Resetting microbiota by *Lactobacillus reuteri* inhibits T reg deficiency–induced autoimmunity via adenosine A_{2A} receptors

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Regulatory T (T reg) cell deficiency causes lethal, CD4$^+$ T cell–driven autoimmune diseases. Stem cell transplantation is used to treat these diseases, but this procedure is limited by the availability of a suitable donor. The intestinal microbiota drives host immune homeostasis by regulating the differentiation and expansion of T reg, Th1, and Th2 cells. It is currently unclear if T reg cell deficiency–mediated autoimmune disorders can be treated by targeting the enteric microbiota. Here, we demonstrate that Foxp3$^+$ T reg cell deficiency results in gut microbial dysbiosis and autoimmunity over the lifespan of scurfy (SF) mouse. Remodeling microbiota with *Lactobacillus reuteri* prolonged survival and reduced multiorgan inflammation in SF mice. *L. reuteri* changed the metabolomic profile disrupted by T reg cell deficiency, and a major effect was to restore levels of the purine metabolite inosine. Feeding inosine itself prolonged life and inhibited multiorgan inflammation by reducing Th1/Th2 cells and their associated cytokines. Mechanistically, the inhibition of inosine on the differentiation of Th1 and Th2 cells in vitro depended on adenosine A_{2A} receptors, which were also required for the efficacy of inosine and of *L. reuteri* in vivo. These results reveal that the microbiota–inosine–A_{2A} receptor axis might represent a potential avenue for combatting autoimmune diseases mediated by T reg cell dysfunction.

INTRODUCTION

Regulatory T (T reg) cells maintain immune homeostasis and play a pivotal role in immune tolerance. Forkhead box protein 3 (Foxp3) is a major transcription factor that is associated with T reg cell development and function (Ouyang et al., 2010). Mutations or deletions of the Foxp3 gene result in IPEX syndrome (immunodysregulation, polyendocrinopathy, and enteropathy, with X-linked inheritance) in humans. IPEX syndrome is an autoimmune disease associated with eczema, severe enteropathy, type I diabetes, thyroiditis, hemolytic anemia, and thrombocytopenia (Bennett et al., 2001). Recently, a Foxp3 mutation has been also identified in a two-generation family with inflammatory bowel disease (IBD; Okou et al., 2014). several other gene defects that affect the function of T reg cells give rise to IPEX-like syndromes, including mutations or deficiency in the α-chain of the IL-2 receptor (CD25), signal transducer and activator of transcription 5b (STAT5b), Itchy E3 ubiquitin protein ligase (ITCH), STAT1, cytotoxic T-lymphocyte–associated protein 4 (CTLA4), and Wiskott-Aldrich syndrome (WAS; Massaad et al., 2013;Verbsky and Chatila, 2013).

The scurfy (SF) mouse, which bears a mutation in the Foxp3 gene, displays a similar clinical phenotype, with early onset dermatitis, progressive multiorgan inflammation, and death within the first month of life caused by a lymphoproliferative syndrome (Godfrey et al., 1991a,b; Sharma et al., 2009). The lethal lymphoproliferative syndrome has been shown to be predominately mediated by CD4$^+$ Th1 and Th2 cell–induced pathology (Blair et al., 1994; Kanangat et al., 1996; Sharma et al., 2009, 2011; Suscovich et al., 2012), similar to what is seen in patients with IPEX syndrome (Zennaro et al., 2012; Baris et al., 2014). To date, treatment with immuno-

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Abbreviations used: cAMP, cyclic adenosine monophosphate; CNT2, concentrative nucleoside transporter 2; EAE, experimental autoimmune encephalomyelitis; ENT1, equilibrative nucleoside transporter 1; IPEX, immunodysregulation, polyendocrinopathy, enteropathy, with X-linked inheritance; *La* DDS, *Lactobacillus acidophilus* DDS; mLN, mesenteric LN; PCoA, principal coordinates analysis; SF, scurfy.

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suppressive drugs in combination with supportive care, such as total parental nutrition (TPN) and blood transfusion, may help to palliate clinical manifestations (Hannibal and Torgerson, 2011). Transplantation of donor T reg cells and stem cells is promising, but the procedure is limited by the availability of a suitable donor; and the ultimate outcome can be fatal or associated with chronic health problems (Rao et al., 2007; Seidel et al., 2009; Burroughs et al., 2010; Nademi et al., 2014).

The intestinal microbiota drives host immune homeostasis by regulating the differentiation and expansion of T reg cells (Round and Mazmanian, 2010; Arpaia et al., 2013; Furusawa et al., 2013; Weng and Walker, 2013; Belkaid and Hand, 2014). Intestinal microbial dysbiosis can develop as a result of an abnormal diet, infection, inflammation, and altered host genetics (Lupp et al., 2007; David et al., 2014; Goodrich et al., 2014; Lukens et al., 2014). Gut microbial dysbiosis can lead to autoimmune diseases, including IBD, autoimmune arthritis, type I diabetes, and experimental autoimmune encephalomyelitis (EAE; Cerf-Bensussan and Gaboriau-Routhiau, 2010; Wu and Wu, 2012; Markle et al., 2013). However, the host–microbiota interactions in monogenic autoimmune diseases largely remain unknown. Therefore, we hypothesized that (i) T reg cell deficiency caused by a Foxp3 mutation disrupted the gut microbiota; and (ii) T reg cell deficiency–mediated autoimmune disease can be treated by targeting gut microbiota.

Probiotics have the capacity to not only induce large-scale changes in the host microbiota composition but also modulate the global metabolic function of intestinal microbiomes (Hemarajata and Versalovic, 2013; Sanders et al., 2013). *Lactobacillus reuteri* (*L. reuteri*) demonstrates beneficial attributes caused by a mutualistic relationship between microbe and host (Walter et al., 2011). *L. reuteri* DSM17938 is effective in treating and preventing diseases that affect infants and children, including necrotizing enterocolitis, diarrhea, and infantile colic (Urbańska and Szajewska, 2014). *L. reuteri* modulates the abnormal microbial communities associated with these diseases (Mai et al., 2006; Rhoads et al., 2009; Torrazza and Neu, 2013; Patel and Denning, 2015).

In this study, we characterize the dynamic changes of autoimmunity and gut microbial dysbiosis over the short lifespan of the Foxp3$^+$ T reg cell–deficient SF mouse. We demonstrate a shift in resident microbiota after treatment with probiotic *L. reuteri* in SF mice, which consequently inhibits the degree of autoimmunity. Protective effects of a microbiota-modulated metabolite, inosine, against T reg cell deficiency–induced autoimmunity were identified. Our study highlights the immunomodulatory mechanism by which remodeling microbiota and inosine counteract T reg cell deficiency–induced autoimmunity.

RESULTS

T reg cell deficiency changes the gut microbiota

Because human genetic factors can shape the gut microbiome (Goodrich et al., 2014), we initially explored whether gut microbiota would be altered by the Foxp3 gene mutation. We conducted a dynamic study of autoimmunity and gut microbiota in Foxp3$^+$ T reg cell–deficient scurfy (SF) mice, comparing WT littermates to SF mice at day 8 (d8), d15, and d22 of age. We observed that SF mice develop chronic inflammation in several tissues (liver, lung, ear, and tail; Fig. S1, A–E) and acquire an increase the frequency of IFN-γ–producing CD4$^+$ T (Th1) and IL-4–producing CD4$^+$ T (Th2) cells in the spleen and mesenteric LNs (MLNs; Fig. 1 A and Fig. S1 G). Also, plasma levels of IFN-γ and IL-4 were increased as early as d8, at a time when a SF clinical phenotype had not been demonstrated (Fig. 1 B). However, Th17 cells were not increased in spleen and MLN of SF mice from d15 to d22 (Fig. S1, F and G), suggesting that Th1 and Th2 responses are the main drivers of autoimmunity in T reg cell–deficient SF mice.

Gut microbial dysbiosis developed over the first 22 d of life in SF mice (compared with WT littermates), as revealed by stool 16S rRNA gene sequencing analysis at d8, d15, and d22. First, Shannon α diversity was significantly decreased in SF mice compared with WT littermates at d22 (Fig. 1 C). Second, weighted UniFrac-based three-dimensional principal coordinates analysis (PCoA) revealed a distinctly shifted gut microbial composition between SF and WT (Fig. 1 D). Longitudinal analysis of the relative abundance of gut microbial communities in these mice indicated broad population changes from the phylum to the genus levels in SF compared with WT mice, suggesting that Foxp3$^+$ T reg cell deficiency shapes gut microbial community structure (Fig. 1 E and Fig. S1 H). For example, the relative abundance of *Lactobacillus* was significantly lower in SF stool compared with that in WT stool at d8 ($P < 0.001$; Fig. 1 F). *Bacteroides* was significantly higher abundance in SF than in WT stool at d22 ($P < 0.001$; Fig. 1 F). In summary, whereas sampling across time, we observed that development of autoimmunity was accompanied by microbial dysbiosis.

Lactobacillus reuteri treatment reprograms gut microbiota in T reg cell deficiency

We further examined whether gut microbiota shaped by T reg cell deficiency can be reprogrammed by oral administration of probiotic *L. reuteri*. Stool microbiota were analyzed when *L. reuteri* was orally fed by gavage, 10^7 CFU/day, daily, starting on d8 for 2 wk (early treatment) or on d15 for 1 wk (late treatment). We found that *L. reuteri* given either early or later produced similar effects in resetting gut microbial dysbiosis in SF mice. The decreased Shannon α diversity associated with T reg cell deficiency was reversed by *L. reuteri* early or late treatment ($P < 0.05$; Fig. 2 A and Fig. S2 F). A three-dimensional PCoA and nonparametrical multiple dimensional scaling analysis revealed that SF mice with *L. reuteri* treatment displayed a shift in microbial community composition, which was distinct from either WT or SF populations (Fig. 2, B and C; and Fig. S2 G). According to our evaluation of predominant bacteria from the phylum to genus level, *L. reuteri* specifically increased the relative abundance of the phylum *Firmicutes* and the genera *Lactobacillus* and *Oscil-*

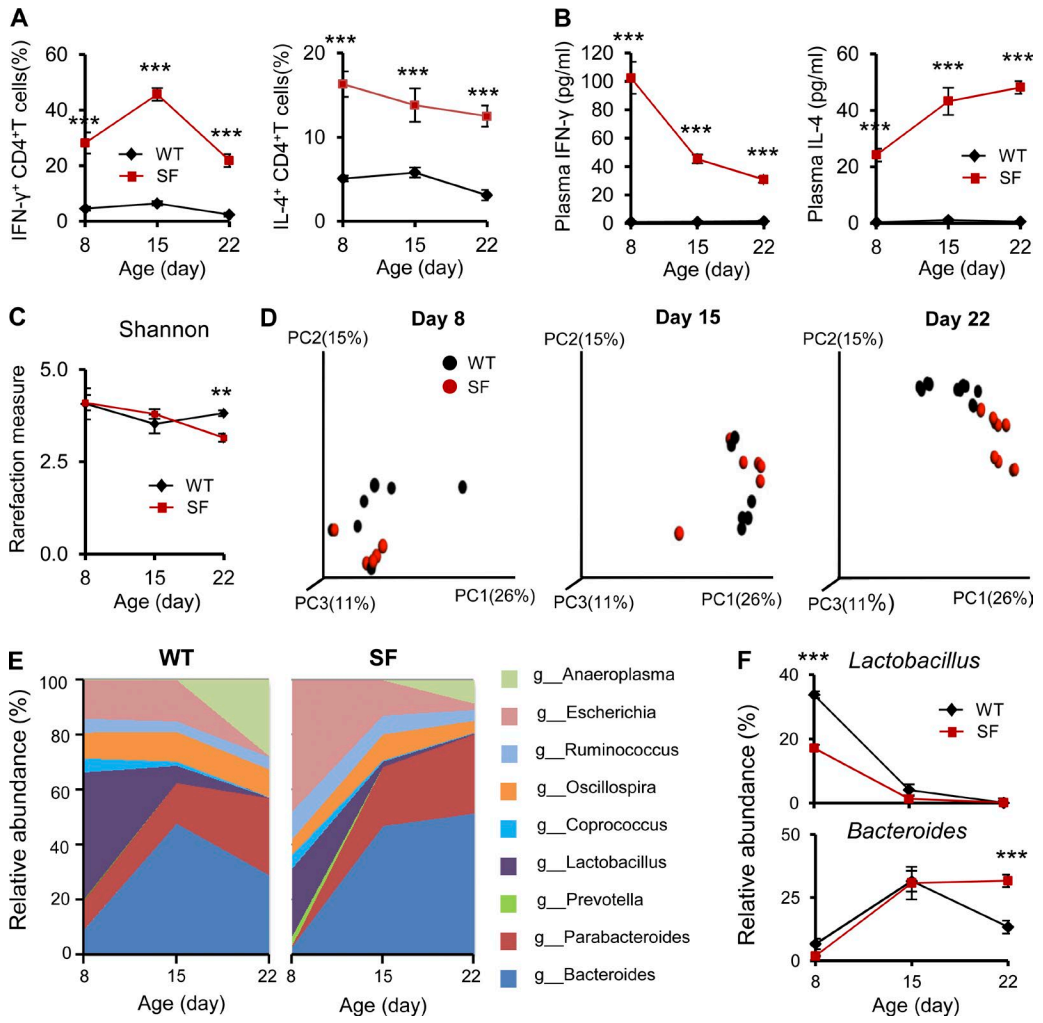

Figure 1. **Dynamics of autoimmunity development and microbiota dysbiosis over 22 d of life in T reg cell–deficient scurfy (SF) mice.** (A) Percentage of IFN-γ– or IL-4–producing-CD4+ T cells in spleen of WT and SF mice at the indicated age (two-way ANOVA; *n* = 6–12 per group). (B) Plasma IFN-γ and IL-4 levels at the indicated age (two-way ANOVA; *n* = 6–12 per group). (C) Gut microbial Shannon diversity analysis (two-way ANOVA; *n* = 6–12 per group). (D) Gut microbial analysis of weighted unifrac-based three-dimensional PCoA plots of WT and SF mice at the indicated age (*n* = 6–12 per group). (E and F) Relative abundance of predominant bacteria (>1% in any sample) at the genus level (E) and relative abundance of total bacteria at the genus level (F; two-way ANOVA; *n* = 6–12 per group). **, P < 0.01; ***, P < 0.001. SF versus WT. Error bars represent mean ± SEM.

lospira, and decreased the relative abundance of the phylum *Tenericutes* and the genus *Bacteroides* (Fig. 2, D–F; and Fig. S2, H and I). Further analysis at the species level of *Lactobacillus* showed that the relative abundances of *L. reuteri* and *L. others* significantly increased after oral feeding of *L. reuteri* to SF mice compared with that of SF mice without *L. reuteri* treatment (Fig. 2 G). These results indicated that gut microbial dysbiosis induced by T reg cell deficiency could be reprogramed by oral administration of *L. reuteri.*

Remodeling gut microbiota by *L. reuteri* suppresses autoimmunity induced by T reg cell deficiency

Gut microbiota play an important role in regulating host immune homeostasis (Wu and Wu, 2012; Belkaid and Hand, 2014). To determine whether remodeling gut microbiota by *L. reuteri* could globally impact T reg cell deficiency–associated autoimmunity, the survival and inflammation of SF mice were evaluated after orally feeding *L. reuteri*, starting on d8 (early treatment, to infinity for survival; and to 2 wk for tissue analysis; Fig. 3 A). SF mice (as expected) died between d21 and d30 as a result of lymphoproliferative syndrome and severe multiorgan inflammation (Fig. 3, B–G). However, the survival rate of SF mice with *L. reuteri* early treatment was significantly increased to at least 125 d (Fig. 3 B). In addition, *L. reuteri* significantly reduced inflammatory infiltration measured by the mean area of lymphocyte infiltration in liver and lung (P < 0.001; Fig. 3, C and D). The frequency of IFN-γ–producing CD4+ T (Th1) and IL-4–producing

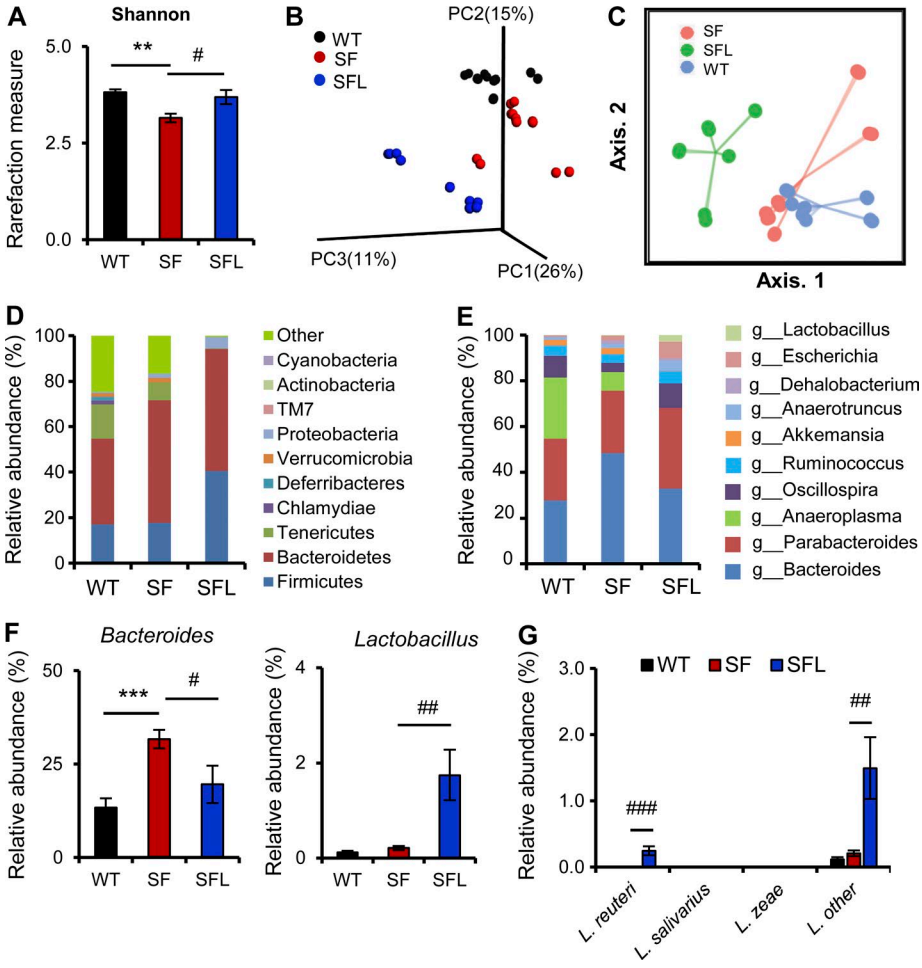

Figure 2. ***L. reuteri* early treatment modulates the intestinal microbiota in SF mice.** (A) Gut microbial Shannon diversity analysis, comparing groups of WT, SF, and SF treated with *L. reuteri* (SFL; one-way ANOVA; n = 10–12 per group). (B) Weighted UniFrac-based three-dimensional PCoA analysis of gut microbiota of WT, SF, and SFL mice (n = 10–12 per group). (C) Nonparametric multiple dimensional scaling analysis of gut microbiota of WT, SF, and SFL mice (n = 10–12 per group). (D–F) Relative abundance of predominant bacteria (>1% in any sample) at the phylum level [D], and at the genus level (E), and relative abundance of total bacteria at genus level (F) in feces (one-way ANOVA; n = 10–12 per group). (G) Relative abundance of *Lactobacillus* at the species level in feces (one-way ANOVA; n = 10–12 per group). **, $P < 0.01$; ***, $P < 0.001$. SF versus WT. #, $P < 0.05$; ##, $P < 0.01$; and ###, $P < 0.001$. SFL versus SF. Error bars represent mean ± SEM.

$CD4^+$ T (Th2) cells in the spleen and MLNs of SF mice were significantly reduced by *L. reuteri* treatment, respectively (Fig. 3, E and F). Furthermore, the levels of IFN-γ and IL-4 in plasma of SF mice were significantly decreased by *L. reuteri* treatment (Fig. 3 G).

We also tested the therapeutic effects of *L. reuteri* in SF mice, beginning once they demonstrated overt clinical symptoms, on d15; we continued *L. reuteri* or *Lactobacillus acidophilus DDS (La DDS)* as a control for 2 wk (late treatment; Fig. S2 A). *La DDS* has been shown to lack the properties of adherence to epithelial cells, induction of mucin expression by intestinal epithelial cells, inhibition of enteropathogenic *E. coli* epithelial cell adherence, and inhibition of NF-κB (Mack et al., 2003; Liu et al., 2012). We found that *L. reuteri* significantly increased the survival of SF mice ($P < 0.0001$; Fig. S2 B). Interestingly, the survival of SF mice was improved even after terminating oral feeding of *L. reuteri* on d29, indicating that *L. reuteri* may induce a persistent shift of gut microbiota in SF mice. In contrast, *La DDS* had a lesser effect on SF survival than *L. reuteri* ($P = 0.0149$ vs. SF mice; $P = 0.0024$ vs. SF+*L. reuteri*; Fig. S2 B). *L. reuteri* but not *La DDS* late treatment reduced inflammatory infiltration in liver and lung, inhibited Th1/Th2 cells in the spleen, and reduced plasma levels of IFN-γ and IL-4 (Fig. S2, C–E). Altogether, these results demonstrate that remodeling gut microbiota by *L. reuteri* suppresses autoimmunity mediated by T reg cell deficiency.

Metabolomic profiles are modulated by T reg cell deficiency and *L. reuteri* treatment

Given that metabolites of commensal bacterial metabolism play a key role in microbe–host interactions (Nicholson et al.,

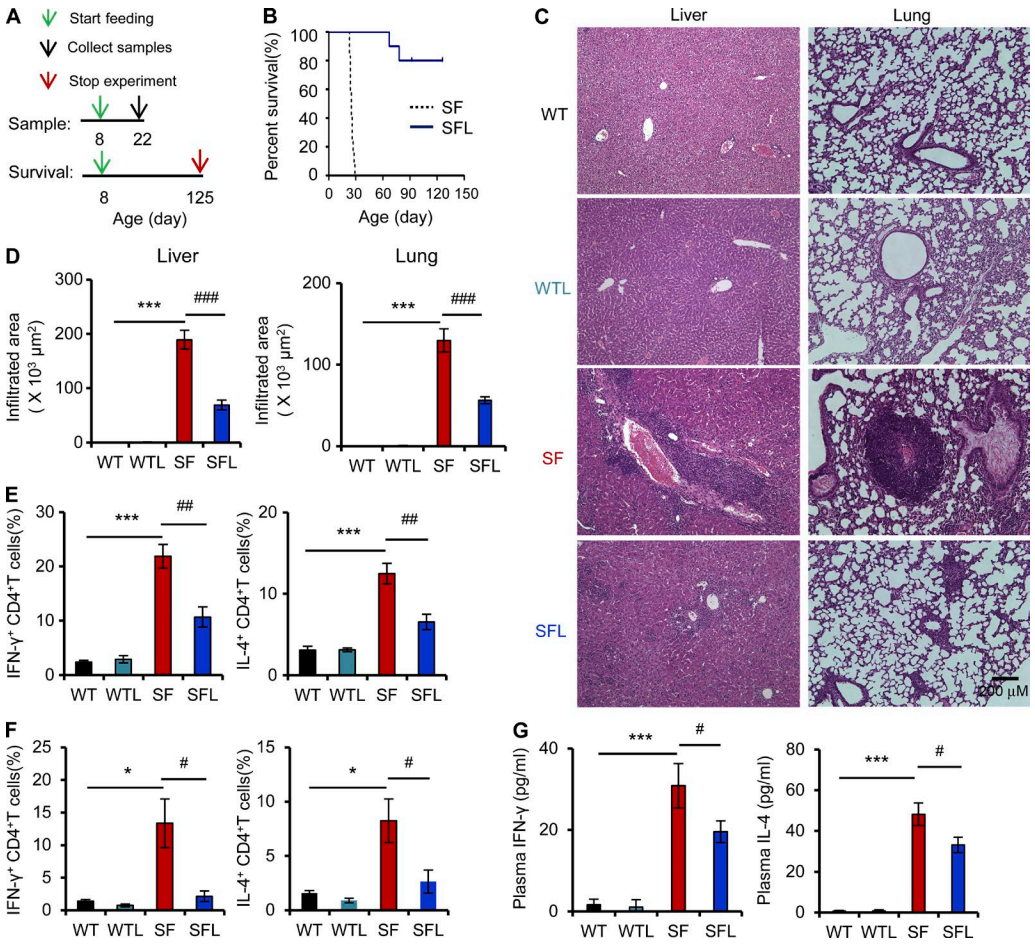

Figure 3. ***L. reuteri* early treatment increases survival and suppresses autoimmunity in SF mice.** (A) Scheme for *L. reuteri* early treatment on WT or SF mice for sample analysis and survival observation. (B) Survival curves of SF and SFL mice (Kaplan-Meier survival curves and log-rank test; $n = 10–17$ per group). (C) Representative H&E staining of liver and lung from WT, WTL, SF, and SFL mice. Bars, 200 μm ($n = 6–12$ per group). (D) Quantitation of inflammatory infiltrates in liver and lung of WT, WTL, SF, and SFL (one-way ANOVA; $n = 6–12$ per group). (E and F) The frequency of IFN-γ or IL-4–producing-CD4$^+$ T cells in spleen (E) and MLNs (F) of mice (one-way ANOVA; $n = 6–12$ per group). (G) Plasma levels of IFN-γ and IL-4 in WT, WTL, SF, and SFL mice (one-way ANOVA; $n = 6–12$ per group). *, $P < 0.05$; ***, $P < 0.001$. SF versus WT. $^{\#}$, $P < 0.05$; $^{\#\#}$, $P < 0.01$; $^{\#\#\#}$, $P < 0.001$. SFL versus SF. Error bars represent mean ± SEM.

2012; Dorrestein et al., 2014; Lee and Hase, 2014), we analyzed metabolomic profiles of plasma and feces obtained from WT, SF, and SF with *L. reuteri* given before overt symptoms (early treatment; SFL). We measured 525 metabolites in the plasma of WT, SF, and SFL mice (Table S1). The PCoA and heat map of the metabolites indicate that T reg cell deficiency affected plasma metabolomic profiles, and that *L. reuteri* treatment had a significant impact on plasma metabolome associated with T reg cell deficiency (Fig. 4, A and B). T reg cell deficiency led to significant alterations in 51% (269/525) of all detected metabolites in plasma, whereas *L. reuteri* treatment changed 5.5% (29/525) of all detected metabolites compared with SF (Fig. 4 C and Table S1). We further studied 29 plasma metabolites that were significantly altered by T reg cell deficiency and focused specifically on which metabolites were restored to WT control levels by *L. reuteri* (Fig. 4 D). We observed that the purine metabolite inosine was decreased fivefold in SF, but was completely restored by *L. reuteri* treatment (Fig. 4, D and E). Other metabolites involved in inosine metabolism, including adenosine, hypoxanthine, and xanthine, were altered by T reg cell deficiency, but were only partially restored by *L. reuteri* treatment (Fig. 4 E and Fig. S3 A).

A total of 657 metabolites in feces of WT, SF, and SFL mice could be detected (Fig. S3 B and Table S2). We found that 11% (76/657) of all detected metabolites in feces were altered by T reg cell deficiency compared with WT, whereas *L. reuteri* treatment changed 6% (42/657) of all detected metabolites compared with SF (Fig. S3 C and Table S2). Notably, the levels of metabolites of inosine metabolism, including adenosine, hypoxanthine, and xanthine, were not changed by either T reg cell deficiency or *L. reuteri* treatment. In feces, adenosine levels trended to be low and inosine was significantly decreased in *L. reuteri*–fed mice (Fig. S3 D). Because plasma inosine levels reverted to normal, we postulated that

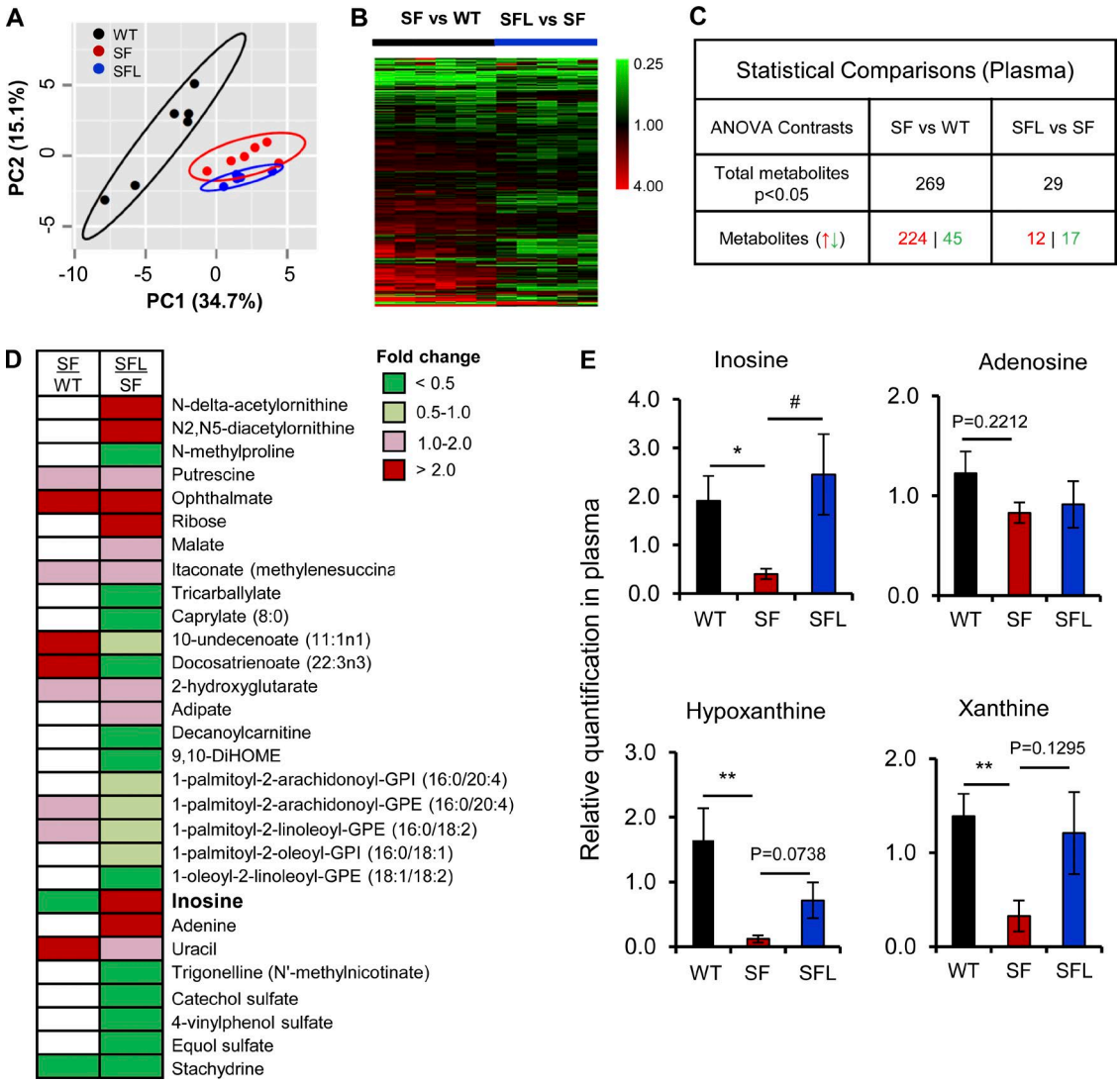

Figure 4. **Plasma metabolomic profiles are modulated by *L. reuteri* early treatment of SF mice.** (A) PCA clustering from plasma metabolites of WT, SF, and SFL (n = 5–6 per group). (B) Heat map of 525 metabolites in plasma of WT, SF, and SFL mice. Each lane represents a different mouse. The scale bar indicates fold change of SF versus WT or SFL versus SF (n = 5–6 per group). (C) Numbers of plasma metabolites affected, either up-regulated (↑) or down-regulated (↓; $P < 0.05$), by SF versus WT or SFL versus SF mice (n = 5–6 per group). (D) Heat map showing the levels of 29 plasma metabolites that were significantly altered by *L. reuteri* treatment in SF mice. Colors indicate fold changes with $P < 0.05$ (SF vs. WT or SFL *vs.* SF). (E) Relative quantification of metabolites related to inosine pathway in plasma (one-way ANOVA; n = 5–6 per group). *, $P < 0.05$; **, $P < 0.01$. SF versus WT. #, $P < 0.05$. SFL versus SF. Error bars represent mean ± SEM.

the reduced inosine in feces might be related to the increased absorption. To address this hypothesis, we measured the villus height and crypt depth, and we examined the expression levels of the intestinal nucleoside transporters *equilibrative nucleoside transporter 1* (*ENT1*) and *concentrative nucleoside transporter 2* (*CNT2*; Ward et al., 2000; Okada et al., 2006). Indeed, we found that *L. reuteri* treatment was associated with improvement of villus height and increased expression of intestinal nucleoside transporters ENT1 and CNT2 (Fig. S4, A–D). The changes of inosine in plasma associated with T reg cell deficiency and reversed by *L. reuteri* suggest that the inosine levels modulated by gut microbiota may play an important role in the mechanism of action of this probiotic.

Inosine inhibits Th1/Th2 differentiation via adenosine A_{2A} receptors

Inosine has been reported to have immunomodulatory effects on immune cells, such as macrophages (Haskó et al., 2004). It is necessary to know the effects of inosine on the differentiation and proliferation of T cells, because T reg cell deficiency results in an early immunopathology driven by Th1/Th2 cells and their associated cytokines in SF mice (Fig. 1, A and B;

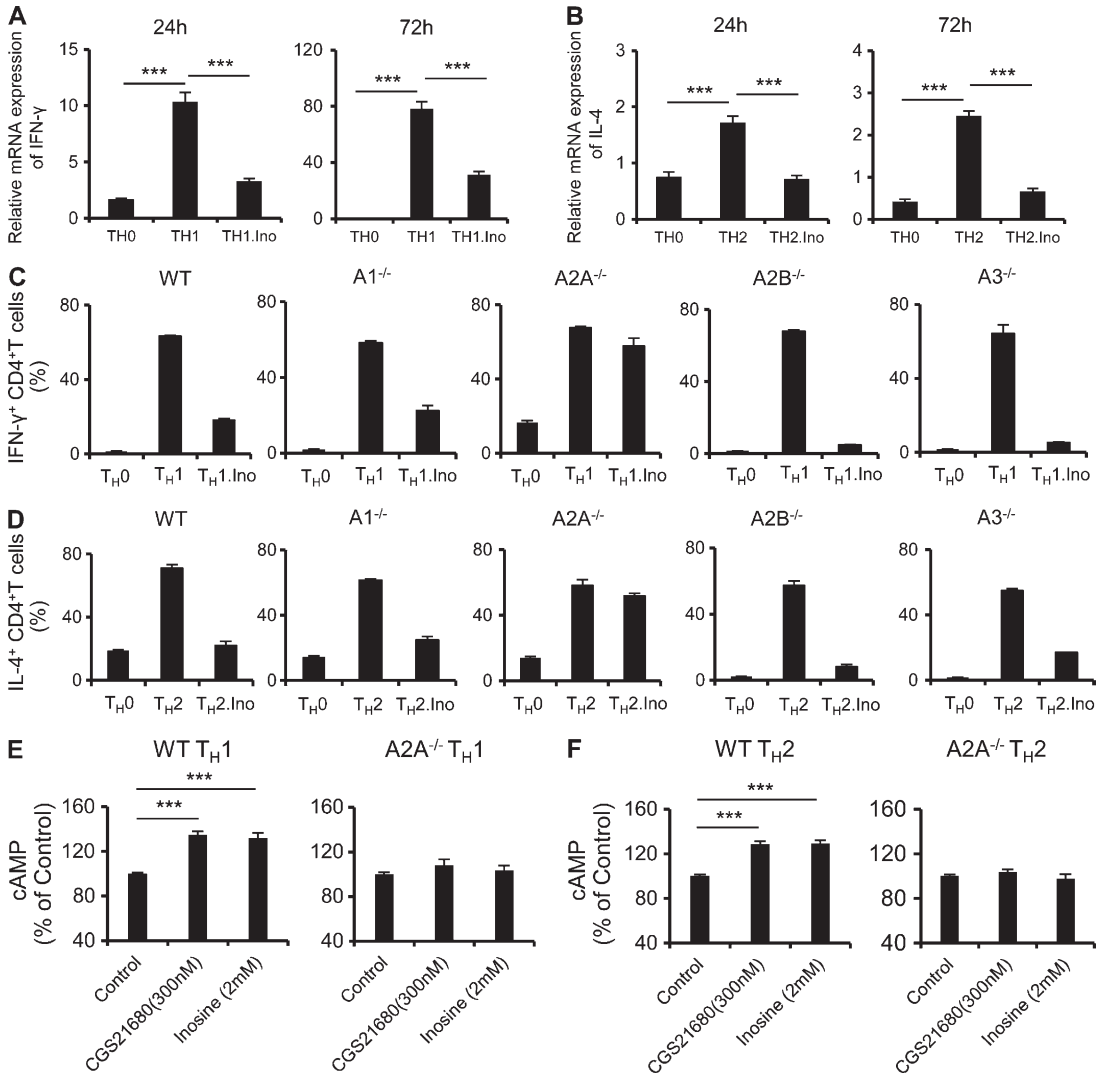

Figure 5. **Inosine suppresses Th1/Th2 cell differentiation via adenosine A_{2A} receptor in vitro.** (A) mRNA expression of IFN-γ in Th1 cells with 2 mM of inosine treatment for 24 or 72 h (one-way ANOVA; *n* = 4 independent experiments). (B) mRNA expression of IL-4 in Th2 cells with 2 mM of inosine treatment for 24 or 72 h (one-way ANOVA; *n* = 4 independent experiments). (C and D) Effect of inosine on Th1 (C) or Th2 (D) cell differentiation from WT, $A_1^{-/-}$, $A_{2A}^{-/-}$, $A_{2B}^{-/-}$, and $A_3^{-/-}$ mice (*n* = 3 independent experiments). (E and F) cAMP level in Th1 cells (E) or cAMP level in Th2 cells (F) of WT or $A_{2A}^{-/-}$ mice after inosine and A_{2A} receptor-selective agonist CGS31680 treatment (one-way ANOVA; *n* = 4 independent experiments). ***, $P < 0.001$. Error bars represent mean ± SEM.

and Fig. S1 G). Naive $CD4^+$ T cells isolated from spleens of C57BL/6J mice can differentiate into Th1 and Th2 cells in vitro in the presence of specific stimulatory/inhibitory cytokines (Zhou et al., 2009). Inosine was added during the process of cell differentiation to test whether inosine affects this process. Results indicated that the relative mRNA expression of IFN-γ was increased in naive T cells with Th1 differentiation media, from 10.3 ± 0.8 at 24 h to 78.3 ± 5.1 at 72 h culture, compared with Th0 in a medium without Th1 differentiation antibodies and cytokines (Fig. 5 A). Increased mRNA expression of IFN-γ was inhibited by inosine (Fig. 5 A). Inosine also inhibited mRNA expression of IL-4 in naive T cells with Th2 differentiation media, at both 24 and 72 h (Fig. 5 B). The frequency of IFN-γ–producing $CD4^+$ Th1 cells and IL-4–producing $CD4^+$ Th2 cells derived from naive $CD4^+$ T cell differentiation and analyzed by flow cytometry also indicated that inosine inhibited Th1/Th2 differentiation (Fig. 5, C and D). However, it appears that inosine had no effects on the proliferation of B- and T-lymphocytes in vitro (Fig. S5, A–C).

Inosine is an agonist of adenosine receptors, which are required for the protective effects of inosine in vivo (Gomez and Sitkovsky, 2003; Haskó et al., 2004; Nascimento et al., 2010; Rahimian et al., 2010; da Rocha Lapa et al., 2013; Muto et al., 2014; Welihinda et al., 2016). One of the major pathways for T reg cells controlling T effector cells (Th1/Th2) is

the adenosine A_{2A} pathway (Deaglio et al., 2007; Csóka et al., 2008; Antonioli et al., 2013). We explored which isoforms of adenosine receptors contribute to the inhibition of inosine on Th1/Th2 differentiation. We initially examined the expression levels of 4 adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3) on $CD4^+$ T cells, and we showed that Th cells mainly express A_1 and A_{2A} receptors. Receptor levels could not be changed by inosine treatment (Fig. S5, D and E). To further examine which adenosine receptors specifically mediate the effect of inosine, we isolated naive $CD4^+$ T cells from mice genetically deficient in the A_1, A_{2A}, A_{2B}, or A_3 receptor ($A_1^{-/-}$, $A_{2A}^{-/-}$, $A_{2B}^{-/-}$, and $A_3^{-/-}$ mice, respectively). Findings indicated that the inhibition by inosine of Th1/Th2 differentiation was absent in mice with A_{2A} receptor deficiency, but not with deficiency of A_1, A_{2B}, or A_3 receptors (Fig. 5, C and D). Thus, we concluded that the immunomodulatory effect of inosine was mediated by A_{2A} receptors.

We further confirmed that inosine increased the downstream mediator of A_{2A} signaling, intracellular cyclic adenosine monophosphate (cAMP). We found that inosine enhanced the cAMP level in WT Th1/Th2 cells, but not in $A_{2A}^{-/-}$ Th1/Th2 cells (Fig. 5, E and F), further supporting the concept that inosine activates adenosine the A_{2A} receptor in Th1/Th2 cells. Altogether, these results provide strong evidence that inosine inhibits Th1/Th2 differentiation in an adenosine A_{2A} receptor–dependent manner.

The therapeutic effect of inosine on T reg cell–deficient SF mice is mediated by A_{2A} receptors

T reg cells control Th1/Th2 mediated by A_{2A} receptors (Deaglio et al., 2007; Csóka et al., 2008; Antonioli et al., 2013). In this T reg cell–deficient model, inosine's inhibition of Th1/Th2 by A_{2A} shown in vitro (Fig. 5) may have therapeutic value in T reg cell deficiency–mediated autoimmunity. We orally fed inosine daily to determine its effects on survival of SF mice (Fig. 6 A). We found that SF mice when fed inosine had significantly prolonged survival to a maximal lifespan of 125 d, compared with 30 d in SF mice without inosine treatment (Fig. 6 B). Tissue inflammation was significantly decreased by inosine treatment, as measured by mean area of lymphocyte infiltration in liver and lung (Fig. 6, C and D). Inosine reduced the frequency of IFN-γ–producing $CD4^+$ T (Th1) and the frequency of IL-4–producing $CD4^+$ T (Th2) cells in the spleen, as well as plasma levels of IFN-γ and IL-4 (Fig. 6, E and F).

To further determine the in vivo role of adenosine A_{2A} receptors, as a mediator of the beneficial effects of inosine, we treated SF mice with inosine and A_{2A} receptor–specific antagonist SCH58261 for 2 wk (SFIS) and examined the inflammatory biomarkers, and compared with the groups of WT mice with inosine (WTI) or inosine+SCh58261 (WTIS), and SF mice with inosine (SFI), or SCH58261 (SFS) or inosine+SCH58621 (SFIS). Our results indicated that in SF mice, SCH58261 itself has no effects on inflammation in SF, but SCH58261 reversed the antiinflammatory effects of inosine in liver and lung (Fig. 6, C and D), the percentage of Th1 or Th2 cells in the spleen, as well as plasma cytokine levels of IFN-γ and IL-4 (Fig. 6, E and F). Our data thus confirmed that the A_{2A} receptor is required for inhibition of inosine on autoimmunity in SF mice.

The inhibition of T reg cell deficiency–associated autoimmunity by *L. reuteri*–remodeled microbiota requires A_{2A} receptors

To further explore whether the adenosine A_{2A} receptors also play a critical role in beneficial effects of the *L. reuteri*–remodeled microbiota on autoimmunity, we fed SF mice with *L. reuteri* in combination with A_{2A} receptor–specific antagonist SCH58261 (Fig. 7 A). We found that the A_{2A} antagonist SCH58261 reversed the beneficial effects of *L. reuteri*–remodeled microbiota on autoimmune damage in liver and lung, the frequencies of Th1 and Th2 cells in the spleen, as well as plasma cytokine levels of IFN-γ and IL-4 in SF mice (Fig. 7, B and E), indicating that the adenosine A_{2A} receptor mediates a substantial proportion of the protection of *L. reuteri*–remodeled microbiota against autoimmunity in this model.

DISCUSSION

Functional T reg cells are of critical importance for the establishment and maintenance of self-tolerance and immune homeostasis. To control inflammation or allergy, T reg cells exert a dominantly negative regulation of other Th subsets (Th1/Th2), which are proinflammatory (Rudensky, 2011). In the absence or malfunction of T reg cells, T effector cell immunity drives inflammation and lethality in humans with IPEX syndrome or IPEX-like syndromes, and in Foxp3-deficient SF mice. Clinical management has not been updated since 1993 (Hannibal and Torgerson, 2011). The life expectancy of patients with IPEX syndrome without hematopoietic stem cell transplant rarely extends beyond infancy. This study aimed to define new therapeutic strategies by targeting microbiota to T reg cell deficiency–induced autoimmunity.

The gut microbiota is not only essential for the development and maturation of the immune system, but also is shaped by the complex host immune system. A critical role for T reg cell–mediated control of inflammation has been studied by using germ-free (GF) mice compared with specific pathogen–free (SPF) mice (Chinen et al., 2010). Their study demonstrated that T reg cell development and suppressive function showed little dependence on gut microbiota. However, in a T reg cell–depleted model (Foxp3-DTR), inflammation in the small intestine of SPF mice was more severe than in GF mice, as shown by significantly increased gut lymphocyte infiltration, decreased body weight, and increased % of IFN-γ–producing T helper cells, indicating that T reg cell deficiency–induced inflammation is related to gut microbiota (Chinen et al., 2010). Reduced diversity of gut microbiota has been shown in immunodeficient mice including mice lacking both B and T cells ($Rag1^{-/-}$) and in mice lacking B cells only ($Ighm^{-/-}$) or T cells only ($Cd3e^{-/-}$; Kawa-

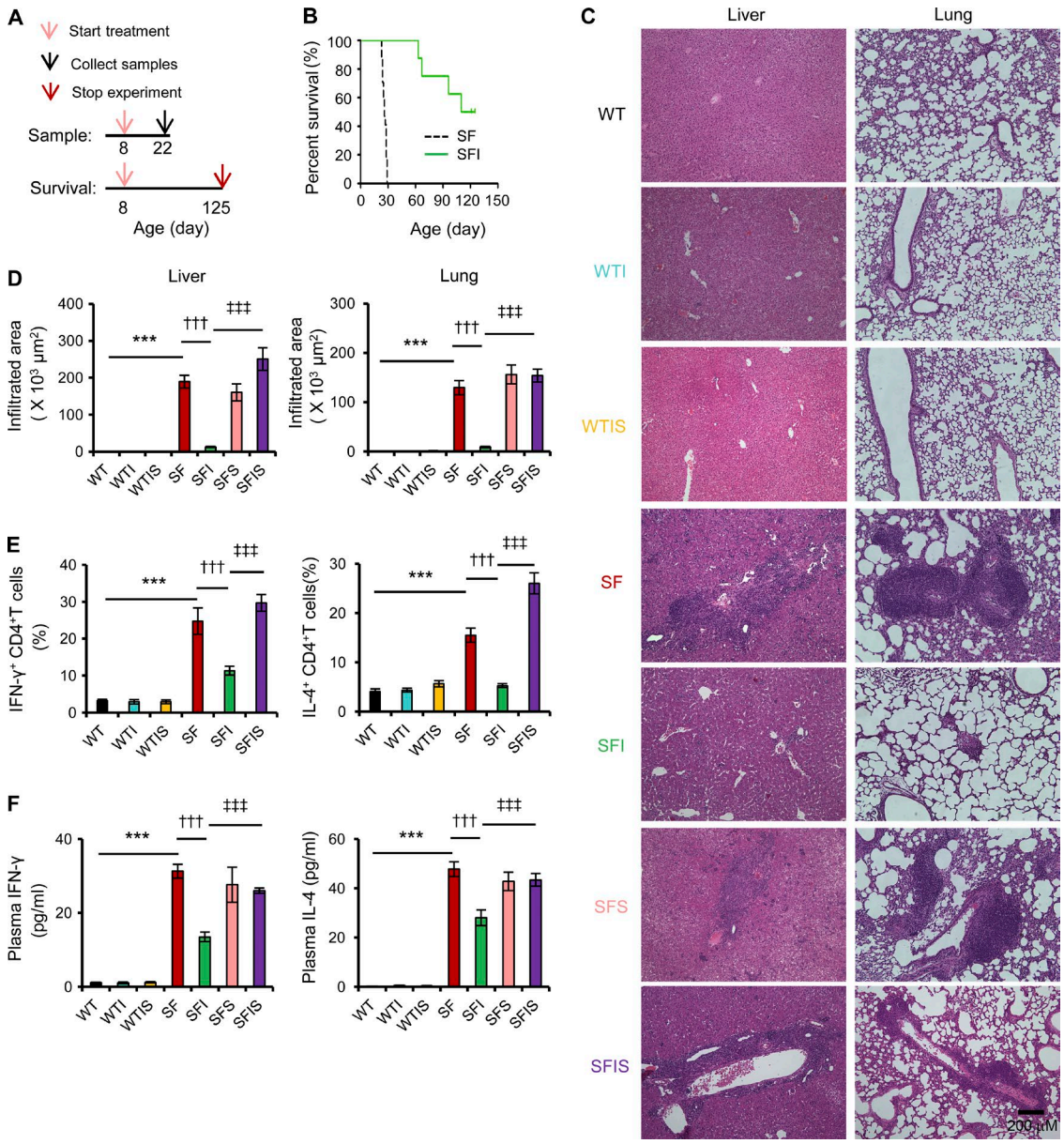

Figure 6. **Inosine inhibits autoimmunity in SF mice via adenosine A_{2A} receptors.** (A) Scheme of WT or SF mice treated with inosine (WTI or SFI) or inosine in combination with an A_{2A} receptor-selective antagonist SCH58261 (WTIS or SFIS) or SF mice treated with only SCH58261 (SFS) for sample analysis and survival observation. (B) Survival curves to compare inosine-fed SF (SFI) with SF without inosine feeding (Kaplan-Meier survival curves and log-rank test; n = 8–17 per group). (C) Representative H&E staining of liver and lung from WT, WTI, WTIS, SF, SFI, SFS, and SFIS; bar, 200 µm (n = 6–12 per group). (D) Quantification of inflammatory infiltrates in liver and lung of WT, WTI, WTIS, SF, SFI, SFS, and SFIS (one-way ANOVA; n = 6–12 per group). (E) The frequency of IFN-γ or IL-4–producing $CD4^+$ T cells in spleen of mice (one-way ANOVA; n = 6–12 per group). (F) Plasma levels of IFN-γ and IL-4 in WT, WTI, WTIS, SF, SFI, SFS, and SFIS mice (one-way ANOVA; n = 6–12 per group). ***, $P < 0.001$. SF versus WT. ††† $P < 0.001$. SFI versus SF. ‡‡‡, $P < 0.001$. SFIS versus SFI. Error bars represent mean ± SEM.

moto et al., 2014). Indeed, our results also showed reduced gut microbial diversity and altered bacterial composition in T reg cell–deficient SF mice, compared with WT mice. It was noted that the microbiota in SF mice could be shifted by oral administration of *L. reuteri* to produce a distinct signature without reversion to that seen in WT mice. The mechanisms by which *L. reuteri* regulates the intestinal microbiota may be multifactorial, including observations that *L. reuteri* produces antimicrobial agents (e.g., reuterin) and other metabolites that suppress the growth of other microorganisms (Spinler et al., 2008); *L. reuteri* also competes for receptors and binding sites with other intestinal microbes on the intestinal mucosa (Col-

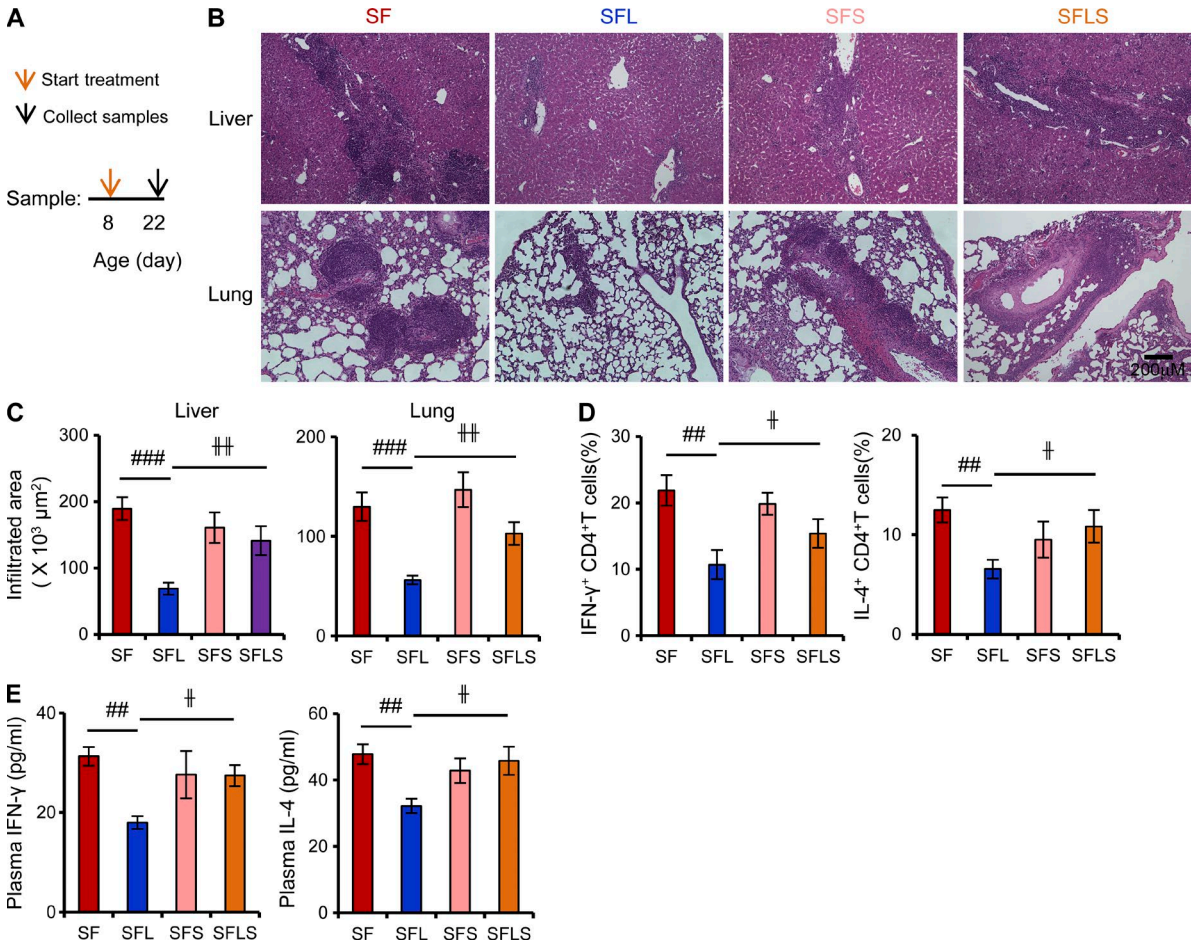

Figure 7. **Inhibition of autoimmunity by *L. reuteri* is mediated by adenosine A_{2A} receptors.** (A) Scheme of SF mice treated with *L. reuteri* (SFL) in the combination with an A_{2A} receptor-selective antagonist SCH58261 (SFLS) or SCH58261 alone (SFS) for sample analysis. (B) Representative H&E staining of liver and lung from SF, SFL, SFS, and SFLS mice; bar, 200 µm (n = 6–12 per group). (C) Quantification of inflammatory infiltrates in liver and lung of SF, SFL, SFS, and SFLS mice (one-way ANOVA; n = 6–12 per group). (D) The frequency of IFN-γ or IL-4–producing $CD4^+$ T cells in spleen of mice (one-way ANOVA; n = 6–12 per group). (E) Plasma levels of IFN-γ and IL-4 in SF, SFL, SFS, and SFLS mice (one-way ANOVA; n = 6–12 per group). $^{\#\#}$, $P < 0.01$; $^{\#\#\#}$, $P < 0.001$. SFL versus SF. †, $P < 0.05$; ††, $P < 0.01$. SFLS versus SFL. Error bars represent mean ± SEM.

lado et al., 2007). The gut microbiota drives host immune homeostasis by regulating the differentiation and expansion of T cells (Hooper et al., 2012). We found that the development of Th1/Th2-drived autoimmunity was accompanied by microbial dysbiosis over the lifespan of SF mice. When this gut microbial dysbiosis was remodeled by *L. reuteri*, the autoimmunity was inhibited, as indicated by prolonged survival, reduced multiorgan inflammation, and decreased Th1/Th2 cytokines in SF mice. We have previously observed the same effects of *L. reuteri* in the setting of experimental necrotizing enterocolitis (Liu et al., 2012, 2013).

Metabolites produced by bacteria promote or suppress immune cell functions (Roelofsen et al., 2010; Arpaia et al., 2013; Furusawa et al., 2013). Microbiota-modulated metabolites accompanying the introduction of *L. reuteri* into T reg cell–deficient mice may play a critical role in regulating immune responses. We discovered that the purine metabolite inosine is reduced in plasma by T reg cell deficiency and is completely restored by *L. reuteri* treatment. *L. reuteri* does not generate large amounts of purine/inosine in culture (unpublished data). Based on the evidence of recovery of plasma levels of inosine to the levels similar to WT, in association with decreased levels in stool of SF mice, we postulate that *L. reuteri* may promote inosine absorption in the intestine by improving overall gut health through multiple mechanisms (for example by improving villus length) and/or by modulating the gut microbial community. As shown, we measured the small intestinal villi in SF mice compared with SF mice after oral feeding and showed that orally feeding *L. reuteri* improves the length of villi and depth of crypts. The increased expression of ENT transporters after *L. reuteri* feeding would also be predicted to correlate with improved absorption.

In addition, the complete genome of *L. reuteri* contains the tRNA-specific adenosine deaminase gene (*tadA*), which

is involved in the biological process of tRNA wobble adenosine-to-inosine editing (UniProt accession no. F8DLR1), and the gene LPXTG-motif cell wall anchor domain protein (HMPREF0538_20056) that belongs to 5′-nucleotidase family which could participate in the conversion of inosine monophosphate into inosine (UniProt accession no. F8DRN6). Therefore, we could not rule out that the gut environment activates these enzymatic functions to help generate more inosine to be absorbed (Muzny et al., 2011). Identity of enzymatic activities related to and direct measurements of inosine absorption will be further explored.

Previous studies demonstrated that inosine treatment reduces levels of inflammatory cytokines produced by LPS-stimulated macrophages in murine models of endotoxic shock (Haskó et al., 2000). Inosine also attenuates the course of chronic autoimmune inflammatory diseases including type I diabetes (Mabley et al., 2003b) and experimental colitis (Mabley et al., 2003a; Rahimian et al., 2010), in association with a reduction of the production of proinflammatory cytokines and chemokines. Recently, investigators demonstrated antiinflammatory effects of inosine in mouse models of pleurisy (da Rocha Lapa et al., 2012) and allergic lung inflammation (da Rocha Lapa et al., 2013). Our results confirm that inosine is sufficient to inhibit Th1/Th2 differentiation in vitro and autoimmunity in vivo by reducing Th1/Th2 cells and the associated cytokines in SF mice.

However, mechanisms of the immunomodulatory effects of inosine are poorly understood. We herein demonstrate that the inhibition of Th1/Th2 differentiation by inosine requires A_{2A} receptors. Mechanistically, the key to T reg cell suppression of T effector cells is the interaction between adenosine produced by T reg cells (mediated by a CD39–CD73 pathway) and the A_{2A} receptor (expressed on T effector cells; Antonioli et al., 2013). It has been reported that lymphocytes predominately express A_{2A} receptors (Gomez and Sitkovsky, 2003). However, during T reg cell deficiency in SF mice or human IPEX syndrome, T effector cells lose regulation by adenosine-A_{2A} mediated signaling. Our findings highlight the possibility that inosine or other A_{2A} agonists could be used therapeutically to control T effector cell-mediated autoimmunity during T reg cell deficiency conditions.

Fredholm et al. (2001) reported that adenosine is the natural ligand at all four adenosine receptors. However, inosine was a potent agonist for A_1 and A_3 receptors, but not for A_2 receptors. This group used in vitro techniques with CHO cells stably transfected with the human A_1, A_{2A}, A_{2B}, and A_3 receptors. The inosine concentrations they tested were between 0.03 and 30 μM. Jin et al. (1997) concluded that physiologically significant concentrations (10–50 μM) of inosine selectively activate A_3, but not A_2, receptors in mast cells. However, several other findings indicate the protective effects of inosine are mediated by interaction with adenosine A_{2A} receptors (Gomez and Sitkovsky, 2003; Mabley et al., 2008; Rahimian et al., 2010; da Rocha Lapa et al., 2012, 2013; Muto et al., 2014). Welihinda et al. (2016) recently reported that inosine is a functional agonist of the A_{2A} receptor. They aimed to solve why the immunomodulatory effects of inosine in vivo, which at least in part, are mediated via A_{2A}, seem to differ from levels needed for in vitro pharmacological effects at the A_{2A} receptor. The research group used a combination of label-free, cell-based, and membrane-based functional assays in conjunction with an equilibrium agonist-binding assay. It provided evidence for inosine engagement of the A_{2A} receptor and subsequent activation of downstream signaling events. EC_{50} values were 12 (4–33) nM for adenosine and 259.9 (104–651) μM for inosine.

Compared with adenosine, inosine has a lower affinity interaction with the A_{2A} receptor, probably because the structure of inosine contains fewer hydrogen bonds than adenosine, which are responsible for the binding to agonist-binding pocket of A_{2A} receptors (Lebon et al., 2011; Xu et al., 2011; Welihinda et al., 2016). Therefore, a high concentration (approximate millimolar) of inosine was previously required in vitro for inosine to inhibit Th1/Th2 differentiation and to activate A_{2A} receptors and increase intracellular cAMP levels (Welihinda et al., 2016). Previous studies showed that inosine has no capacity to activate A_{2A} or displace the binding of a high-affinity agonist to A_{2A} (Fredholm, 1995; Jin et al., 1997; Fredholm et al., 2001), which may be caused by its low affinity for the A_{2A} receptor, to low levels of A_{2A} expression, or to differential cell types that affect the binding between inosine and A_{2A} receptors when studied using insufficient concentrations of inosine for those in vitro experiments. Methods focusing on target identification of bioactive small molecules will further provide tools to elucidate the molecular details of inosine–A_{2A} interaction (Futamura et al., 2013; Ziegler et al., 2013).

Our current study focused on the effects of inosine in T cells. T cells differ from mast cells, platelets, or macrophages in that T cells express A_{2A} as their main adenosine receptor (Csóka et al., 2008). A_{2A} knockout mice data strongly suggest the effects of inosine are dependent on the A_{2A} receptor on T cells. We believe our studies using knockout models provide more solid evidence than in vitro studies with A_{2A} antagonists.

We could not rule out that inosine modulates A_{2A} activation via the salvage pathway in vivo (Kolassa et al., 1977). In this pathway, inosine is converted to hypoxanthine, which can be converted into inosine 5-monophosphate by hypoxanthine-guanine phosphoribosyltransferase, to adenylosuccinate, and then to adenosine 5-monophosphate (AMP), finally generating adenosine. Adenosine could activate A_{2A} at very low concentrations, and then rapidly degrade into inosine due to its short biological half-life (∼10 s). Adenosine would be rapidly cleared in plasma in vivo (in ∼30 s (Möser et al., 1989), whereas inosine has a much longer in vivo half-life than adenosine (∼15 h; Viegas et al., 2000).

In clinical trials in humans, investigators have concluded that inosine, when given orally at 3,000 mg per day, was safe and well-tolerated (Schwarzschild et al., 2014). The dosages of oral inosine used in mouse models varied in different dis-

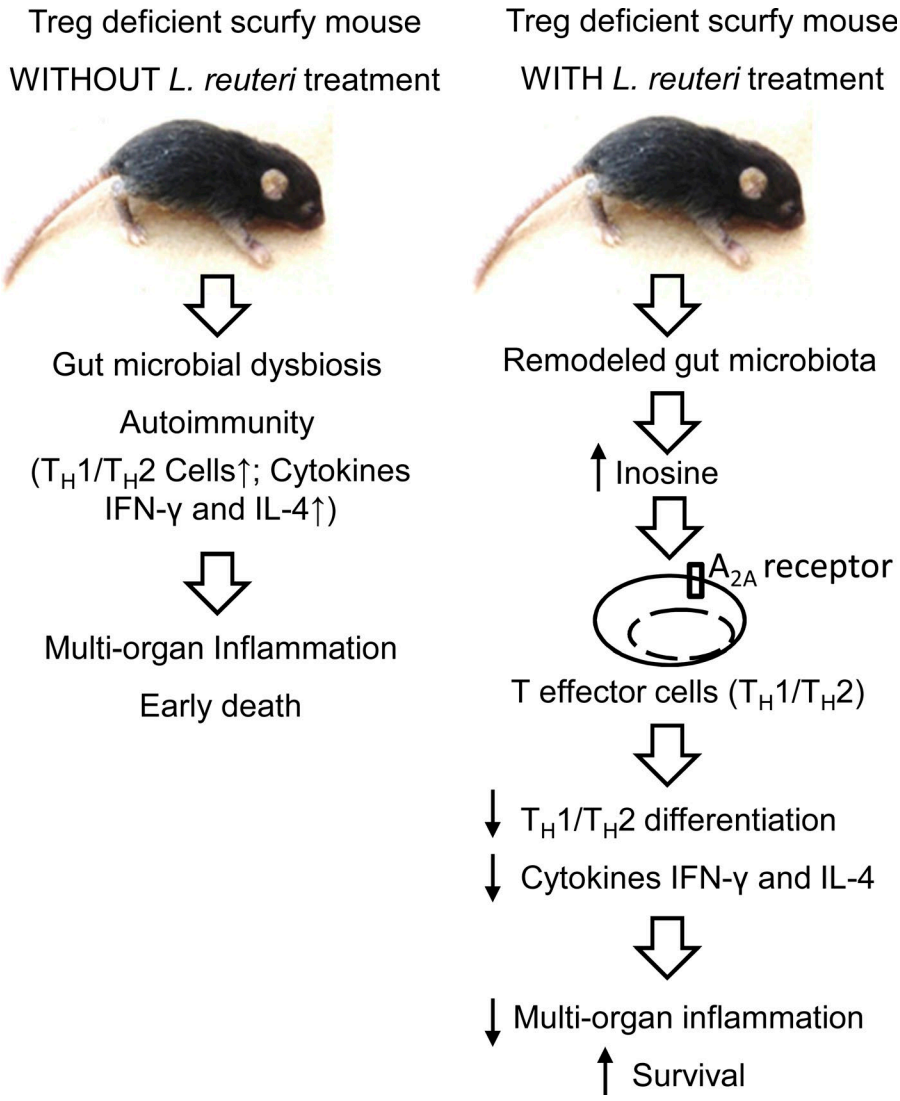

Figure 8. **Mechanisms of *Lactobacillus reuteri* protection against T reg cell deficiency–mediated autoimmunity.** T reg cell deficiency shapes gut microbiota and induces autoimmunity resulting in multiorgan inflammation and early death (left). *L. reuteri* remodels gut microbiota, alters the metabolites, and protects against T reg cell deficiency–induced autoimmunity by suppressing Th1/Th2 cells via inosine–adenosine A_{2A} interaction (right).

ease models, with the highest dose being 750 mg/kg/bw/day (Mabley et al., 2003b; Hou et al., 2007; Muto et al., 2014), and toxic effects were not demonstrated. We used a dosage of inosine of 800 mg/kg/day for our survival study because the phenotype and manifestations of IPEX in SF mice were severe. Drug dosages used in mouse studies can be translated into human dosages based on surface area (Reagan-Shaw et al., 2008); accordingly, the dosage of 800 mg/kg/bw/day used in mice would be approximately equivalent to 65 mg/kg/bw/day in humans. Our dosage is thus much lower than the dosage in the aforementioned safety clinical trial (Schwarzschild et al., 2014). Moreover, the inosine plasma level in SF mice is significantly lower than its level in WT mice, according to our plasma metabolomics analysis.

In conclusion, our work reveals that microbial dysbiosis may be implicated in the pathogenesis of T reg cell deficiency–induced autoimmunity and that microbiota remodeling greatly influences outcome in autoimmune diseases via inosine–A_{2A} receptor signaling, and we have summarized our findings in Fig. 8. Even though the data do not formally rule out noninosine-dependent effects of *L. reuteri* in suppressing inflammation in SF mice, this study may broaden the concept of how immunodeficiency diseases evolve during early development, with key contributions by the microbiota and their metabolites acting through receptors on immune cells. This proof-of-principle study will spur future application of probiotic *L. reuteri*, inosine, and A_{2A} receptor agonists in other autoimmune diseases, such as IPEX syndrome, many of which are linked to inborn immune defects (Uhlig et al., 2014).

MATERIALS AND METHODS

Mice

WT C57BL/6 and heterozygous B6.Cg-Foxp3^{sf}/J mice were purchased from BioGaia AB and allowed to acclimatize for 2 wk before experimentation. SF mice with hemizygous

B6.Cg-Foxp3^{sf}/Y were generated by breeding heterozygous B6.Cg-Foxp3^{sf}/J female to C57BL/6J male mice. Because the Foxp3 gene is on the X chromosome, in each litter of breeding pairs, all males are either SF used as the experiments or WT littermates as the controls. Adenosine receptor–deficient mice with B6 background, including $A_1^{-/-}$, $A_{2A}^{-/-}$, $A_{2B}^{-/-}$, and $A_3^{-/-}$ knockout mice, were provided by M.R. Blackburn (The University of Texas Health Science Center at Houston McGovern Medical School, UT Health, Austin, TX). Animal numbers used in each group of different experiments are indicated in the figures and figure legends. All mice were housed in animal facility at UT Health. All experimental procedures were approved by the IACUC (protocol number: AWC-14-056).

L. reuteri preparation and treatment of SF mice

Human breast milk–derived *Lactobacillus reuteri* DSM17938 (*L. reuteri*) was provided by BioGaia AB. *Lactobacillus acidophilus DDS* (*La DDS*) was provided by D.R. Mack (Children's Hospital of Eastern Ontario, Ontario, Canada). *L. reuteri* was prepared as previously described (Liu et al., 2014). In brief, *L. reuteri* was anaerobic cultured in deMan-Rogosa-Sharpe (MRS) medium at 37°C for 24 h, and then plated in MRS agar at specific serial dilution and grown anaerobically at 37°C for 48–72 h. Quantitative analysis of bacteria in culture media was performed by comparing absorbance at 600 nm of cultures at known concentrations, using a standard curve of bacterial CFU/ml grown on MRS agar. Each male mouse was given either MRS as control, or *L. reuteri* (WTL or SFL) or *La DDS* (WTDDS or SFDDS) in cultured media (10^7 CFU/day) by gavage, daily, starting from 8 d of age (d8; early treatment) or d15 (late treatment), until the date as indicated in Fig. 3 A and Fig. S2 A, respectively. For treatment with *L. reuteri* in combination with A_{2A} receptor antagonist SCH58261 (Sigma-Aldrich) to examine the immunological biomarkers, each SF mouse was orally administered 10^7 CFU of *L. reuteri* and i.p. injected 2 mg/kg of SCH58261 daily (SFLS) or was i.p. injected 2 mg/kg of SCH58261 daily (SFS) from d8 to the date as indicated in Fig. 3 A, Fig. S2 A, and Fig. 7 A, respectively.

Inosine preparation and treatment of SF mice

Inosine (Sigma-Aldrich) was dissolved in sterilized water at the concentration of 40 mg/ml. For determining the effect of inosine on autoimmunity in SF mice, 800 mg/kg of inosine per day was orally administered to WT (WTI), SF mice (SFI) from d8 until the date as indicated in Fig. 6 A. For treatment with inosine in the combination with SCH58261 to examine the immunological biomarkers, WT (WTIS) SF (SFIS) mice were orally administered 800 mg/kg of inosine and 2 mg/kg of SCH58261 i.p. once daily from d8 to the date as indicated in Fig. 6 A.

Histopathology

All tissues were fixed and processed by the Cellular and Molecular Morphology Core Lab (Texas Medical Center Digestive Diseases Center, Houston, TX) and stained with hematoxylin and eosin (H&E) for histological evaluation. The area of lymphocyte infiltration of liver and lung and the villus height and crypt depth of small intestine were measured using ImageJ (National Institutes of Health) morphometry software.

In vitro tissue preparation and stimulation for flow cytometry analysis

Single-cell suspensions from the spleen and MLNs were obtained by gently fragmenting and filtering the tissues through 40-μm cell strainers (BD) into MACS buffer consisting of PBS, 0.5% BSA (Hyclone Laboratories), and 2 mM EDTA (Lonza). For in vitro stimulation, cells were stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) in the presence of Brefeldin A (5 μ/ml) for 4 h to analyze IFN-γ–producing (Th1) and IL-4–producing (Th2) $CD4^+$ T cells by flow cytometry.

Naive $CD4^+$ T cell isolation and in vitro differentiation to Th1 and Th2

Naive $CD4^+$ T cells were isolated from the spleens of 6–8-wk-old C57BL/6J or adenosine receptor knockout mice by magnetic cell sorting, using a naive $CD4^+$ T cell isolation kit (MACS; Miltenyi Biotec). For Th1 differentiation, naive T cells (5×10^5 cells/well) were plated in 24-well plates containing 1 μg/ml anti-CD3, 2 μg/ml anti-CD28, 20 ng/ml IL-2, 10 μg/ml anti–IL-4–neutralizing antibody, and 20 ng/ml recombinant mouse IL-12 in RPMI-1640 complete medium at 37°C for 5 d with or without 2 mM of inosine. For Th2 differentiation, naive T cells were cultured in the presence of 1 μg/ml anti-CD3, 2 μg/ml anti-CD28, 20 ng/ml IL-2, 10 μg/ml anti–IFN-γ neutralizing antibody and 10 ng/ml recombinant mouse IL-4 in RPMI-1640 complete medium at 37°C for 5 d with or without 2 mM of inosine. At day 5, the cells were stimulated with PMA and ionomycin in the presence of Brefeldin A. The detailed antibodies and cytokines used are listed in Table S3.

Staining cells for flow cytometry analysis

For evaluation of the purity of naive $CD4^+$ T cells, after sorting, cells were stained using fluorescein-labeled CD44, CD45RB, CD4, and CD62L. For characterization of Th1 and Th2 cells, cells were surface-stained by fluorescein labeled–CD4 and intracellularly stained with IFN-γ for Th1 and IL-4 for Th2. Intracellular staining was performed with a fixation/permeabilization kit, according to the manufacturer's protocol (eBioscience). The data from all samples were acquired on FACSCalibur (BD) and analyzed using FlowJo software (Tree Star). The detailed antibodies used are listed in Table S3.

qPCR analyses

Total RNA was extracted from treated cells and animal tissues by using TRIzol (Sigma-Aldrich) and RNAeasy Mini kit (QIAGEN), according to the manufacturer's protocol. RNA (2 μg) was reverse transcribed using amfiRivert Platinum ONE cDNA Synthesis Master Mix (GEnDEPOT). Quan-

titative RT-PCR was performed using amfiQSYBR Green PCR Master Mix (GenDEPOT) with CFX96 RT-PCR detection system (Bio-Rad Laboratories). All qPCR primers used are listed in Table S4.

Lymphocyte proliferation test

Splenic lymphocytes were seeded into 96-well plates at an initial density of 2×10^4 cells per well and were incubated with different doses of inosine under control, 40 μg/ml of LPS or 10 μg/ml of phytohemagglutinin (PHA), respectively. After 96 h, cell viability was measured by TACS XTT cell proliferation assay kit (Trevigen, Inc.).

cAMP assay

Differentiated Th1 or Th2 cells from naive $CD4^+$ T cells isolated from WT and adenosine A_{2A} receptor knockout mice were seeded (10^4 cells/well) into 96-well plates. Cells were incubated in the absence or presence of inosine (2 mM) or adenosine A_{2A} receptor agonist CGS21680 (300 nM) at 37°C. The cAMP levels in Th1 and Th2 cells were measured by cAMP-Glo assay kit (Promega) after treatment for 15 min.

Plasma cytokine assays

Plasma cytokine levels of IFN-γ, IL-4, IL-2, IL-1β, and IL-10 were assessed using a mouse multi-spot proinflammatory panel kit from Meso Scale Discovery (MSD), according to the manufacturer's protocol.

Stool microbial community analysis

Feces from cecum to rectum of mice were collected. Stool DNA was extracted by using Quick Stool DNA Isolation kit (QIAGEN). The composition of the stool microbiota was analyzed using high-throughput sequencing analysis of PCR-amplified 16s rRNA genes as previous described (Gupta et al., 2013). Bacterial diversity, species composition, and abundance were assessed using QIIME (Caporaso et al., 2010).

Plasma and stool metabolomic analysis

Plasma and stool metabolites were measured by Metabolon Inc. A total of 657 metabolites in stool and 525 metabolites in plasma were detected by a nontargeted metabolomic analysis platform, including UPLC-MS/MS and GC/MS, respectively. The metabolomic data were analyzed by pattern recognition analyses (unsupervised principal component analysis and Heat-map), revealing the biochemical perturbations induced by T reg cell deficiency or *L. reuteri* treatment, as previously described (He et al., 2015).

Statistical analysis

Data are presented as mean ± SEM. Significance was determined using one-way ANOVA corrected for multiple comparisons with Tukey and Dunnett's posttests, or two-way ANOVA for multiple comparisons with a Bonferroni test. Kaplan-Meier survival curves were graphed, and the comparison was analyzed using Logrank with χ^2 test. The statistical analysis was performed using Prism version 4.0 (GraphPad Software). Differences were noted as significant at $P < 0.05$.

Online supplemental material

Fig. S1 shows the dynamic changes of autoimmunity and gut microbiota over 22 d of life in SF mice. Fig. S2 shows that *L. reuteri* late treatment prolongs survival, inhibits autoimmunity, and modulates gut microbiota in SF mice, but *L. acidophilus DDS* does not. Fig. S3 shows that *L. reuteri* early treatment reprograms fecal metabolomic profiles in SF mice. Fig. S4 shows that *L. reuteri* early treatment improves villus height and decreases crypt depth, and increases the expression of nucleoside transporters in small intestine of SF mice. Fig. S5 shows that inosine does not inhibit the proliferation of B and T cells or change the expression of adenosine receptors in Th1/Th2 cells. Table S1 shows 525 plasma metabolites and their relative quantification in WT, SF, and SF with *L. reuteri* treatment (SFL) mice. Table S2 shows 657 fecal metabolites and their relative quantification in WT, SF and SF with *L. reuteri* early treatment (SFL) mice. Table S3 lists antibodies and cytokines used in the study. Table S4 lists sequences of qPCR primers used in the study. Tables S1–S4 are available as Excel files.

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B. He, S. Roos, J.M. Rhoads, and Y. Liu have a patent application pending on use of inosine and A_{2A} agonists in IPEX syndrome. The authors declare no additional competing financial interests.

Author contributions: B. He, J.M. Rhoads, and Y. Liu conceived the project, designed the experiments, and wrote the manuscript. B. He, T.K. Hoang, T. Wang, F. Luo, J.G. Molina, T.H. Gomez, and Y. Liu. performed all experiments and analysis. J. Zhou and N. Tatevian performed and assisted the pathological analysis of tissue inflammation. M. Ferris, C.M. Taylor, X. Tian, and M. Luo performed stool microbiota analysis. X. Tian and S. Roos contributed to metabolomics analysis. J.M. Rhoads, M.R. Blackburn, D.Q. Tran, and Y. Liu guided experimental design and data interpretation and edited the manuscript.

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