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Induction of innate lymphoid cell-derived interleukin-22 by the transcription factor STAT3 mediates protection against intestinal infection

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Summary

Inhibitors of the transcription factor STAT3 target STAT3-dependent tumorigenesis but patients often develop diarrhea from unknown mechanisms. Here we showed that STAT3 deficiency increased morbidity and mortality after *Citrobacter rodentium* infection with decreased secretion of cytokines including IL-17 and IL-22 associated with the transcription factor RORγt. Administration of the cytokine IL-22 was sufficient to rescue STAT3-deficient mice from lethal infection. Although STAT3 was required for IL-22 production in both innate and adaptive arms, using conditional gene deficient mice we observed that STAT3 expression in RORγt$^+$ innate lymphoid cells (ILC3s), but not T cells, was essential for the protection. However, STAT3 was required for RORγt expression in T helper cells, but not in ILC3s. Activated STAT3 could directly bind to the *Il22* locus. Thus, cancer therapies that utilize STAT3 inhibitors increase the risk for pathogen-mediated diarrhea through direct suppression of IL-22 from gut ILCs.

INTRODUCTION

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that regulates many genes involved in apoptosis, proliferation, migration and survival in different cell types. STAT3 signaling is constitutively active in many types of tumor cells and tumor-associated immune cells during tumorigenesis, and this dysregulation promotes tumor growth and suppresses antitumor immune responses (Yu et al., 2007). Thus, STAT3 inhibitors have been explored in clinical trials for different cancer patients (Page et al., 2011). Sunitinib, an oral multitargeted tyrosine kinase inhibitor used for the treatment of several types of cancers including gastrointestinal tumors, also suppressed STAT3 activity

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SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, one table and Supplemental Experimental Procedures.

in host immune cells (Xin et al., 2009). However, nearly half of the patients developed diarrhea after Sunitinib treatment with unclear pathogenesis (Schwandt et al., 2009). Mutations of *STAT3* have been shown to be related to Hyper-immunoglobulin E syndrome patients with recurrent mucosal infections (Minegishi et al., 2007). Therefore, STAT3-related diarrhea could be linked with increased susceptibility to mucosal infections such as intestinal infections.

Citrobacter rodentium is a natural mouse extracellular enteric pathogen that mimics human Enterohaemorrhagic *E. coli* and Enteropathogenic *E. coli*, which are major causes of diarrheal disease worldwide that result in hundreds of thousands of deaths each year. Clearance of *C. rodentium* requires both the innate and adaptive immune responses (Bry and Brenner, 2004; Maaser et al., 2004). Both RORγt$^+$ group 3 innate lymphoid cells (ILC3s) and T helper cells (Th17, Th22) are important for the host to control *C. rodentium* infection (Basu et al., 2012; Ivanov et al., 2009; Qiu et al., 2011; Tumanov et al., 2011). Transferring either wild-type ILC3s cells (Sonnenberg et al., 2011) or Th22 cells (Basu et al., 2012) protects the mice from *C. rodentium* infection. However, it is not clear whether the innate or adaptive RORγt$^+$ lymphocytes are essential for protection against infection.

ILCs represent a family of immune cells with morphological characteristics of lymphocytes, yet lack rearranged antigen receptors. ILCs can produce an array of effector cytokines that correspond to the cytokine profiles of the T helper cell subsets; for example, IFN-γ by group 1 ILCs (NK cells and ILC1) and Th1 cells, IL-5 and IL-13 by group 2 ILCs (ILC2) and Th2 cells, and IL-17 and IL-22 by ILC3s (including LTi, NCR$^+$ ILC3 and NCR$^-$ ILC3) and Th17 and Th22 cells (Spits et al., 2013; Spits and Cupedo, 2012; Spits and Di Santo, 2010). The current dogma is that development and function of innate and adaptive lymphoid cells are under the control of analogous transcription factors. T-bet is involved in the development of NK, ILC1 and Th1 cells (Fuchs et al., 2013; Gordon et al., 2012; Szabo et al., 2000), and GATA3 is critical for the development of both ILC2 and Th2 cells (Furusawa et al., 2013; Hoyler et al., 2012; Klein Wolterink et al., 2013; Mjosberg et al., 2012; Moro et al., 2010; Zheng and Flavell, 1997), whereas RORγt is required for the development of both ILC3s and Th17 cells (Eberl et al., 2004; Ivanov et al., 2006). STAT proteins are transcription factors involved in the differentiation of T helper cells (O'Shea et al., 2011). However, whether ILCs and T helper cells also share STAT protein for their development and downstream cytokines production is still unknown.

Previous studies have shown that deletion of STAT3 in Th17 cells impairs their expression of RORγt and development (Ivanov et al., 2006; Laurence et al., 2007; O'Shea et al., 2011; Veldhoen et al., 2008; Yang et al., 2007; Yang et al., 2008; Zhou et al., 2007), but its role in ILCs has not been examined yet. Because innate and adaptive IL-17 producers share many transcriptional networks, it is expected that STAT3 also regulates RORγt expression in ILCs, analogous to their adaptive counterparts. However, in contrast to the STAT3-dependent development of Th17 and Th22 cells, we revealed that STAT3 does not control RORγt expression on ILCs, but rather directly regulates their production of IL-22 in response to infection.

RESULTS

Sunitinib impairs the host defense against intestinal *C. rodentium* infection

To determine whether tyrosine kinase (including STAT3 pathway) inhibitor treatment increases the risk of gut infection, *C. rodentium* infected mice were orally administered Sunitinib at clinically relevant doses (Xin et al., 2009). After *C. rodentium* infection, the mice with high-dose Sunitinib (40 mg/Kg) treatment developed severe diarrhea and rapidly lost body weight, with nearly fifty percent mortality by 3 weeks; low-dose Sunitinib (4 mg/

Kg) treated mice also developed diarrhea but lost less body weight, with few mortality by 3 weeks; however, neither weight loss nor death was observed in the control treatment mice (Figure 1A and 1B). Sunitinib treatment also resulted in increased bacterial titers in both feces and blood at day 8 and 11 post-infection (Figure 1C). Compared with control mice, the Sunitinib treated mice exhibited severe colonic pathology with severely disruption of the epithelial layer and infiltration of inflammatory cells (Figure S1). Together, these data indicate that oral Sunitinib administration could increase morbidity and mortality following intestinal *C. rodentium* infection.

Previous studies have shown that the Th17 and Th22 cell-related cytokines, IL-17, IL-22 and IL-23, are important for the host defense against *C. rodentium* infection (Ishigame et al., 2009; Zheng et al., 2008). To determine whether the increased risk of intestinal infection by tyrosine kinase inhibition was due to impaired host immune responses, the colonic cytokine expression profile was examined at day 5 post infection. In contrast to Th1 and Th2 cell-related cytokines, the Th17 and Th22 cell-related cytokines (IL-17 and IL-22) were up-regulated after infection but were suppressed by both in vivo and in vitro Sunitinib treatment (Figure 1D, 1E and **data not shown**). Although IL-23 from DC or macrophages is a major inducer for IL-22 production, Sunitinib had no effect on IL-23 production (Figure 1D and 1E). Taken together, these data indicate that Sunitinib impairs the host defense against intestinal *C. rodentium* infection. It raises the possibility that STAT3 might contribute to IL-22-dependent protection.

STAT3 signaling from RORγt$^+$ lymphocytes controls *C. rodentium* infection

IL-17 and IL-22 are known to be regulated by RORγt (encoded by *Rorc*) and are mainly produced by RORγt$^+$ ILCs and T cells (Ivanov et al., 2006; Qiu et al., 2011). To determine whether Sunitinib's adverse effect was due to STAT3 inhibition on ILC3s and T cells, *Stat3*-floxed mice were crossed with *Rorc-cre* transgenic mice to achieve specific deletion of STAT3 in RORγt-expressing cells (*Rorc-cre-Stat3*$^{fl/fl}$). Because RORγt is transiently expressed at high levels at the double positive stage of T cell development, *Rorc-cre-Stat3*$^{fl/fl}$ mice not only lack STAT3 expression by RORγt$^+$ ILC3s, but also most αβ T cells (Figure S2A–S2C).

We next tested whether STAT3 signaling is necessary for controlling the *C. rodentium* infection. After infection, *Rorc-cre-Stat3*$^{fl/fl}$ mice rapidly lost body weight and died around day 10, whereas no weight loss or death was observed in their littermate control wild-type (*Stat3*$^{fl/fl}$) mice (Figure 2A and 2B) or *Rorc-cre-Stat3*$^{fl/+}$ heterozygous mice (Figure S2D). To test whether the increased morbidity and mortality were associated with bacterial colitis, we checked bacterial titer and diarrhea status. *Rorc-cre-Stat3*$^{fl/fl}$ mice had 10–100 times higher bacterial titers in the feces compared to WT mice at day 5 and 8 post-infection (Figure 2C and S2E). *Rorc-cre-Stat3*$^{fl/fl}$ mice also showed increased bacterial titers in the blood, liver and spleen (Figure 2D and S2E), suggesting systemic dissemination of *C. rodentium*. The *Rorc-cre-Stat3*$^{fl/fl}$ mice exhibited severe diarrhea, colonic shortening, severe inflammation, and tissue injury in the colon and severely disruption of the epithelial layer compared with WT mice (Figure 2E, 2F and S2F). Together, these data indicate that STAT3 signaling in RORγt$^+$ cells is essential for host defense against a mucosal bacterial pathogen.

STAT3 signaling from RORγt$^+$ lymphocytes controls both IL-22 and IL-17 production during *C. rodentium* infection

We have shown that tyrosine kinase (including STAT3 pathway) inhibition suppressed both IL-22 and IL-17 expression after infection, which are required for the protection against *C. rodentium* infection (Ishigame et al., 2009; Ivanov et al., 2009; Wang et al., 2010; Zheng et al., 2008). We also measured IL-22 and IL-17 amounts in the colon of WT and *Rorc-cre-*

Stat3$^{fl/fl}$ mice early after *C. rodentium* infection. Compared to WT mice, expression of IL-22 and IL-17 was significantly reduced in the colon of *Rorc-cre-Stat3*$^{fl/fl}$ mice on day 5 post infection (Figure 3A). RegIIIγ and RegIIIβ, two antimicrobial proteins dependent on IL-22 (Pickert et al., 2009; Zheng et al., 2008), were also reduced in the colon of *Rorc-cre-Stat3*$^{fl/fl}$ mice after infection (Figure 3A). These results suggest that the STAT3 signaling is essential for IL-22 and IL-17 production in the gut after mucosal bacterial infection.

Exogenous IL-22 but not IL-17 rescues STAT3 deficiency mice during *C. rodentium* infection

Because both IL-17 and IL-22 are controlled by STAT3 signaling, we wondered whether administration of exogenous IL-17 or IL-22 is sufficient to rescue the death of *Rorc-cre-Stat3*$^{fl/fl}$ mice from *C. rodentium* infection. To address this question, IL-17A and IL-22 expressing plasmids were hydrodynamically injected into *Rorc-cre-Stat3*$^{fl/fl}$ mice at the same day of *C. rodentium* infection. All of the *Rorc-cre-Stat3*$^{fl/fl}$ mice treated with IL-22 survived after infection and lost weight only slightly, whereas vector and IL-17 alone treated mice rapidly lost body weight and succumbed to *C. rodentium* infection (Figure 3B and 3C). Accordingly, IL-22 treated *Rorc-cre-Stat3*$^{fl/fl}$ mice had reduced bacterial counts in their feces and blood compared to vector and IL-17 alone treated mice (Figure 3D). Collectively, these data demonstrate that exogenous IL-22 but not IL-17 is able to rescue *Rorc-cre-Stat3*$^{fl/fl}$ mice from a lethal *C. rodentium* infection and IL-22 is an essential and sufficient pathway downstream of STAT3 signaling to protect mice against mucosal bacterial pathogens.

STAT3 signaling is required for both innate and adaptive IL-22 production

Previous studies have shown that *C. rodentium* infection induces early wave of IL-22 production by ILC3s and second wave by CD4$^+$ T cells (Th22) (Basu et al., 2012; Qiu et al., 2011; Tumanov et al., 2011). To test whether STAT3 control innate or adaptive IL-22 production, we examined cytokine expression in the intestinal lamina propria lymphocytes (LPLs) isolated from both naïve and infected *Rorc-cre-Stat3*$^{fl/fl}$ and wild type mice by intracellular cytokine staining. Consistent with the previous study (Basu et al., 2012; Qiu et al., 2011), CD3$^-$ innate LPLs were the main IL-22 producers compared to CD3$^+$ T cells in naïve and infected WT mice at an early stage of infection (day 4), while there were more IL-22 producing T cells than ILCs at a later stage of infection (day 8) in WT mice (Figure 3E). However, *Rorc-cre-Stat3*$^{fl/fl}$ mice had severely decreased expression of IL-22 by intestinal LPLs, regardless of CD3$^-$ innate cells or CD3$^+$ T cells (Figure 3E, S3A and S3B). Moreover, Sunitinib treatment also suppressed IL-22 production in innate LPLs from wild type mice (Figure S3E). In a similar manner as IL-22, the IL-17 production was also significantly reduced in both innate and adaptive lymphocytes (Figure S3C and S3D). Since PMA and ionomycin are not physiological stimuli, the LPLs were also stimulated with anti-CD3 and anti-CD28 to mimic TCR signaling. Similar to PMA and ionomycin treatment, STAT3 deficient T cells also showed little IL-22 and IL-17 production after anti-CD3 and anti-CD28 stimulation (Figure S3F). Together, our data indicate that STAT3 signaling is essential for IL-22 and IL-17 expression in both ILC3s and T cells.

STAT3 signaling and IL-22 from ILC3s but not from T cells are essential for the control of *C. rodentium* infection

Because STAT3 signaling regulates IL-22 expression in both innate and adaptive lymphocytes, we wondered whether innate or adaptive IL-22 is more important for the protection from *C. rodentium* infection. To test whether the STAT3 signaling from ILC3s is sufficient for the protection against *C. rodentium* infection in the transient absence of T cells, CD4 mAb was administered to infected *Rorc-cre-Stat3*$^{fl/fl}$ and WT mice to deplete the

$CD4^+$ T cells. To avoid depletion of $CD4^+$ LTi cells, we used low dose of CD4 mAb to transiently remove $CD4^+$ T cells but not $CD4^+$ ILC3s. As shown in Figure S4A, 90% of peripheral $CD4^+$ T cells were depleted, while there was no proportional reduction of ILC3s in the lamina propria after multiple CD4 mAb injection. Because $CD4^+$ T cells are required for host adaptive immunity especially for antibody responses against *C. rodentium* (Bry et al., 2006), depletion of $CD4^+$ T cells provided another model to study whether the innate immune response is sufficient against this pathogen in the absence of adaptive responses. Treatment with CD4 mAb resulted in reduction of *C. rodentium* specific IgG in WT mice and all of them died at 3 weeks after *C. rodentium* infection (Figure S4B, 4A and 4B), which is the same as *Rag1*$^{-/-}$ mice, suggesting that adaptive immunity is responsible for the late phase of protection. Treatment of *Rorc-cre-Stat3*$^{fl/fl}$ mice with additional CD4 mAb did not aggravate or ameliorate the *C. rodentium* infection compared with Rat IgG (control for CD4 mAb) treatment group. All of the *Rorc-cre-Stat3*$^{fl/fl}$ mice with or without depletion of $CD4^+$ T cells still died on day 8 post-infection (Figure 4A–4C). These data suggest that STAT3 signaling and IL-22 from $CD4^+$ T cells are not essential for protecting mice in the early phase of *C. rodentium* infection.

To further test whether STAT3 signaling in T cells is required for the later T and B cell response and prevention of chronic infection, we rescued *Rorc-cre-Stat3*$^{fl/fl}$ mice by exogenous IL-22 and examined the antibody production. IL-22-rescued *Rorc-cre-Stat3*$^{fl/fl}$ mice produced the same amount of *C. rodentium* specific IgG as WT mice (Figure S4C). Moreover, 60 days after first *C. rodentium* infection, IL-22-rescued *Rorc-cre-Stat3*$^{fl/fl}$ mice were challenged with high dose of *C. rodentium* again. Interestingly, none of the IL-22-rescued *Rorc-cre-Stat3*$^{fl/fl}$ mice showed diarrhea (data not shown) and weight loss (Figure S4D), suggesting IL-22-rescued *Rorc-cre-Stat3*$^{fl/fl}$ mice developed normal protection. Together, these data demonstrate that STAT3 signaling from $CD4^+$ T cells is not essential for normal IgG production and protecting mice after *C. rodentium* infection.

To further test whether the STAT3 signaling from RORγt^+ ILCs is sufficient for the protection against *C. rodentium* infection, we crossed the *Stat3-floxed* mice with *Cd4-cre* mice to generate mice lacking STAT3 only in T cells (*Cd4-cre-Stat3*$^{fl/fl}$) but not in $CD4^+$ RORγt^+ ILCs (Eberl and Littman, 2004) (data not shown). Even after high dose of *C. rodentium* infection, the *Cd4-cre-Stat3*$^{fl/fl}$ mice did not lose body weight and all survived (Figure 4D). Furthermore, the fecal titers of *C. rodentium* were not significant higher in *Cd4-cre-Stat3*$^{fl/fl}$ mice than WT mice 5–11 days after infection (Figure 4E). Similar to the *Rorc-cre-Stat3*$^{fl/fl}$ mice, *Cd4-cre-Stat3*$^{fl/fl}$ mice also lacked IL-22 producing $CD4^+$ T cells in the gut (Figure 4F and 4G). However, the IL-22 producing ILCs in *Cd4-cre-Stat3*$^{fl/fl}$ mice were not reduced compared with WT mice, which were different with *Rorc-cre-Stat3*$^{fl/fl}$ mice (Figure 3E, 4F and 4G). Furthermore, CD4 mAb was also administered to *Cd4-cre-Stat3*$^{fl/fl}$ mice and *Stat3*$^{fl/fl}$ mice. As shown in Figure S4E and S4F, both CD4 mAb treated *Stat3*$^{fl/fl}$ or *Cd4-cre-Stat3*$^{fl/fl}$ mice lost body weight and died around day 20 post infection, while untreated mice survived, suggesting that $CD4^+$ T cells are important for the protection against *C. rodentium* infection, but STAT3 signaling from T cells is not essential. Together, these data demonstrate that STAT3 signaling and IL-22 from ILC3s, but not $CD4^+$ T cells, is essential for protecting mice from death during *C. rodentium* infection.

$CD90^{hi}CD45^{lo}$ ILC3s protect *Rorc-cre-Stat3*$^{fl/fl}$ mice during *C. rodentium* infection

Because we have shown that STAT3 signaling from ILC3s but not T cells is essential for the protection from *C. rodentium* infection, we wonder whether adoptive transfer of ILC3s could rescue *Rorc-cre-Stat3*$^{fl/fl}$ mice from infection. However, because of extremely low number of ILC3s and limited *Rorc*$^{gfp/+}$ mice, it was difficult to get enough ILC3s. To find better cell surface marker to identify ILC3s, we screened a large panel of surface markers

and found ILC3s could be strictly identified by CD45 and CD90. As shown in Figure 5A, after gating with CD45 and CD90, $CD90^+$ LPLs from the large intestine and small intestine of *Rag1*$^{-/-}$ mice fell into three separate populations, $CD90^{hi}CD45^{lo}$, $CD90^{lo}CD45^{int}$ and $CD90^{lo}CD45^{hi}$. Most of $CD90^{hi}CD45^{lo}$ LPLs (96%) were RORγt$^+$ ILC3s, and most of $CD90^{lo}CD45^{int}$ LPLs (95%) were $NK1.1^-$RORγt$^-$ ILCs, whereas nearly three fourths of $CD90^{lo}CD45^{hi}$ were $NK1.1^+$ NK cells. Similar to *Rag1*$^{-/-}$ mice, $CD90^+$ LPLs from both large intestine and small intestine of C57BL/6 WT mice fell into two separate populations, $CD90^{hi}CD45^{lo}$ and $CD90^{lo}CD45^{hi}$. Most of $CD90^{hi}CD45^{lo}$ LPLs (94%) were RORγt$^+$ ILC3s, whereas $CD90^{lo}CD45^{hi}$ were $CD3^+$ T cells and other RORγt$^-$ ILCs (Figure S5). These data suggest that $CD90^{hi}CD45^{lo}$ are specific cell surface markers for intestinal RORγt$^+$ ILC3s.

Then we sorted $CD90^{hi}CD45^{lo}$ ILC3s and transferred small number of them into *Rorc-cre-Stat3*$^{fl/fl}$ mice with *C. rodentium* infection. Compared with untreated *Rorc-cre-Stat3*$^{fl/fl}$ mice, ILC3s-transfered *Rorc-cre-Stat3*$^{fl/fl}$ mice lost less body weight and about one third of the mice survived and recovered from *C. rodentium* infection (Figure 5B and 5C). Accordingly, ILC3s-transferred *Rorc-cre-Stat3*$^{fl/fl}$ mice exhibited reduced bacterial counts in their feces and blood compared to untreated mice (Figure 5D). Collectively, these data demonstrate that $CD90^{hi}CD45^{lo}$ ILC3s are sufficient to protect mice against *C. rodentium* infection.

The development of RORγt$^+$ Th cells, but not RORγt$^+$ ILC3s, requires STAT3 signaling

Because *Rorc-cre-Stat3*$^{fl/fl}$ mice showed reduced IL-22 in both ILCs and T cells, we next determined how STAT3 regulates IL-22 production. IL-22 is mainly produced in RORγt$^+$ cells, and RORγt directly regulates IL-22 production at the transcriptional level. Previous studies have shown that STAT3 regulates the development of RORγt$^+$ Th17 cells (Ivanov et al., 2006; Laurence et al., 2007; Yang et al., 2007). To determine whether STAT3 signaling is shared by both innate and adaptive colonic RORγt$^+$ cells for their development, we examined the RORγt expression in the intestinal LPLs isolated from *Rorc-cre-Stat3*$^{fl/fl}$ mice and their littermate control wild-type mice. Consistent with *Cd4-cre-Stat3*$^{fl/fl}$ mice (Figure S6A), both the percentage and number of RORγt$^+$ Th cells ($CD3^+CD4^+$RORγt$^+$) in the colon were much lower in *Rorc-cre-Stat3*$^{fl/fl}$ mice compared with the WT mice (Figure 6A–6C and S6B). Unexpectedly, there were no differences in the percentages and numbers of the total RORγt$^+$ ILC3s (Figure 6D–6F), the subsets of ILC3s (LTi, NCR^+ ILC3 and NCR^- ILC3; Figure 6D and S6C) or other ILCs (ILC1, ILC2; Figure S6C) between *Rorc-cre-Stat3*$^{fl/fl}$ and WT mice, suggesting that STAT3 was required for RORγt expression in T cells but not ILC3s or maintenance of ILC3s. Moreover, similar to naïve mice, there were no differences in percentages and total numbers of the ILC3s after *C. rodentium* infection between *Rorc-cre-Stat3*$^{fl/fl}$ and WT mice (Figure 6E and 6F). Taken together, our data indicate that STAT3 signaling is essential for the development of RORγt$^+$ Th cells, but not ILC3s, and that STAT3-controlled IL-22 production is not through regulating RORγt expression in innate cells, which also suggests that RORγt is not sufficient to induce IL-22 production in the absence of STAT3 signaling.

STAT3 directly binds to the *Il22* locus and regulates IL-22 production

Previous studies have shown that IL-23 is an inducer of IL-22 (Zheng et al., 2008). IL-23 interacts with the IL-23 receptor (IL-23R) and activates STAT3, which has multiple target genes, such as *Il23r, Rorc, Ahr* in T cells (Ghoreschi et al., 2010; Ivanov et al., 2006; Veldhoen et al., 2008). RORγt and Ahr can bind to the *Il22* locus and directly promote IL-22 production (Qiu et al., 2011). As the expression of RORγt in ILCs was not reduced in *Rorc-cre-Stat3*$^{fl/fl}$ mice, we began to explore other pathways, such as IL23R or Ahr, defects in which could result in a defective response to IL-23 and subsequently reduced expression

of IL-22. To test this hypothesis, we first examined the expression of these related genes in the colon of WT and *Rorc-cre-Stat3*$^{fl/fl}$ mice before or early after *C. rodentium* infection. Unexpectedly, there were no significant reductions of IL-23p19, p40, IL23R and Ahr in the *Rorc-cre-Stat3*$^{fl/fl}$ mice compared with WT mice, and the expression of RORγt was actually increased in the STAT3-deficient mice, likely as a secondary response to the increased infection (Figure 7A). Interestingly, IL-6 was increased in *Rorc-cre-Stat3*$^{fl/fl}$ mice after infection, indicating increased inflammation. Even with higher expression of RORγt, the IL-22 production was still impaired, suggesting that the regulation of IL-22 production by STAT3 signaling may not be mediated through these related pathways.

To further study the mechanism of how STAT3 regulates IL-22, ILC3s were purified from both *Rorc*$^{gfp/+}$ and *Rorc-cre-Stat3*$^{fl/fl}$ *Rorc*$^{gfp/+}$ mice by flow cytometric sorting and stimulated with IL-23. Consistent with the previous results, STAT3 is required for IL-22 production in ILC3s after IL-23 stimulation. However, there were no significant differences in IL-23R, RORγt and Ahr between STAT3 deficient and WT ILC3s (Figure 7B). Together, these data suggest that STAT3 regulation of IL-22 is not through IL-23R, RORγt and Ahr.

STAT3 is a transcription factor that directly regulates IL-17 production (Yang et al., 2011). Therefore we wondered whether STAT3 can also directly regulate IL-22 production at the transcriptional level. Using an *in silico* approach, we found multiple STAT3 binding sites at the promoter and intron 1 of the *Il22* gene (Hutchins et al., 2012), suggesting that the *Il22* locus may be a direct target of STAT3 (Figure 7C). Indeed, using EL4 lymphoma cells stably expressing the double flag peptide (DTFC) and double flag-tagged STAT3C (DFTC-STAT3C), an active form of STAT3 (Zhou et al., 2007), a chromatin immunoprecipitation (ChIP) assay using anti-flag antibodies showed enhanced recruitment of STAT3 to the *Il22* locus in cell lines stably expressing DFTC-STAT3C, suggesting that *Il22* is a direct target gene of STAT3 (Figure 7D). To further confirm binding of STAT3 to the *Il22* locus in primary cells, we sorted ILC3s from the intestinal LPLs of *Rag1*$^{-/-}$ mice. ChIP assay with anti-STAT3 antibody also showed enhanced recruitment of STAT3 to the *Il22* locus in ILC3s (Figure 7E). Together, these data suggest that STAT3 could directly bind to the *Il22* locus and promote IL-22 production in RORγt^{+} cells.

DISCUSSION

IL-22-producing innate lymphoid cells were shown to control the dissemination of commensal bacteria and prevent systemic inflammation in naïve mice, which correlates with chronic infectious diseases in humans, particularly in patients undergoing prolonged treatments that increase their risk for nosocomial infections of bacterial origins (Sonnenberg et al., 2012). However how IL-22 is regulated by various transcriptional factors is not fully understood. Our results show the production of IL-22 in both innate and adaptive lymphoid cells is dependent on STAT3, suggesting that STAT3 inhibition or deficiency may not only increase the chance of pathogen infection in the gut, but also result in the disruption of normal gut homeostasis.

Th22 cells are an important source of IL-22 for host protection against low dose *C. rodentium* infection (Basu et al., 2012). Here, we carefully utilized a complementary collection of conditional gene deficient mice combined with T cell-specific depletion to show that IL-22 from T cells is not necessary for conferring protection even after high doses of *C. rodentium* inoculation. Although adoptive transfer of IL-22 producing T cells rescues IL-22 deficient mice from lethal infection (Basu et al., 2012), this suggests that additional IL-22-producing T cells can sufficiently control infection but does not prove that Th22 is essential for the infection. Consistently, exogenous IL-22 can sufficiently rescue many different gene deficient mice from *C. rodentium* infection (Ota et al., 2011; Qiu et al., 2011;

Tumanov et al., 2011; Zheng et al., 2008). Our data suggest that STAT3 and IL-22 from ILCs, but not Th22 cells, are essential for protecting the host in normal physiology state.

It is thought that the development and function of ILCs and Th cells are under the control of corresponding transcription factors (Hoyler et al., 2012; Zhou, 2012). However, our data suggest that the transcriptional regulation of ILC3s and Th17 and Th22 cells is complex, and may be distinct between innate and adaptive lymphocytes. In contrast to the essential role of controlling RORγt expression in T cells (Yang et al., 2007; Zhou et al., 2007), STAT3 has little impact on the development of RORγt$^+$ ILC3s. This finding could explain the major differences in ontogeny between RORγt$^+$ Th cells and ILC3s. ILC3s first appear as fetal LTi cells that are generated on E12.5 during ontogeny in the liver, while RORγt$^+$ Th17 and Th22 cells are differentiated from naive CD4$^+$ T cells after birth dependent on the commensal microbiota (Eberl, 2012). Germ-free mice mostly lack RORγt$^+$ Th cells. Colonization of germ-free mice with segmented filamentous bacterium is sufficient to induce Th17 cells in the small intestinal (Ivanov et al., 2009). In contrast, the development of ILC3s is independent of gut flora, although there are debates about the influence of symbiotic microbiota on some subsets or function of ILC3s (Sanos et al., 2009; Satoh-Takayama et al., 2008; Sawa et al., 2011; Sonnenberg and Artis, 2012). Our data suggest that STAT3 signaling is necessary for the host to sense the environmental microbiota and subsequently generate RORγt$^+$ Th cells, but not to “program” the expression of RORγt in microbiota-independent ILCs. Thus, the mechanisms underlying the differential regulation RORγt expression by STAT3 in ILCs and T cells remain to be defined.

STAT3 is one of the downstream adaptors of IL-23R signaling and regulates the expression of RORγt, Ahr, and IL-23R in T cells (Ghoreschi et al., 2010; Ivanov et al., 2006; Veldhoen et al., 2008). Presumably, IL-23 activates the STAT3 signaling, which induces RORγt, Ahr, and IL-23R expression, then subsequent induction of RORγt and Ahr regulates IL-22 production. However, our data show that the STAT3 deficiency in ILC3s has minimal impact on the expression of RORγt, Ahr and IL-23R, but rather activated STAT3 directly binds to the *Il22* locus to regulate IL-22 transcription, suggesting that the expression of RORγt in ILC3s is not sufficient to promote IL-22 production in the absence of STAT3. Both RORγt and STAT3 are required for IL-22 production, and RORγt regulating IL-22 production is dependent on STAT3 signaling in ILCs, raising the questions whether RORγt controls STAT3 signaling or how STAT3 and RORγt cooperate on regulating IL-22 production in ILCs.

Together, our studies have demonstrated several important findings. 1) In contrast to the essential role of controlling the expression of RORγt in T cells, STAT3 has little impact on the development of RORγt$^+$ ILC3s, but rather directly regulates the function of innate RORγt$^+$ ILC3s, which is essential for gut immunity. 2) Although STAT3 regulates the function of both innate and adaptive RORγt$^+$ cells, our data suggest that STAT3 signaling and IL-22 from innate RORγt$^+$ ILC3s but not from adaptive T cells, are essential in the response to acute mucosal bacterial infections. 3) Previous studies have shown that STAT3 signaling controls IL-22 production by T cells through regulating RORγt expression. Our data show that STAT3 can directly control IL-22 production. 4) STAT3 inhibition in a major anti-tumor therapy also impairs the host protection from intestinal bacterial infection. Exogenous IL-22 administration may be beneficial for reducing side effects in the cancer patients undergoing STAT3 inhibitor treatment.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 and *Rag1*$^{-/-}$ mice were purchased from Harland Teklad. *Cd4-Cre* (Lee et al., 2001) mice were purchased from Taconic. *Rorc*$^{gfp/+}$ (Eberl et al., 2004) mice were purchased from The Jackson Lab. *Rorc-cre-Stat3*$^{fl/fl}$, *Rorc-cre-Stat3*$^{fl/+}$, and *Cd4-cre-Stat3*$^{fl/fl}$ mice were generated by crossing *Stat3-floxed* mice (Takeda et al., 1998) with *Rorc-cre* (Eberl and Littman, 2004) and *Cd4-cre* transgenic mice, respectively. *Rorc-cre-Stat3*$^{fl/fl}$ *Rorc*$^{gfp/+}$ mice were generated by crossing *Rorc-cre-Stat3*$^{fl/fl}$ mice with *Rorc*$^{gfp/+}$ mice. All mice are on C57BL/6 background. Animal care and use were in accordance with institutional and National Institutes of Health guidelines and all studies were approved by the Animal Care and Use Committee of the University of Chicago and Northwestern University.

Infection with *C. rodentium* and Treatment

Mice were orally gavaged with *C. rodentium* strain DBS100 (ATCC 51459; American Type Culture Collection) and body weight, survival, colony-forming units (CFUs) counts, tissue histology were assessed as previously described (Tumanov et al., 2011; Wang et al., 2010). Colon culture was done as previously described (Zheng et al., 2008). Where indicated, Sunitinib (SU11248; SUTENT) or control was gavaged orally, once a day, at doses of 4 or 40 mg/Kg body weight after infection. Where indicated, mice were injected intraperitoneally with mouse IL-22-Ig fusion protein on 0, 2, 4 day after *C. rodentium* infection at a dose of 50 μg per mouse each time. Control groups received isotype control immunoglobulin. For depletion of CD4$^+$ T lymphocytes, mice were injected intraperitoneally with mAb GK1.5 or Rat IgG on −5, 0, 5, 10, 15 day after *C. rodentium* infection at a dose of 50 μg per mouse each time. Where indicated, IL-17 and IL-22-expressing plasmids were introduced into mice using a hydrodynamic tail vein injection-based gene transfer technique (Tumanov et al., 2011).

Isolation of Intestinal LPLs

The isolation of intestinal lamina propria cells was done as previously described (Wang et al., 2010). In brief, mice were killed and colons were removed and placed in ice-cold PBS. The intestines were cut open longitudinally, thoroughly washed in ice-cold PBS, and cut into 1.5 cm pieces. Intestines were incubated with shaking in RPMI 1640 (Invitrogen) containing 3% FBS, 1mM DTT, 5 mM EDTA and 10 mM HEPES at 37°C for 30 min. After washing with RPMI 1640 containing 2 mM EDTA, the tissues were then digested in RPMI 1640 containing 0.05% DNase I (Sigma) and Liberase (Roche) (0.1 mg/ml) at 37°C for 30 min with slow rotation. The digested tissues were homogenized by vigorous shaking and passed through 100 μm cell strainer. Mononuclear cells were then harvested from the interphase of an 80% and 40% Percoll gradient after a spin at 2500 rpm for 20 min at room temperature.

Flow Cytometry, Antibodies and ELISA

Antibodies against CD3, CD4, CD8, CD45, CD90, Nkp46, Gr-1, CD11b, KLRG1, TCRβ, NK1.1 and Streptavidin-APC were purchased from BioLegend. Antibodies against RORγt and IL-17 were purchased from eBioscience. Antibodies against STAT3 and Rabbit IgG were purchased from Cell Signaling Technology. Anti-IL-22 antibody was a gift from Genentech. For nuclear staining, cells were fixed and permeabilized using a Transcription Factor Staining Buffer Set (eBioscience). For cytokine production, cells were stimulated ex vivo by IL-23 (25 ng/ml, R&D), PMA (50 ng/ml, Sigma) and ionomycin (750 ng/ml, Calbiochem) or anti-CD3 (plate coated, 1 μg/ml) and anti-CD28 (2 μg/ml) for 4 hr and Brefeldin A (10 μg/ml, eBioscience) was added 2 hr before cells were harvested for

analysis. IC Fixation Buffer and Permeabilization Buffer were used according to the manufacturer's instructions (eBioscience) for intracellular cytokine staining. Flow cytometry analysis was performed on FACSCanto (BD Biosciences) instruments and analyzed with FlowJo software (Tree Star Inc.). IL-22 (R&D Systems), IL-17, IL-23 (eBioscience) in supernatants were measured by ELISA according to the manufacturer's recommendations.

Quantitative Real-Time RT-PCR

RNA from tissues was isolated with Trizol reagent (Invitrogen) and reverse transcribed with AMV Reverse Transcriptase (Promega). RNA from sorted cells was isolated with the RNeasy Micro Kit (Qiagen) and was reverse transcribed with Seniscript Reverse Transcription Kit (Qiagen). Real-time RT-PCR was performed using SSoFast EvaGreen supermix (Bio-Rad) and different primer sets (Table S1) on StepOne Plus (Applied Biosystems). Samples were normalized to HPRT and reported according to the $2^{-\Delta\Delta C_T}$ method.

Adoptive Transfer Experiments

Rag1$^{-/-}$ mice were infected with *C. rodentium*. CD90^{hi}CD45^{lo} ILC3s were sorted from both the colon and small intestine LPLs on a FACS Aria III instrument (BD Bioscience) at day 1 post infection. Sorted ILC3s were stimulated with IL-23 (25 ng/ml) for 1 hour, then were transferred by i.v. injection (2×10^5 cells per mouse) into recipients inoculated with *C. rodentium* at day 0 and day 3.

Chromatin Immunoprecipitation

ChIP assays with EL4 cells were performed as previously described (Qiu et al., 2011). Briefly, 5×10^7 EL4 cells were first fixed in 1% formaldehyde in RPMI1640 media for 10 min at room temperature. Then the reaction was stopped by adding glycine solution. Fixed cells were washed with PBS and resuspended in lysis buffer. Chromatin was sheared by sonication on ice, and the insoluble fraction was removed by centrifugation. The lysate was precleared with Protein A beads (Santa Cruz Biotechnology) and then incubated with anti-Flag beads (Sigma) at 4°C for 5 hrs. After washing, precipitated chromatin fragments were eluted with flag peptide (Sigma). Then the samples were reverse cross-linked at 65°C by adding Tris-EDTA with 1% SDS. After proteinase K digestion, DNA was extracted using Phenol-Chloroform, and precipitated for quantitative real-time PCR analyses using specific primers. CD90^{hi}CD45^{lo} ILC3s were sorted from *Rag1*$^{-/-}$ mice and ChIP assays were performed with anti-STAT3 rabbit mAb (D3Z2G) or Rabbit IgG (Cell Signaling Technology). MegnaChIP protein A magnetic beads (Millipore) were used to isolate immune complexes. Crosslinks were reversed by heating at 65ting at 65rosslproteinase K treatment. DNA was purified with Qiaquick PCR purification kit (QIAGEN).

Statistical Methods

Statistical analysis was performed by two-tailed Student's t test using GraphPad Prism 5.0 program. Data from such experiments are presented as mean values ± SEM; $p < 0.05$ was considered significant. For survival curves, statistics were done using the log rank (Mantel-Cox) test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

Basu R, O'Quinn DB, Silberger DJ, Schoeb TR, Fouser L, Ouyang W, Hatton RD, Weaver CT. Th22 cells are an important source of IL-22 for host protection against enteropathogenic bacteria. Immunity. 2012; 37:1061–1075. [PubMed: 23200827]

Bry L, Brenner MB. Critical role of T cell-dependent serum antibody, but not the gut-associated lymphoid tissue, for surviving acute mucosal infection with Citrobacter rodentium, an attaching and effacing pathogen. J Immunol. 2004; 172:433–441. [PubMed: 14688352]

Bry L, Brigl M, Brenner MB. CD4+-T-cell effector functions and costimulatory requirements essential for surviving mucosal infection with Citrobacter rodentium. Infect Immun. 2006; 74:673–681. [PubMed: 16369024]

Eberl G. Development and evolution of RORgammat+ cells in a microbe's world. Immunol Rev. 2012; 245:177–188. [PubMed: 22168420]

Eberl G, Littman DR. Thymic origin of intestinal alphabeta T cells revealed by fate mapping of RORgammat+ cells. Science. 2004; 305:248–251. [PubMed: 15247480]

Eberl G, Marmon S, Sunshine MJ, Rennert PD, Choi Y, Littman DR. An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. Nat Immunol. 2004; 5:64–73. [PubMed: 14691482]

Fuchs A, Vermi W, Lee JS, Lonardi S, Gilfillan S, Newberry RD, Cella M, Colonna M. Intraepithelial Type 1 Innate Lymphoid Cells Are a Unique Subset of IL-12- and IL-15-Responsive IFN-gamma-Producing Cells. Immunity. 2013

Furusawa J, Moro K, Motomura Y, Okamoto K, Zhu J, Takayanagi H, Kubo M, Koyasu S. Critical role of p38 and GATA3 in natural helper cell function. J Immunol. 2013; 191:1818–1826. [PubMed: 23851685]

Ghoreschi K, Laurence A, Yang XP, Tato CM, McGeachy MJ, Konkel JE, Ramos HL, Wei L, Davidson TS, Bouladoux N, et al. Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling. Nature. 2010; 467:967–971. [PubMed: 20962846]

Gordon SM, Chaix J, Rupp LJ, Wu J, Madera S, Sun JC, Lindsten T, Reiner SL. The transcription factors T-bet and Eomes control key checkpoints of natural killer cell maturation. Immunity. 2012; 36:55–67. [PubMed: 22261438]

Hoyler T, Klose CS, Souabni A, Turqueti-Neves A, Pfeifer D, Rawlins EL, Voehringer D, Busslinger M, Diefenbach A. The Transcription Factor GATA-3 Controls Cell Fate and Maintenance of Type 2 Innate Lymphoid Cells. Immunity. 2012; 37:634–648. [PubMed: 23063333]

Hutchins AP, Poulain S, Miranda-Saavedra D. Genome-wide analysis of STAT3 binding in vivo predicts effectors of the anti-inflammatory response in macrophages. Blood. 2012; 119:e110–119. [PubMed: 22323479]

Ishigame H, Kakuta S, Nagai T, Kadoki M, Nambu A, Komiyama Y, Fujikado N, Tanahashi Y, Akitsu A, Kotaki H, et al. Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses. Immunity. 2009; 30:108–119. [PubMed: 19144317]

Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, Wei D, Goldfarb KC, Santee CA, Lynch SV, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell. 2009; 139:485–498. [PubMed: 19836068]

Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell. 2006; 126:1121–1133. [PubMed: 16990136]

Klein Wolterink RG, Serafini N, van Nimwegen M, Vosshenrich CA, de Bruijn MJ, Fonseca Pereira D, Veiga Fernandes H, Hendriks RW, Di Santo JP. Essential, dose-dependent role for the transcription factor Gata3 in the development of IL-5+ and IL-13+ type 2 innate lymphoid cells. Proc Natl Acad Sci U S A. 2013; 110:10240–10245. [PubMed: 23733962]

Laurence A, Tato CM, Davidson TS, Kanno Y, Chen Z, Yao Z, Blank RB, Meylan F, Siegel R, Hennighausen L, et al. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. Immunity. 2007; 26:371–381. [PubMed: 17363300]

Lee PP, Fitzpatrick DR, Beard C, Jessup HK, Lehar S, Makar KW, Perez-Melgosa M, Sweetser MT, Schlissel MS, Nguyen S, et al. A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. Immunity. 2001; 15:763–774. [PubMed: 11728338]

Maaser C, Housley MP, Iimura M, Smith JR, Vallance BA, Finlay BB, Schreiber JR, Varki NM, Kagnoff MF, Eckmann L. Clearance of Citrobacter rodentium requires B cells but not secretory immunoglobulin A (IgA) or IgM antibodies. Infect Immun. 2004; 72:3315–3324. [PubMed: 15155635]

Minegishi Y, Saito M, Tsuchiya S, Tsuge I, Takada H, Hara T, Kawamura N, Ariga T, Pasic S, Stojkovic O, et al. Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome. Nature. 2007; 448:1058–1062. [PubMed: 17676033]

Mjosberg J, Bernink J, Golebski K, Karrich JJ, Peters CP, Blom B, te Velde AA, Fokkens WJ, van Drunen CM, Spits H. The transcription factor GATA3 is essential for the function of human type 2 innate lymphoid cells. Immunity. 2012; 37:649–659. [PubMed: 23063330]

Moro K, Yamada T, Tanabe M, Takeuchi T, Ikawa T, Kawamoto H, Furusawa J, Ohtani M, Fujii H, Koyasu S. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. Nature. 2010; 463:540–544. [PubMed: 20023630]

O'Shea JJ, Lahesmaa R, Vahedi G, Laurence A, Kanno Y. Genomic views of STAT function in CD4+ T helper cell differentiation. Nat Rev Immunol. 2011; 11:239–250. [PubMed: 21436836]

Ota N, Wong K, Valdez PA, Zheng Y, Crellin NK, Diehl L, Ouyang W. IL-22 bridges the lymphotoxin pathway with the maintenance of colonic lymphoid structures during infection with Citrobacter rodentium. Nat Immunol. 2011; 12:941–948. [PubMed: 21874025]

Page BD, Ball DP, Gunning PT. Signal transducer and activator of transcription 3 inhibitors: a patent review. Expert Opin Ther Pat. 2011; 21:65–83. [PubMed: 21114420]

Pickert G, Neufert C, Leppkes M, Zheng Y, Wittkopf N, Warntjen M, Lehr HA, Hirth S, Weigmann B, Wirtz S, et al. STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. J Exp Med. 2009; 206:1465–1472. [PubMed: 19564350]

Qiu J, Heller JJ, Guo X, Chen ZM, Fish K, Fu YX, Zhou L. The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. Immunity. 2011; 36:92–104. [PubMed: 22177117]

Sanos SL, Bui VL, Mortha A, Oberle K, Heners C, Johner C, Diefenbach A. RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. Nat Immunol. 2009; 10:83–91. [PubMed: 19029903]

Satoh-Takayama N, Vosshenrich CA, Lesjean-Pottier S, Sawa S, Lochner M, Rattis F, Mention JJ, Thiam K, Cerf-Bensussan N, Mandelboim O, et al. Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. Immunity. 2008; 29:958–970. [PubMed: 19084435]

Sawa S, Lochner M, Satoh-Takayama N, Dulauroy S, Berard M, Kleinschek M, Cua D, Di Santo JP, Eberl G. RORgammat+ innate lymphoid cells regulate intestinal homeostasis by integrating negative signals from the symbiotic microbiota. Nat Immunol. 2011; 12:320–326. [PubMed: 21336274]

Schwandt A, Wood LS, Rini B, Dreicer R. Management of side effects associated with sunitinib therapy for patients with renal cell carcinoma. Onco Targets Ther. 2009; 2:51–61. [PubMed: 20616894]

Sonnenberg GF, Artis D. Innate lymphoid cell interactions with microbiota: implications for intestinal health and disease. Immunity. 2012; 37:601–610. [PubMed: 23084357]

Sonnenberg GF, Monticelli LA, Alenghat T, Fung TC, Hutnick NA, Kunisawa J, Shibata N, Grunberg S, Sinha R, Zahm AM, et al. Innate lymphoid cells promote anatomical containment of lymphoid-resident commensal bacteria. Science. 2012; 336:1321–1325. [PubMed: 22674331]

Sonnenberg GF, Monticelli LA, Elloso MM, Fouser LA, Artis D. CD4(+) lymphoid tissue-inducer cells promote innate immunity in the gut. Immunity. 2011; 34:122–134. [PubMed: 21194981]

Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, Koyasu S, Locksley RM, McKenzie AN, Mebius RE, et al. Innate lymphoid cells--a proposal for uniform nomenclature. Nat Rev Immunol. 2013; 13:145–149. [PubMed: 23348417]

Spits H, Cupedo T. Innate lymphoid cells: emerging insights in development, lineage relationships, and function. Annu Rev Immunol. 2012; 30:647–675. [PubMed: 22224763]

Spits H, Di Santo JP. The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. Nat Immunol. 2010; 12:21–27. [PubMed: 21113163]

Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell. 2000; 100:655–669. [PubMed: 10761931]

Takeda K, Kaisho T, Yoshida N, Takeda J, Kishimoto T, Akira S. Stat3 activation is responsible for IL-6-dependent T cell proliferation through preventing apoptosis: generation and characterization of T cell-specific Stat3-deficient mice. J Immunol. 1998; 161:4652–4660. [PubMed: 9794394]

Tumanov AV, Koroleva EP, Guo X, Wang Y, Kruglov A, Nedospasov S, Fu YX. Lymphotoxin controls the IL-22 protection pathway in gut innate lymphoid cells during mucosal pathogen challenge. Cell Host Microbe. 2011; 10:44–53. [PubMed: 21767811]

Veldhoen M, Hirota K, Westendorf AM, Buer J, Dumoutier L, Renauld JC, Stockinger B. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. Nature. 2008; 453:106–109. [PubMed: 18362914]

Wang Y, Koroleva EP, Kruglov AA, Kuprash DV, Nedospasov SA, Fu YX, Tumanov AV. Lymphotoxin beta receptor signaling in intestinal epithelial cells orchestrates innate immune responses against mucosal bacterial infection. Immunity. 2010; 32:403–413. [PubMed: 20226692]

Xin H, Zhang C, Herrmann A, Du Y, Figlin R, Yu H. Sunitinib inhibition of Stat3 induces renal cell carcinoma tumor cell apoptosis and reduces immunosuppressive cells. Cancer Res. 2009; 69:2506–2513. [PubMed: 19244102]

Yang XO, Panopoulos AD, Nurieva R, Chang SH, Wang D, Watowich SS, Dong C. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. J Biol Chem. 2007; 282:9358–9363. [PubMed: 17277312]

Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y, Ma L, Shah B, Panopoulos AD, Schluns KS, et al. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. Immunity. 2008; 28:29–39. [PubMed: 18164222]

Yang XP, Ghoreschi K, Steward-Tharp SM, Rodriguez-Canales J, Zhu J, Grainger JR, Hirahara K, Sun HW, Wei L, Vahedi G, et al. Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. Nat Immunol. 2011; 12:247–254. [PubMed: 21278738]

Yu H, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. Nat Rev Immunol. 2007; 7:41–51. [PubMed: 17186030]

Zheng W, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell. 1997; 89:587–596. [PubMed: 9160750]

Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, Abbas AR, Modrusan Z, Ghilardi N, de Sauvage FJ, et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. Nat Med. 2008; 14:282–289. [PubMed: 18264109]

Zhou L. Striking similarity: GATA-3 regulates ILC2 and Th2 cells. Immunity. 2012; 37:589–591. [PubMed: 23084352]

Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, Egawa T, Levy DE, Leonard WJ, Littman DR. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat Immunol. 2007; 8:967–974. [PubMed: 17581537]

Highlights

STAT3 and IL-22 from innate but not adaptive RORγt$^+$ cells are crucial for protection.

STAT3 controls the development of RORγt$^+$ T helper cells but not ILC3s.

STAT3 can directly control IL-22 production in both ILC3s and T cells.

Inhibition of IL-22 production could cause pathogen-induced diarrhea.

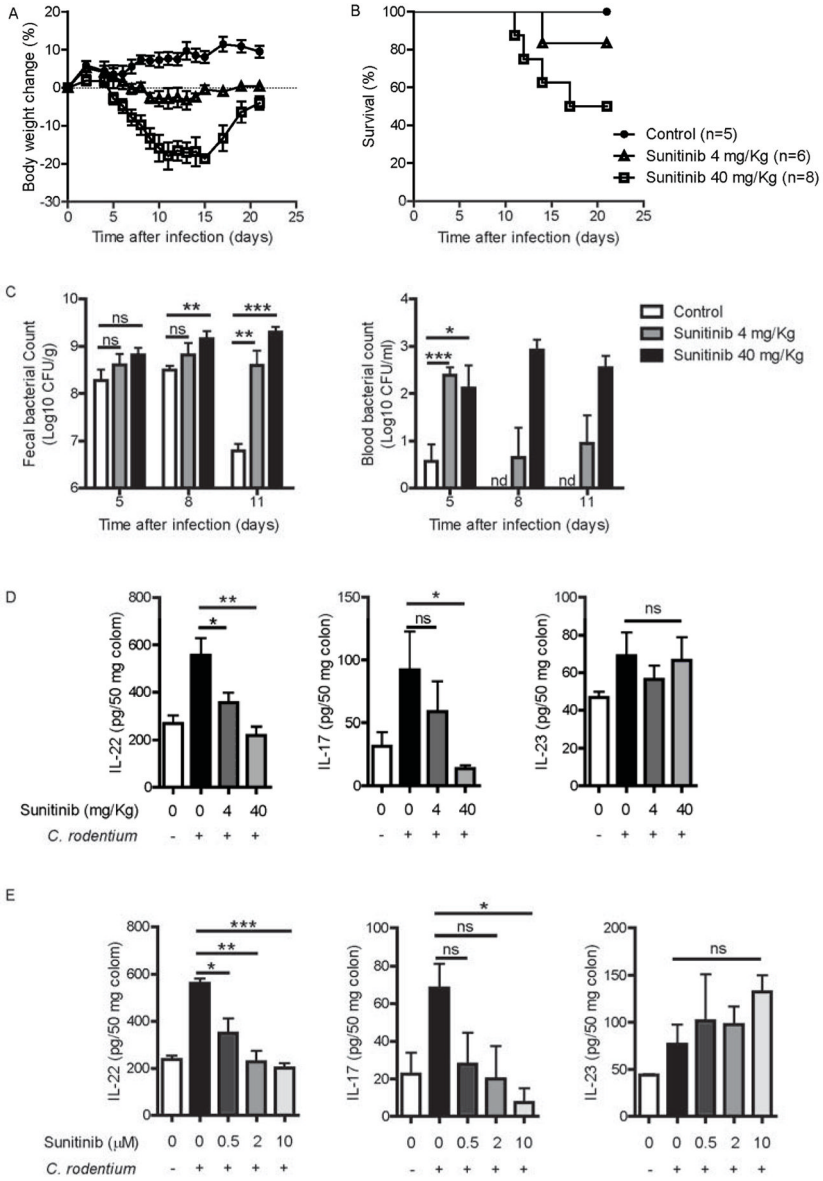

Figure 1. Sunitinib impairs the host defense against intestinal *C. rodentium* infection

(**A–C**) 8 weeks old wild type mice were orally inoculated with 2×10^9 CFU of *C. rodentium.* Sunitinib (4 mg/Kg, n=6 or 40 mg/Kg, n=8) or control (n=5) was administered orally from day 0 to day 7, once daily. Body weight was measured at the indicated time points (**A**) and survival rates (**B**) are shown.

(**C**) Bacterial titers from blood and fecal homogenate cultures at indicated day post infection in the mice in **A, B**.

(**D**) IL-22, IL-17 and IL23 levels from the colon culture supernatants of Sunitinib or control treated mice at day 5 after infection (n=5 each group).

(**E**) The colons from untreated mice (n=3) were removed at day 5 after infection, and cultured with Sunitinib at indicated concentrations for 24 hours in vitro. IL-22, IL-17 and IL23 levels were examined in the colon culture supernatants.

*P<0.05, **P<0.01, ***P<0.001; ns, no significant difference (Student's *t*-test); nd, nondetectable. Data are representative of two independent experiments (mean ± s.e.m.). See also Figure S1.

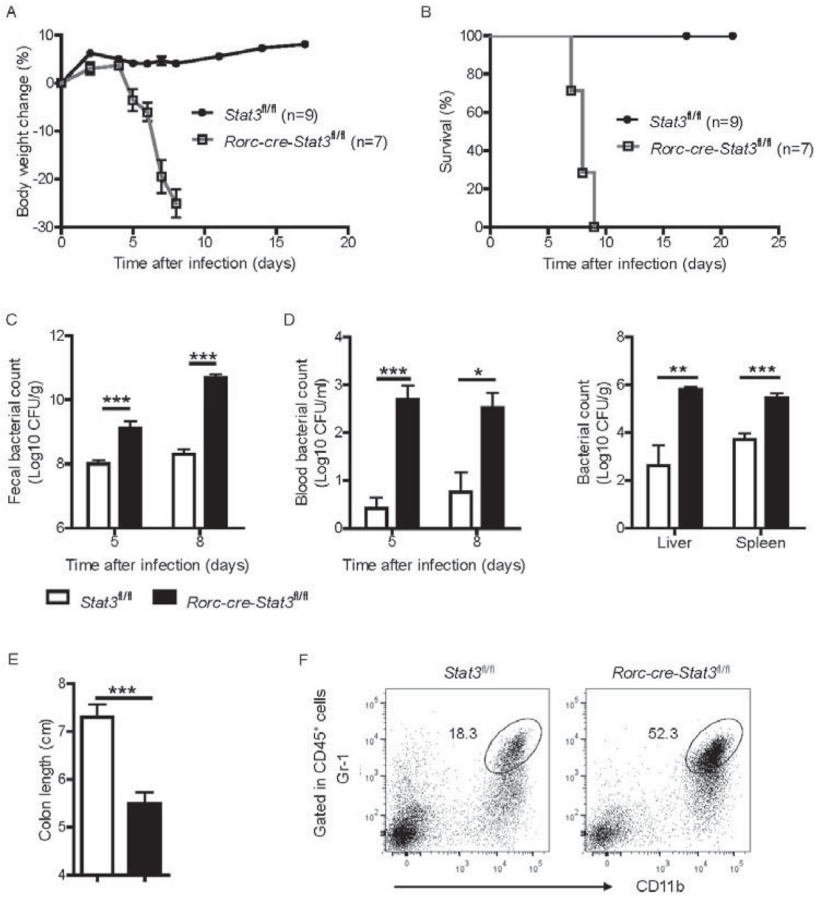

Figure 2. STAT3 signaling is essential for control of intestinal *C. rodentium* infection

(**A**–F) 7 weeks old *Rorc-cre-Stat3*$^{fl/fl}$ (n = 7) and their littermate wild type *Stat3*$^{fl/fl}$ (n = 9) mice were orally inoculated with *C. rodentium* (2×10^9 CFU) and body weight was measured at the indicated time points. Body weight change (**A**) and survival rates (**B**) are shown.

(**C**) Bacterial titers from fecal homogenate cultures at indicated day post infection in the mice in **A, B**.

(**D**) Bacterial titers from blood and spleen, liver homogenate cultures at indicated day post infection are shown.

(**E**) *Rorc-cre-Stat3*$^{fl/fl}$ mice show colon shortening at day 8 post infection.

(**F**) *Rorc-cre-Stat3*$^{fl/fl}$ mice show more inflammatory monocytes or neutrophils infiltration in the colon. LPLs were isolated from colon and gated in CD45$^+$ cells.

*P<0.05, **P<0.01, ***P<0.001 (Student's *t*-test). Data are representative of three independent experiments (**A–C**; mean ± s.e.m.) or two independent experiments (**D–E**; mean ± s.e.m.). See also Figure S2.

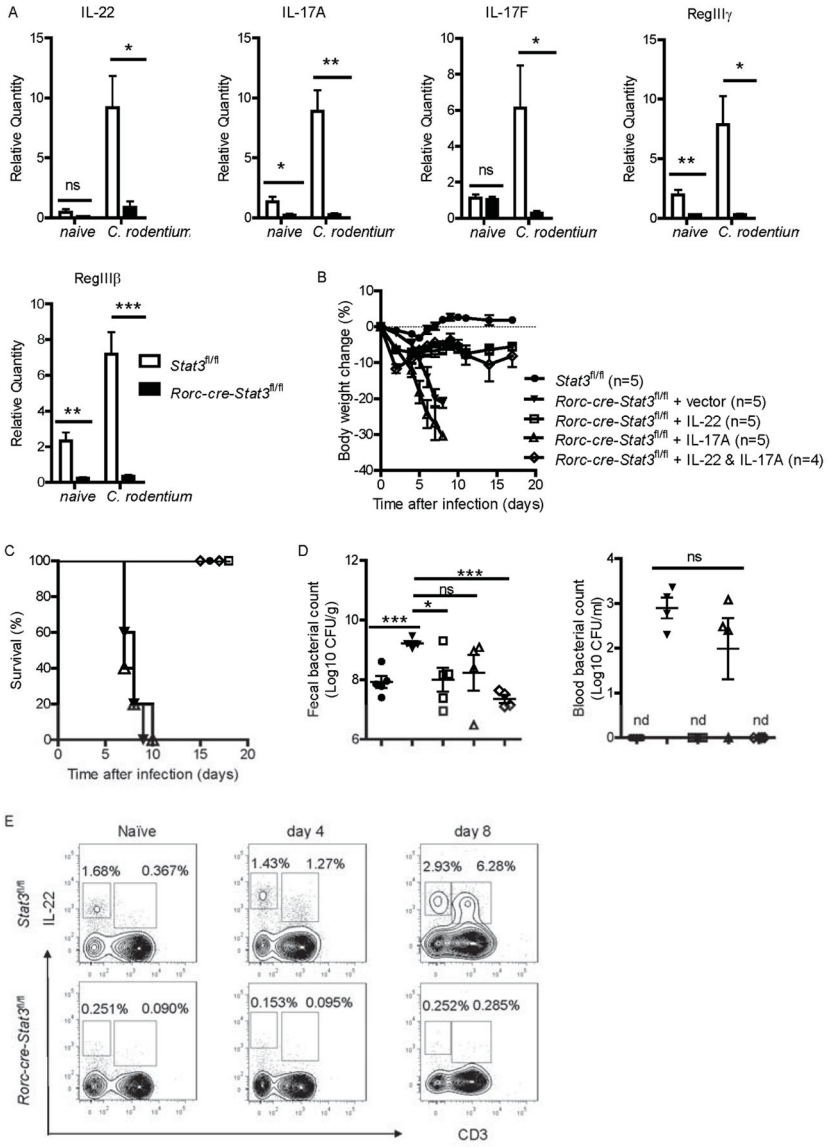

Figure 3. IL-22, but not IL-17, is an essential and sufficient pathway downstream of STAT3 signaling to protect mice against *C. rodentium* infection

(**A**) *Rorc-cre-Stat3*$^{fl/fl}$ and wild type *Stat3*$^{fl/fl}$ littermate mice (n = 5 per group) were infected with 2 × 10^9 CFU of *C. rodentium*. The mRNA expression of IL-22, IL-17A, IL-17F and IL-22- dependent RegIIIγ and RegIIIβ antimicrobial proteins in the colon of naïve or 5 days infected mice were measured by real-time. *P<0.05, **P<0.01, ***P<0.001 (Student's *t*-test). Data are representative of two independent experiments (**A**; mean ± s.e.m.). (**B–D**) *Stat3*$^{fl/fl}$ (n = 5) and *Rorc-cre-Stat3*$^{fl/fl}$ mice were orally inoculated with 2 × 10^9 CFU of *C. rodentium*. 10 μg control vector (n = 5), IL-17 (n = 5), IL-22 (n = 5), or both IL-17 and IL-22 (n = 4) expressing plasmids were hydrodynamic injected into *Rorc-cre-Stat3*$^{fl/fl}$ mice through the tail vein 6 hr after *C. rodentium* infection. Body weight changes (**B**) and survival rates (**C**) were monitored at indicated time points. (**D**) CFU counts of *C. rodentium* in the fecal pellets and blood on day 5 after infection are shown. Data are representative of two independent experiments (mean ± s.e.m.). *P<0.05, ***P<0.001; ns, no significant difference (Student's *t*-test). nd, nondetectable.

(**E**) STAT3 signaling is required for both innate and adaptive IL-22 production. IL-22 expression in $CD3^-$ and $CD3^+$ cells were analyzed by intracellular cytokine staining followed by flow cytometry. *Rorc-cre-Stat3*$^{fl/fl}$ and wild type *Stat3*$^{fl/fl}$ littermate mice were infected with 2×10^9 CFU of *C. rodentium*. Intestinal LPLs were isolated from the colons of *Stat3*$^{fl/fl}$ or *Rorc-cre-Stat3*$^{fl/fl}$ mice at day 4 and day 8 post-infection. Cells were stimulated with IL-23 (25 ng/ml), PMA (50 ng/ml) and ionomycin (750 ng/ml) for 4 hours and gated in $Thy1^+$ lymphocytes. Naïve mice were used as control. Data are representative of three independent experiments. See also Figure S3.

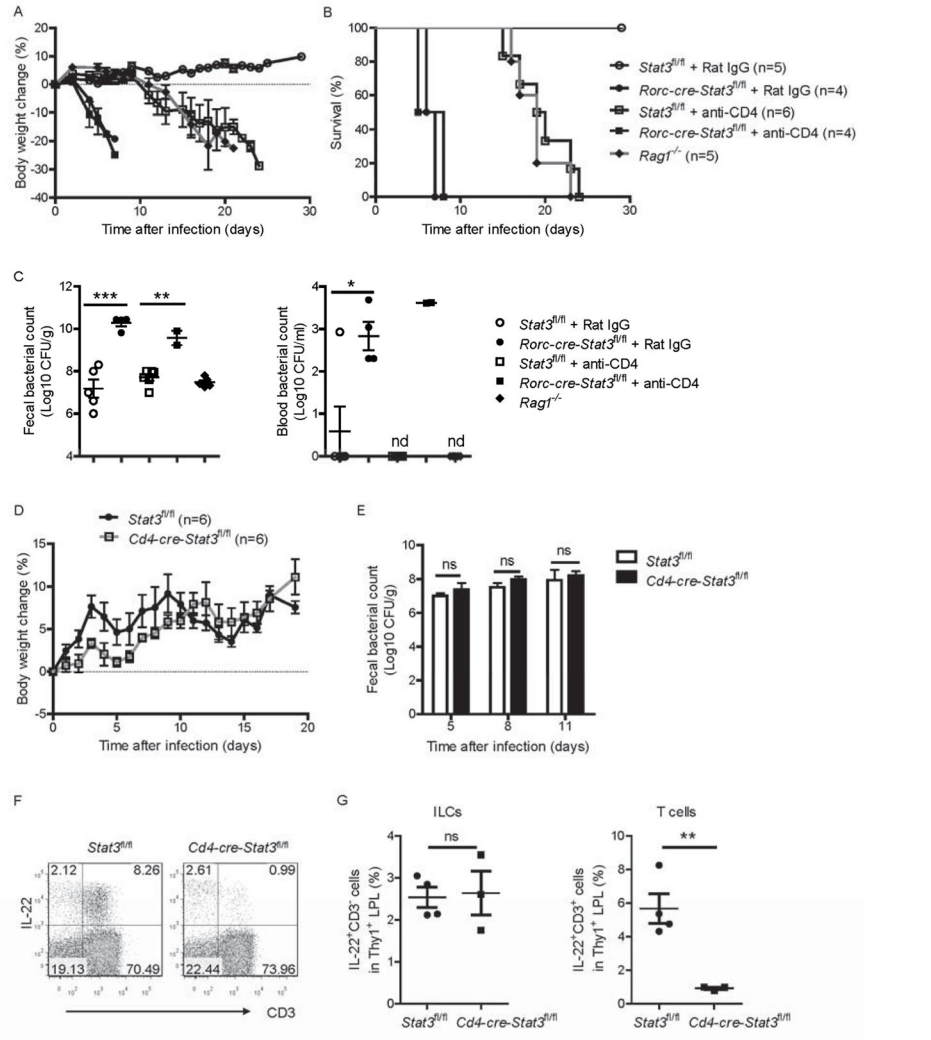

Figure 4. STAT3 signaling and IL-22 from RORγt$^+$ ILCs, but not T cells, are essential for the protection from *C. rodentium* infection

(A–**C**) *Rorc-cre-Stat3*$^{fl/fl}$ and wild type *Stat3*$^{fl/fl}$ mice were infected with 5 × 10^6 CFU of *C. rodentium* and injected intraperitoneally with mAb GK1.5 or Rat IgG (50 μg per mouse each time) at day −5, 0, 5, 10 and 15 post infection for depletion of CD4$^+$ T cells (4 to 6 mice per group). Average body weight change (**A**) and survival rates (**B**) at the indicated time points are shown. (**C**) Bacterial titers from blood and fecal homogenate cultures at day 5 post infection. Each dot represents one individual mouse.

(**D**, **E**) *Cd4-cre-Stat3*$^{fl/fl}$ (n=6) and wild type *Stat3*$^{fl/fl}$ mice (n=6) were orally infected with 2 × 10^9 CFU of *C. rodentium.* (**D**) Body weight changes are shown at the indicated time points. (**E**) Bacterial titers from fecal homogenate cultures at indicated day post infection in the mice in **D**.

(**F**, **G**) IL-22 expression in CD3$^-$ and CD3$^+$ cells were analyzed by intracellular cytokine staining followed by flow cytometry. *Cd4-cre-Stat3*$^{fl/fl}$ and wild type *Stat3*$^{fl/fl}$ littermate mice were infected with 2 × 10^9 CFU of *C. rodentium*. LPLs were isolated from the colons at day 8 post-infection. Cells were stimulated with IL-23 (25 ng/ml), PMA (50 ng/ml) and ionomycin (750 ng/ml) for 4 hours and gated in Thy1$^+$ lymphocytes. Each dot represents one individual mouse.

*P<0.05, **P<0.01, ***P<0.001; ns, no significant difference (Student's *t*-test). nd, nondetectable. Data are representative of two independent experiments (**A–G**; mean ± s.e.m.). See also Figure S4.

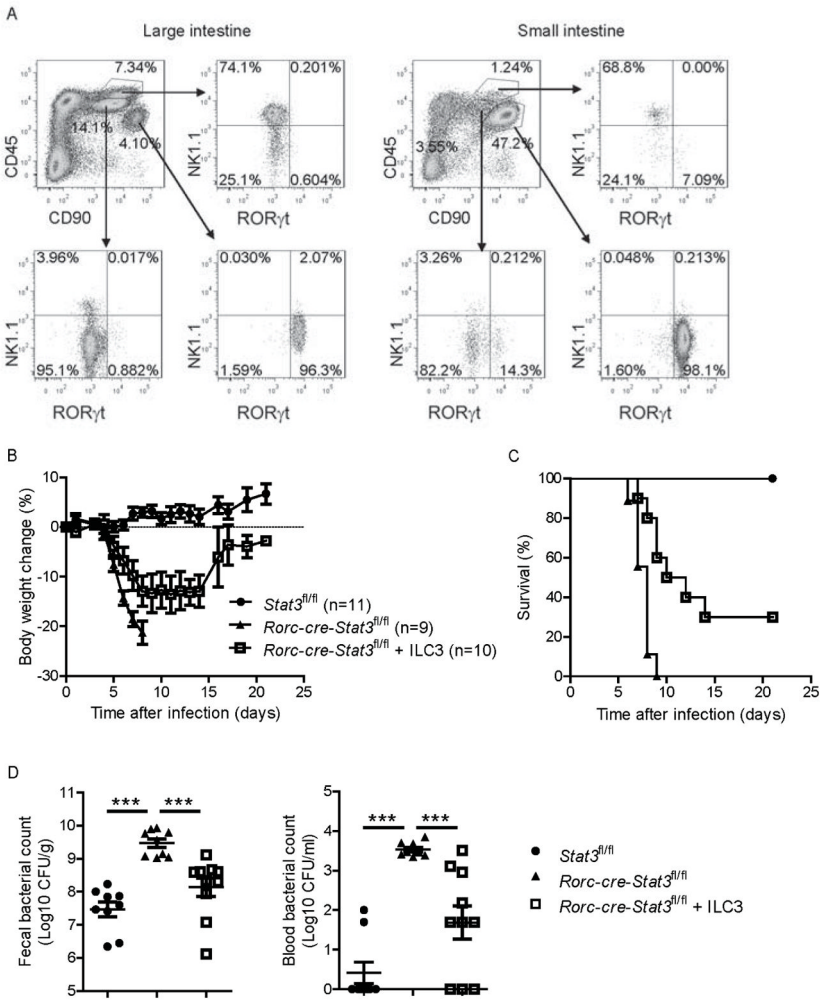

Figure 5. CD90^{hi}CD45^{lo} ILC3s protect *Rorc-cre-Stat3*$^{fl/fl}$ mice from *C. rodentium* infection

(**A**) Intestinal CD90^{hi}CD45^{lo} LPLs are RORγt^{+} ILCs. LPLs were isolated from both the large intestine and small intestine of *Rag1*$^{-/-}$ mice and gated by CD45 and CD90 expression. Three separate populations, CD90^{hi}CD45^{lo}, CD90^{lo}CD45^{int} and CD90^{lo}CD45^{hi} were further analyzed with the expression of NK1.1 and RORγt. Data are representative of two independent experiments.

(**B–D**) *Rag1*$^{-/-}$ mice were infected with 2 × 10^{9} CFU of *C. rodentium* and CD90^{hi}CD45^{lo} ILC3s were sorted from both the colon and small intestine LPLs at day 1 post infection. Sorted ILC3s were stimulated with IL-23 (25 ng/ml) for 1 hour, then were transferred by i.v. injection (2 × 10^{5} cells per mouse) into *Rorc-cre-Stat3*$^{fl/fl}$ recipients at day 0 and day 3 post inoculated with 5 × 10^{6} CFU of *C. rodentium*. Untreated *Rorc-cre-Stat3*$^{fl/fl}$ and wild type *Stat3*$^{fl/fl}$ littermate mice were as control. Body weight changes (**B**) and survival rates (**C**) were monitored at indicated time points. (**D**) CFU counts of *C. rodentium* in the fecal pellets and blood on day 5 after infection are shown. Data are pooled from two independent experiments with nine to eleven mice per group (mean ± s.e.m.). ***P<0.001 (Student's *t*-test). See also Figure S5.

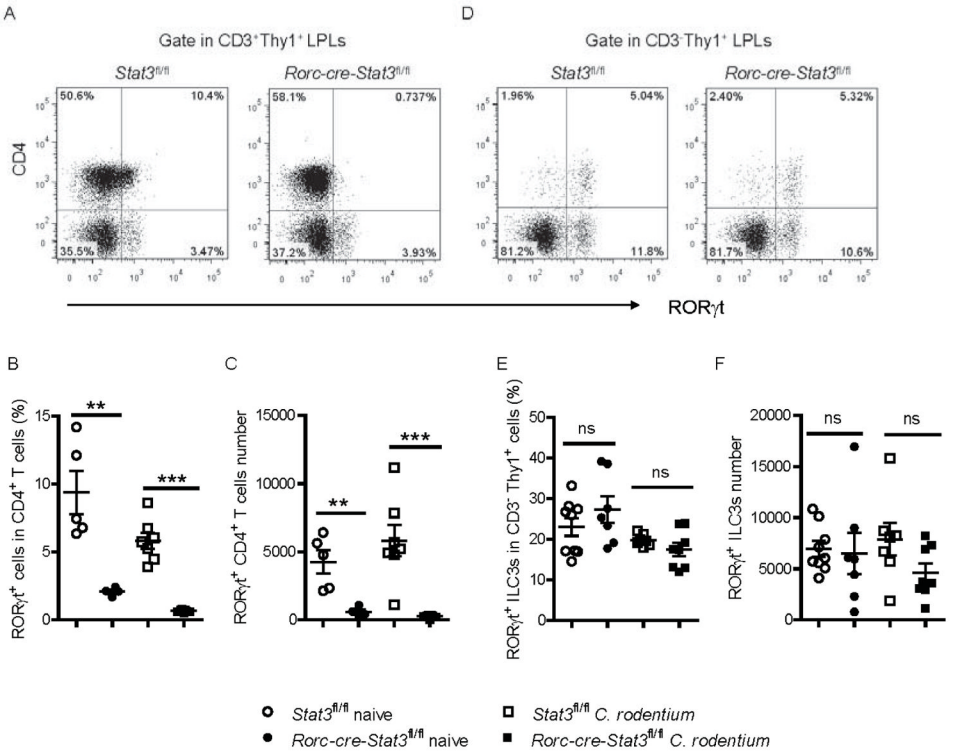

Figure 6. The development of RORγt$^+$ T helper cells, but not RORγt$^+$ ILCs is dependent on STAT3 signaling

(**A–F**) Colonic LPLs were isolated from *Stat3*$^{fl/fl}$ and *Rorc-cre-Stat3*$^{fl/fl}$ littermate mice before or 5 days after *C. rodentium* infection.

(**A**, **D**) RORγt and CD4 expression were analyzed in colonic LPLs from naïve mice by flow cytometry after gating on Thy1$^+$ CD3$^+$ adaptive lymphocytes and Thy1$^+$ CD3$^-$ innate lymphoid cells.

(**B**, **C**) Percentage of RORγt$^+$ cells in the CD4$^+$ CD3$^+$ T cell population, as well as the absolute numbers of RORγt$^+$ CD4$^+$ T cells in the colons of naïve and *C. rodentium* infected *Stat3*$^{fl/fl}$ and *Rorc-cre-Stat3*$^{fl/fl}$ mice are shown.

(**E**, **F**) Percentage of RORγt$^+$ cells in the CD3$^-$ Thy1$^+$ ILCs population, as well as the absolute numbers of RORγt$^+$ ILCs in the colons of naïve and *C. rodentium* infected *Stat3*$^{fl/fl}$ and *Rorc-cre-Stat3*$^{fl/fl}$ mice are shown.

P<0.01, ***P<0.001; ns, no significant difference (Student's *t*-test). Data are representative of three independent experiments (mean ± s.e.m.). Each dot represents one individual mouse (B, C, E, F**). See also Figure S6.

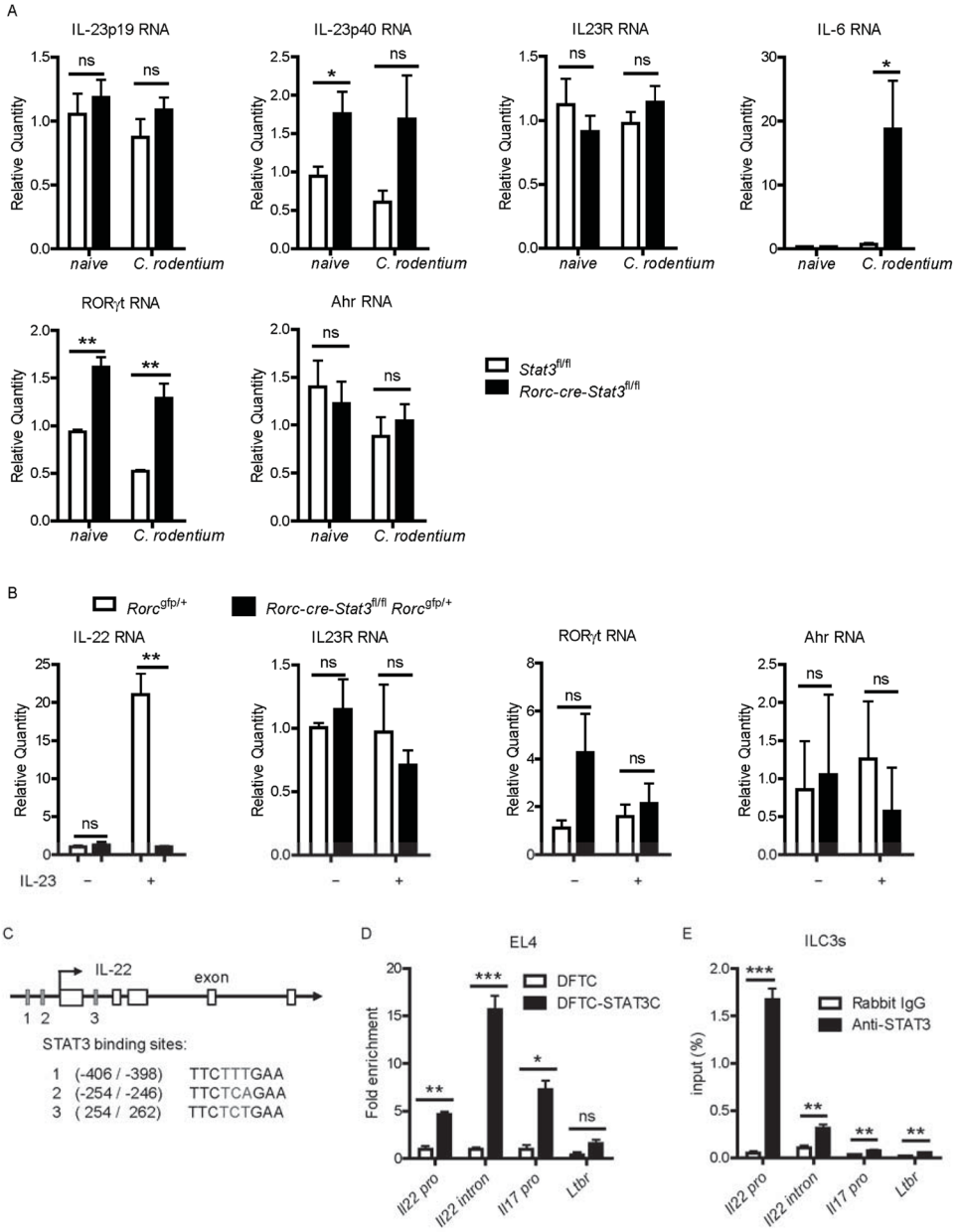

Figure 7. STAT3 directly binds to the *Il22* locus and regulates IL-22 production

(**A**) *Rorc-cre-Stat3*$^{fl/fl}$ and *Stat3*$^{fl/fl}$ littermate mice (n = 5 per group) were infected with 2 × 10^9 CFU of *C. rodentium*. The mRNA expression of IL-23 p19, IL-23 p40, IL23R, IL-6, RORγt and Ahr in the colon were measured by real-time PCR before and 5 days after infection. Data are representative of two independent experiments (mean ± s.e.m.).

(**B**) RORγt$^+$ ILCs were purified by flow cytometric sorting from colonic LPLs of *Rorc-cre-Stat3*$^{fl/fl}$ *Rorc*$^{gfp/+}$ and *Rorc*$^{gfp/+}$ mice and stimulated with or without IL-23 (25 ng/ml) for 1 hour. The mRNA expression of IL-22, IL-23R, RORγt and Ahr were measured by real-time PCR. Data are representative of two independent experiments (mean ± s.e.m.).

(**C**) STAT3 binding sites at the *Il22* locus are shown.

(**D**) STAT3 binding at the *Il22* locus in EL4 stable cell lines stably expressing either flag peptide (DFTC) or flag-tagged STAT3C (DFTC-STAT3C) was monitored using a ChIP

assay. The anti-flag antibody immunoprecipitates were analyzed by real-time PCR. The fold enrichment of STAT3C binding at each locus was normalized to DFTC-empty EL4 cells. *Ltbr* and *Il17* loci were used as negative and positive controls in the ChIP assays, respectively. Data are representative of three independent experiments (mean ± s.e.m. of triplicate samples of real-time PCR).

(**E**) $CD90^{hi}CD45^{lo}$ ILC3s were sorted from *Rag1*$^{-/-}$ mice and stimulated with IL-23 (25 ng/ml) for 30 minutes. ChIP assays with anti-STAT3 mAb showed enhanced enrichment of STAT3 at the *Il22* locus in primary ILC3s. *Ltbr* loci lacking STAT3 binging sites was used as negative controls. Data are representative of two independent experiments (mean ± s.e.m. of quadruplicate samples of real-time PCR).

*P<0.05, **P<0.01, ***P<0.001; ns, no significant difference (Student's *t*-test).