphorylation of STAT3, we found that IL-1 signaling increased the phosphorylation of Ser727 of STAT3 via the p38 kinase pathway (Supplementary Fig. 7a,b), an effect that has been shown to potentiate the actions of the STAT3 homodimer28. However, this effect was more transient than the IL-1-induced tyrosine phosphorylation of STAT3 and was independent of a requirement for co-signaling via IL-23 (or IL-6) (Supplementary Fig. 7a,b). This suggested that any effects on the serine-phosphorylation of STAT3 would probably be secondary to the principal effects on tyrosine-phosphorylation modulated by the NF-kB–SOCS3 axis.

Finally, we directly established a role for IL-1-mediated repression of SOCS3 in overriding the RA-mediated inhibition of TH17 differentiation, as the inhibitory effect of RA was largely mitigated by specific
IL-1β modulates STAT3 and STAT5 binding to consensus sequences

Given the dominant effect of IL-1 in overriding RA- and IL-2-induced repression of T_{H}17 cell development, we reasoned that the actions of IL-1 signaling in enhancing the phosphorylation of STAT3 might overcome competition by phosphorylated STAT5 for binding to shared STAT-binding consensus sequences induced by RA-dependent IL-2 signaling. We performed chromatin-immunoprecipitation analyses to quantify the relative binding of phosphorylated STAT3 and STAT5 to consensus regulatory sites in the Il17a-Il17f and Foxp3 loci under various conditions of the addition RA and IL-1β, in T_{H}17-polarized cells activated by IL-6 alone (STAT3) or IL-6 plus IL-2 (STAT5). As a control for the STAT5-dependent effects of RA, we included T_{H}17-polarized cells deficient in STAT5 (Stat5^{-/-}) cells. We found that, with the exception of the intergenic enhancer 10 kilobases (kb) downstream of the Il17a transcription start site, all STAT3-STAT5 consensus sites in the Il17a-Il17f locus of T_{H}17 cells treated with RA alone showed reciprocal decreased binding of STAT3 and increased binding of STAT5 compared with their binding in the absence of RA (Fig. 7a). The decrease in STAT3 binding caused by the addition of RA was reversed in STAT5-deficient cells (Fig. 7a), consistent with the actions of RA in enhancing IL-2-dependent signaling via STAT5. The addition of IL-1β reversed both the RA-induced increase in binding of STAT5 and decrease in binding of STAT3 (Fig. 7a), consistent with the augmented phosphorylation of STAT3 induced by IL-1 signaling (Fig. 5b–f). At the intergenic enhancer downstream of Il17a noted above, the binding of both STAT3 and STAT5 was unaffected by addition of RA, although both were significantly affected by IL-1β (Fig. 7a), which indicated that additional regulatory mechanisms might confer enhanced binding of STAT3 at this cis-regulatory element regardless of IL-2-induced phosphorylation of STAT5.

Competition between phosphorylated STAT5 and STAT3 in binding to the CNS2 intronic enhancer of Foxp3 has been proposed as regulating the heritable maintenance of Foxp3 expression and, thus, the stability of the T_{reg} cell developmental program. We found that RA, by enhancing the binding of STAT5, also restricted the IL-6-mediated binding of STAT3 at CNS2 in the Foxp3 locus during T_{H}17 differentiation but was outcompeted by increased binding of STAT3 promoted by IL-1 (Fig. 7b). Consistent with those results, while IL-1 alone had no effect on repressing Foxp3 expression during iT_{reg} cell development (Supplementary Fig. 8a), it substantially augmented the limited extinction of Foxp3 expression induced by IL-6 in secondary cultures of sorted Foxp3^{+} T cells derived from Il17f^{−/−}/Foxp3^{GFP} dual-reporter mice (Supplementary Fig. 8b). Indeed, even in the presence of exogenous RA, which promoted stability of Foxp3 expression, greater than 50% of cells extinguished their expression of Foxp3, with a significant population converting into IL-17F-producing cells (Supplementary Fig. 8b). Thus, IL-1 acted cooperatively with IL-6 to subvert the Foxp3^{+}-iT_{reg} cell program in favor of T_{H}17 cell development due at least in part to its actions in altering the relative binding of phosphorylated STAT3 and STAT5 at the CNS2 enhancer element in the Foxp3 locus.

**DISCUSSION**

The early developmental programs of iT_{reg} cells and T_{H}17 cells are intimately linked, reflected in the coexpression of the lineage-specifying transcription factors Foxp3 and RORγt downstream of TGF-β signaling. The balance between the antagonistic effects of RA and IL-2, which promote iT_{reg} cell development while suppressing T_{H}17 cell development, and those of IL-6 and IL-1, which promote T_{H}17 cell development while suppressing iT_{reg} cell development, is particularly critical in determining homeostatic immunity versus host-protective immunity to microbes in the intestines, where production of RA by resident DCs normally favors antimicrobial tolerance. Here we found that IL-1 signaling override the effects of...
the RA–IL-2 axis in promoting iTreg cell differentiation to allow a host-protective T_{H17} response to an enteric pathogen, and we defined a mechanism by which IL-1 signaling modulated the T_{H17} cell–iTreg cell developmental balance. By enhancing the amplitude and duration of phosphorylated STAT3 activity induced by T_{H17}-specifying cytokines (for example, IL-6 and IL-23), IL-1 altered the ratio of phosphorylated STAT3 to phosphorylated STAT5 in developing T cells to enhance T_{H17} cell development at the expense of iTreg cell development. Indeed, by favoring the binding of phosphorylated STAT3 over that of phosphorylated STAT5 at the CNS2 intronic enhancer in the Foxp3 locus, IL-1-dependent potentiation of STAT3 signaling directly subverted the T_{reg} cell–stabilizing function of phosphorylated STAT3 to phosphorylated STAT5 in developing T cells to promote T_{H17} cell development.

Cytokines to promote T_{H17} cell development.

Although the contribution of IL-1 to T_{H17} differentiation is well established, the mechanism of its action has been unclear. Here we identified a link between IL-1 signaling and repression of SOCS3 expression as a mechanism for specifically augmenting signaling by STAT3 but not that of STAT5. Published studies have detailed the structural basis by which SOCS3 interacts with the complex composed of the signal-transducing receptor gp130 and kinase Jak1 following ligand-mediated trans-phosphorylation of the cytoplasmic domain of receptors of the IL-6 family, which explains the specificity of SOCS3 for this family of receptors, and presumably non-gp130 receptors such as the structurally related IL-23R and gp130, but not the IL-2 receptor. The rapid induction of SOCS3 expression downstream of signaling via IL-6 (or IL-23) provides a negative feedback loop that rapidly terminates signaling via the IL-6 receptor or IL-23 receptor by inhibiting receptor-bound Jak1 or Jak2, respectively. Through repression of SOCS3 expression, IL-1 indirectly sustains Jak-STAT interactions that potentiate phosphorylated STAT3 actions. Absent comparable effects on IL-2 receptor–induced phosphorylation of STAT5, IL-1 signaling effectively shifts the ratio of phosphorylated STAT3 to phosphorylated STAT5 in favor of phosphorylated STAT3, providing a competitive advantage for the binding of phosphorylated STAT3 to shared DNA target sequences, as demonstrated in our chromatin-immunoprecipitation studies.

Interestingly, IL-1 augmented the IL-21-induced phosphorylation of STAT3 similarly to that induced by IL-6 and IL-23. Although the IL-21 receptor is unlike other common γ-chain cytokine receptors in activating mainly STAT3, not STAT5, as far as we are aware, a direct role for SOCS3 in modulating signaling via the IL-21 receptor has not been defined. Additional studies will be needed to determine whether the IL-21 receptor is unusual among common γ-chain cytokine receptors in recruiting SOCS3 or whether other effects of IL-1 signaling contribute to the increased phosphorylation of STAT3 induced by IL-21. IL-1 is notable that TGF-β has been reported to contribute to T_{H17} cell development through the inhibition of IL-6 and IL-21-induced SOCS3 expression, although another study has found that TGF-β also increases STAT5 phosphorylation, an effect we did not observe here for IL-1 signaling. Thus, although both IL-1 and TGF-β might repress SOCS3 expression in concert with T_{H17}-polarizing cytokines, albeit via fundamentally different signaling pathways, IL-1 would seem to ‘preferentially’ alter the ratio of phosphorylated STAT3 to phosphorylated STAT5 in favor of phosphorylated STAT3. In this context, it is of interest that T_{H17} cell development can occur in the absence of TGF-β signaling, contingent on co-signaling by IL-1β with either IL-6 or IL-23 (ref. 37). It would therefore be of interest to determine whether this effect is mediated by IL-1-induced inhibition of SOCS3.

In addition to positioning SOCS3 for inhibition of the Jak catalytic domain, docking of the SH2 domain of SOCS3 onto phosphorylated Tyr759 of the IL-6-activated gp130 cytoplasmic domain inhibits binding.

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while promoting expression of the gene encoding RORγt and other genes associated with the Th17 lineage that contain consensus sequences regulated by competitive antagonism of STAT5 binding versus STAT3 binding and thereby deviate nascent or immature iTreg cells toward Th17 cell development. These findings extend the role of proinflammatory cytokines in overriding a dominant program of iTreg cell development in the intestines to enable the recruitment of Th17 cell–mediated host defense and provide a rationale for the consideration of IL-1 blockade to reestablish homeostasis in the treatment of chronic inflammatory diseases mediated by the Th17 pathway.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

R.B., S.K.W., R.D.H. and C.T.W. designed the studies; R.B., S.K.W., S.R. and R.D.H. performed the experiments; C.L.Z. advised on design and execution of figures; T.R.S. performed analyses of pathology; E.N.B. assisted in the design and comments; G. Frankel and S. Wiles (Imperial College, London) for the bioluminescent imaging; and B.J. Parsons for technical assistance; G. Gaskins for editorial assistance; the University of Alabama at Birmingham Small Animal Imaging Facility for imaging studies; the University of Alabama at Birmingham Center for AIDS Research Flow Cytometry Core for sorting cells by flow cytometry; and the University of Alabama at Birmingham Epitope Recognition and Immunoregulant Core Facility for antibody preparation. Supported by the US National Institutes of Health (PO1DK71176 and R01DK93015 to C.T.W. and R.D.H., and R01AI047833 to W.S.P.) and the Crohn’s and Colitis Foundation of America (C.T.W. and R.B.).

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. The following mouse strains used were from the Jackson Laboratory and/or were bred at the University of Alabama at Birmingham (UAB) animal facility: C57BL/6 (B6); B6.129P2-Trexp1(Thy1)Weav (Th1); B6.129S-Ii(3)129 Fl/J (IIb−/−); B6;Cg-Tg(-Il17f–κ alleles(TcRb)1Fls/J (OT-II); B6.129(Cg)-Foxp3–GFP+YFP+ (Foxp3-GFP+); B6.129S7-Il11r1tm1(Cng)J (Ultr1−/−); B6.129S-Stat5a−/−Stat5b−/− (Mjmax (Stat5b)°); B6.129S1-RelaFL−/− (RedbFL−); B6.Cg-Tg(Cd4-Cre)1Cre (Cd4-Cre) mice were from Taconic, and B6.CD4.1 mice were from Frederick Cancer Center. All strains were on a C57BL/6 (B6) background. All intercrosses to generate additional strains, such as wild-type and IL-1R-deficient dual reporter (IIbFBy−/−-foxp3-GFP) mice, IIbFBy−/− CD4.5+1 CD45.2+ mice and Stat5b−/− Cd4-Cre mice, were generated by crosses in UAB's breeding facility. Animals were bred and maintained under specific pathogen–free condition in accordance with institutional animal care and use committee regulations.

C. rodentium infection and mAb treatments. C. rodentium strain DBS100 (American Type Culture Collection) was used for all inoculations, with the exception of experiments in which whole-body imaging was performed. For imaging experiments, the bioluminescent C. rodentium strain ICC180 (derived from DBS100) was used. This strain has a constitutive lucD2Bae operon that encodes luciferase of the nematode C. elegans. For infection experiments using DBS100, C. rodentium strain DBS100 was grown at 37 °C in LB broth, and C. rodentium strain ICC180 was grown in LB broth containing kanamycin (100 µg/mL). Mice were inoculated with 2 × 10^8 colony-forming units of C. rodentium (a high dose) in a total volume of 200 µl of PBS via gastric gavage.

For depletion of IIbFBy−/−-1 cells in vivo, mice were given intraperitoneal injection of 400 µg anti-Thy-1.1 (19E1.2; University of Alabama–Birmingham Epitope Recognition and Immunoreagent Core Facility) on days 3 and 7 after infection. In all studies, age- and sex-matched mice 6–12 weeks of age were used.

Bioluminescence imaging. Mice were anesthetized with isoflurane and were placed in the supine position in a custom-built chamber for imaging with the IVIS-100 system and Living Image Software (Xenogen). Baseline images were obtained before gavage with 2 × 10^8 colony-forming units of C. rodentium strain ICC180, and whole-body images were obtained at a bimonthly rate of 4 of 1 for 3 min at the appropriate times after infection with C. rodentium ICC180. Luminescence emitted from the same gate in individual mice was quantified as counts per second, and pseudocolor images represent light intensity generated as a measure of colonization of the luminescent bacterial strain.

Cell isolation, in vitro T cell differentiation, protein transduction and inhibitor treatment. Naive CD4+ T cells from spleens or lymph nodes of 8- to 10-week-old mice were purified by sorting on a FACSAria II (BD Bioscience) with gating on the CD4+CD25−CD62L−CD44− fraction. Isolated CD4+ T cells were cultured in R-10 medium (RPMI medium containing 10% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate, nonessential amino acids, 50 µM β-mercaptoethanol and 2 mM L-glutamine). For stimulation with plate-bound polyclonal antibodies, sorted CD4+ T cells were stimulated with 5 µg of plate-bound anti-CD3 (145-2C11; BD Pharmingen) and 1 µg/mL of soluble anti-CD28 (37.51; BD Pharmingen). For polyclonal feeder-based stimulation, sorted CD4+ T cells were stimulated with 1 µg/mL of soluble anti-CD3 (identified above) in presence of in presence of populations of feeder cells obtained from the spleen or MLNs of IIb−/− mice, then irradiated and depleted of T cells. For antigen-specific stimulation, sorted naive OT-II CD4+ T cells (which have transgenic expression of an ovalbumin-specific TCR) were activated with 5 µg/mL ovalbumin peptide in presence of populations of splenic feeder cells obtained from IIb−/− mice, then irradiated and depleted of T cells. In all cases in which feeder cells were used for T cell polarization, T cells and feeder cells were added at a ratio of 1:1. All cultures were done in a volume of 200 µl in 96-well U-bottomed plates. For T cell or T cell polarization, the following exogenous cytokines or antibodies were added (concentrations in parentheses), unless stated otherwise: IL-6 (20 ng/mL; R&D Systems); TGF-β (2 ng/mL; R&D Systems); IL-21 (10 ng/mL; R&D Systems); IL-23 (5 ng/mL; R&D Systems); IL-1β (20 ng/mL; R&D Systems), human IL-2 (1–100 U; Roche), anti-IL-4 (10 µg/mL; eBioscience); prepared in-house); anti-IFN-γ (10 µg/mL; 11B11; prepared in-house); and anti-IL-2 (10 µg/mL; JES6-1A2; eBioscience). All-trans RA (Sigma) was added at various concentrations (1–100 nM), and the RA antagonist LE540 (Wako) was added at a concentration of 1 µM.

For the purification of Foxp3+ CD4+ T cells, Foxp3-GFP+ cells were sorted by flow cytometry and subjected to various conditions. For the purification of CD4+ single-positive thymocytes, CD8+ T cells were initially removed by depletion via magnetic-activated cell sorting (Mouse CD8 MicroBeads; Miltenyi Biotec), and CD4+ single-positive cells were further purified by isolation of the flow-through by magnetic-activated cell sorting (Mouse CD4+ T Cell Isolation Kit II; Miltenyi Biotec).

In experiments in which naive CD4+ T cells were treated by TAT-Cre protein, sorted naive CD4+ T cells from Rosa26RdTomato (Rd) reporter mice with a lovix-flanked STOP cassette were initially standardized by transduction of TAT-Cre. Cells were incubated for 15–20 min at 37 °C in 1 mL of 100 µg/mL TAT-Cre peptide in serum-free RPMI medium, then were washed and resuspended in RPMI 3% albumin medium containing IL-7 (10 ng/mL) plus 10% FCS, followed by incubation for 6, 12, 24 or 48 h; maximum expression of the reporter was observed at 24 h. Subsequently, sorted naive CD4+ T cells from A14 td-Tomato reporter and Rosa26RdTomato (Rd) reporter were treated in tandem with TAT-Cre or control peptide and were cultured for 24 h in IL-7-containing medium, followed by polarization under T cell conditions, with the additional culture modifications indicated in figure legends.

Flow cytometry, including flow cytometry with phosphorylation-specific antibodies. The following mAbs were used for sorting cells by flow cytometry: anti CD4-eFlour 780 (eBioscience, Clone RM4-5); anti-CD25-FTTC (BD Pharmingen, Clone 7D4); anti-CD25-PE (BD Pharmingen, Clone M17). For flow cytometry and intracellular staining, the following mAbs were used: fluorescein isothiocyanate– or peridinin chlorophyll protein–cyanine 5.5–conjugated anti-CD3 (145-2C11; eBioscience); phycoerythrin–indotricarbocyanine–conjugated anti-CD4 (RM4-5; eBioscience); allophycocyanin–conjugated anti-IL-17A (1B7B; eBioscience); peridinin chlorophyll protein–cyanine 5.5–conjugated anti-CD25 (PC61.5; eBioscience); peridinin chlorophyll protein–cyanine 5.5–conjugated anti-CD45.1 (A20; eBioscience) and phycoerythrin–conjugated anti-RO57 (AFKJS-9; eBioscience). Differentiated T cell17 cells were directly stained with anti-Thy-1.1 (OX-7; BD Pharmingen) without secondary staining. Only in cases in which intracellular IL-17A was detected, cells were stimulated for 4 h with PMA (phorbol 12-myristate 13-acetate; 50 ng/mL; Sigma) and ionomycin (750 ng/mL; Calbiochem) in the presence of Golgi Plug (BD Pharmingen). For detection of intracellular IL-17A and Foxp3, cells were resuspended in Foxp3 staining buffer (eBioscience). In all cases, LIVE/Dead Fixable Near-IR Dead Cell Stain (Invitrogen) was included before surface staining to exclude dead cells in flow cytometry.

For flow cytometry with phosphorylation-specific antibodies, sorted naive CD4+ T cells were differentiated with plate-bound anti-CD3 and soluble anti-CD28 (both identified above) under TAT17-polarizing conditions alone or in presence of RA or RA plus IL-1β. After 4 d, cells were allowed to `rest’ briefly in medium at a neutral pH before being stimulated for 20 min with IL-6 alone (50 ng/mL; R&D Systems) or with IL-2 (500 U/mL). The cells were then washed and resuspended in prewarmed PBS before being fixed for 10 min, in an incubator at 37 °C, with 4% paraformaldehyde. The cells were washed again and resuspended in ice-cold phospho-wash buffer (PBS containing 1× HALT protease inhibitor (78437; Thermo Scientific)) before the addition of chilled 100% methanol in drops and incubation for 30 min, followed by two washes in PBS containing 0.05% Tween. Cells were preincubated for 15 min in PBS-Tween containing 3% goat serum and 1 µl Fc block before incubation for 1 h with phycoerythrin–conjugated antibody to STAT3 phosphorylated at Tyr705 (612569; BD Biosciences) or Alexa Fluor 647–conjugated...
antibody to STAT3 phosphorylated at Tyr694 (612599; BD Pharmingen). Samples were acquired on an LSRII (BD Biosciences), and data were analyzed with FlowJo software (TreeStar).

Isolation and staining of LPLs. For isolation of LPLs, the large intestine was removed and cleared of luminal contents and fat, then was cut into small pieces and washed in chilled Hank's balanced-salt solution without Ca²⁺ or Mg²⁺. Minced tissue pieces were incubated for 30 min in presence of EDTA and were vortexed thoroughly to remove epithelial cells, then were incubated in RPMI medium containing collagenase IV (1 mg/ml; Sigma-Aldrich), dispase (0.5 mg/ml; Gibco, Invitrogen) and DNase I (0.25 mg/ml; Sigma-Aldrich). LP lymphocytes were collected by centrifugation. In all experiments with Il17f⁻/⁻ Foxp3⁺ reporter mice, unmanipulated LPLs were analyzed after undergoing surface staining with anti-CD3, anti-CD4, anti-CD25, anti-CD45.1, anti-TCRβ and anti-Thy-1.1 (all antibodies identified above). For analyses of Il1r1⁻/⁻ Il17f⁻/⁻ (CD45.1⁺CD45.2⁻) and Il1r1⁻/⁻ Il17f⁻/⁻ (CD45.2⁺) recipient Tcrb⁻/⁻ mice, unmanipulated LPLs were analyzed after undergoing surface staining with anti-TCRβ, anti-CD25, anti-CD45.1 and anti-CD90.1 (all antibodies identified above) and were subsequently fixed in fixation buffer (Foxp3-staining buffer; eBioscience) before intracellular staining with mAb to Foxp3 (identified above).

Adoptive transfer and in vivo treatment with LE135. For co-transfer of CD4⁺ T cells from congenically marked (donor) Il1r1⁻/⁻ Il17f⁻/⁻ (CD45.1⁺CD45.2⁻) and Il1r1⁻/⁻ Il17f⁻/⁻ (CD45.2⁺) reporter mice into Tcrb⁻/⁻ recipient mice, CD4⁺ T cells were isolated directly ex vivo by depletion of cells via magnetic-activated cell sorting using phycoerythrin-labeled anti-CD8ε (53-6.7; eBioscience), anti-CD11b (M1/70; eBioscience), anti-CD11c (N418; eBioscience), anti-CD19 (I3D1, eBioscience), anti-CD45R (B220) (RA3-6B2; eBioscience), anti-CD49b (DX5) (DX5; eBioscience), anti-CD105 (M17/18; eBioscience), anti–MHC class II (M5/114.15.2; Miltenyi Biotec), anti-γδ TCR (eBioGL3; eBioscience) and anti-Ter-119 (TER-119; eBioscience), captured by anti-TCR (eBioGL3) and anti-CD90.1 (all antibodies identified above) and were subsequently washed in chilled Hank's balanced-salt solution without Ca²⁺ or Mg²⁺. Unmanipulated LPLs were analyzed after fixing in Foxp3-staining buffer; eBioscience) before intracellular staining with mAb to Foxp3 (identified above).

ELISA. For determination of cytokine concentrations in vivo, supernatants of differentiated T cells were collected on day 3 of culture and analyzed by capture ELISA. For determination of cytokine concentrations ex vivo, colonic tissues from infected or C. rodentium–infected mice were collected at the appropriate time after infection, then were finely minced and were cultured for 24 h in 24-well plates in R-10 medium supplemented with 10 μg/ml gentamicin. Supernatants were collected after infection and were analyzed by capture ELISA. ELISA of IL-2, IFN-γ, IL-17A and IL-22 used mouse quantitk ELISA kits according to the manufacturer’s recommendations (R&D Systems).

Chromatin-immunoprecipitation assay. CD4⁺ single-positive thymocytes from wild-type or Stat5β⁻/⁻ C57BL/6 mice were polarized for 3–4 d under T H0 conditions (no cytokine) or T H17-polarizing conditions (IL-6 plus TGF-β), with or without RA alone or RA plus IL-1β, followed by 20 min of stimulation in CO₂ incubator by the addition of IL-6 (50 ng/ml; R&D Systems) or IL-2 (500 U/ml). The cells were crosslinked for 5 min with 1% (vol/vol) formaldehyde, quenched with 125 mM glycine and then suspended in HALT protease inhibitor ‘cocktail’ (78437; Thermo Scientific) containing PBS. Chromatin immunoprecipitation was performed with an ExactChip Kit in accordance with the manufacturer’s instruction (R&D Systems). Cells were suspended overnight at 4 °C in lysis buffer containing protease inhibitor and were immunoprecipitated with biotinylated anti-STAT3 (ECP1799; R&D Systems) or anti-STAT5 (ECP2168; R&D Systems). Bound DNA was collected through the use of agarose-streptavidin beads and was purified with a QIAprep Spin Miniprep Kit (Qiagen). Immunoprecipitated DNA was quantified by real-time PCR with IQTM SYBR Green Supermix (Bio-Rad) on a Bio-Rad Cycler with the following primer pairs specific for elements in the Il17a–Il17f locus and Foxp3 locus: Il17a–5′, 5′-CAGGTGATTATTTCTCAGGGCTTGG-3′ (forward) and 5′-TTGCCAATGGTTCTTTTCTTTTG-3′ (reverse); Il17a–3′ promoter, 5′-CACCTCTACAGGAGCACAG-3′ (forward) and 5′-ATGT TTTGGCGCTCTCGATC-3′ (reverse); Il17a+10, 5′-GGATTAAGGGACCAGC GCTTGG-3′ (forward) and 5′-TTCTCCCACTCTGTCTTTCCA-3′ (reverse); Il17a+28, 5′-TCTACGCTGTCCTCACAAGAG-3′ (forward) and 5′-GAGGCTGCTTACAGGGCTT-3′ (reverse); and Foxp3 5′-GACAGAGATGAGAAGAGATGATAAGTGA-3′ (forward) and 5′-TTTCTCTTGCAAAGGATGTTGGGGG-3′ (reverse). Reactions were run in duplicate and samples were normalized to 18S rRNA as a induction relative to expression in control samples unless stated otherwise.
TCACA-3′. Data are presented as relative values normalized to the input DNA samples (percentage of input).

**Histopathological evaluation.** Tissue samples obtained from proximal, middle and distal portions of the large intestine were fixed in 10% neutral buffered formalin and embedded in paraffin for the preparation of sections 5 µm in thickness that were stained with hematoxylin and eosin. The tissue sections were examined and assigned scores for tissue pathology as described\(^48\). In all score assignments, the identity of specimens was concealed from the pathologist.

**Statistical analysis.** For statistical analyses, \( P \) values were calculated by a paired or unpaired Student's \( t \)-test, unless stated otherwise in figure legends. A \( P \) value of <0.05 was considered significant.

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