Immunity
Article

The Cytokines Interleukin 27 and Interferon-γ Promote Distinct Treg Cell Populations Required to Limit Infection-Induced Pathology

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SUMMARY

Interferon-γ (IFN-γ) promotes a population of T-bet$^+$ CXCR3$^+$ regulatory T (Treg) cells that limit T helper 1 (Th1) cell-mediated pathology. Our studies demonstrate that interleukin-27 (IL-27) also promoted expression of T-bet and CXCR3 in Treg cells. During infection with *Toxoplasma gondii*, a similar population emerged that limited T cell responses and was dependent on IFN-γ in the periphery but on IL-27 at mucosal sites. Transfer of Treg cells ameliorated the infection-induced pathology observed in *Il27*$^{-/-}$ mice, and this was dependent on their ability to produce IL-10. Microarray analysis revealed that Treg cells exposed to either IFN-γ or IL-27 have distinct transcriptional profiles. Thus, IFN-γ and IL-27 have different roles in Treg cell biology and IL-27 is a key cytokine that promotes the development of Treg cells specialized to control Th1 cell-mediated immunity at local sites of inflammation.

INTRODUCTION

IL-27 is a member of the IL-6 and IL-12 family of cytokines. Early studies described it as an inducer of the T helper 1 (Th1) cell-associated transcription factor T-bet, which enhances Th1 differentiation (reviewed in Hall et al., 2012). However, IL-27 is also an antagonist of inflammation associated with Th1, Th2, and Th17 cell responses in multiple settings (Stumhofer and Hunter, 2008) and the regulatory properties of IL-27 can be explained in part by its ability to limit IL-2 production, antagonize Th2 and Th17 cell responses, and promote T cell production of IL-10. However, questions remain about the mechanisms used by IL-27 to limit immune pathology associated with Th1 cell responses (reviewed in Stumhofer and Hunter, 2008; Yoshida and Miyazaki, 2008).

CD4$^+$ T cells that express the transcription factor Foxp3 (Treg cells) are an important means of immune suppression. Recent studies have demonstrated that during inflammation, specialized populations of Treg cells emerge that express transcriptional profiles similar to their effector cell counterparts (Esposito et al., 2010; Fujimoto et al., 2011; Koch et al., 2009). It has been suggested that this heterogeneity allows for regulation of specific types of immunity. For example, Treg cell expression of STAT3 is critical for limiting Th17 cell responses (Chaudhry et al., 2009), whereas expression of IRF4 allows control of Th2 cells (Zheng et al., 2009). During infections dominated by Th1 cells, Treg cells express *Tbx21* and *Cxcr3*, genes associated with the presence of IFN-γ, and expression of T-bet is required for their survival and proliferation (Koch et al., 2009). Whereas IFN-γ has been implicated in the development of this specialized Treg cell population, whether other environmental cues influence this program is unclear.

A subset of natural Treg (nTreg) cells expresses high levels of the IL-27Rα (Villarino et al., 2005). Paradoxically, there are reports that IL-27 is a direct antagonist of Treg cell conversion (Cox et al., 2011; Huber et al., 2008; Neufert et al., 2007; Stumhofer et al., 2007). The data presented here reveal that, contrary to these reports, IL-27 does not limit Treg cells and directly promotes Treg cell expression of T-bet and CXCR3. In mice challenged with *T. gondii*, or other intracellular pathogens, a population of Treg cells emerged that expressed T-bet, CXCR3, and IL-10 and limited T effector responses. In mice that lack IL-27, this population is reduced at primary sites of infection but not at peripheral sites, such as in the spleen, where IFN-γ had a more prominent role. However, transcriptional profiling highlighted that IL-27 appeared to have a more dominant impact than IFN-γ on Treg cell expression of

immunosuppressive genes such as *Il10*. Together, these studies identify distinct roles for IL-27 and IFN-γ in driving the T-bet$^+$ subset of Treg cells that are specialized to control regional pathology during Th1 cell responses.

RESULTS

IL-27 Promotes the Expression of T-bet and CXCR3 in Inducible Treg Cells and Natural Treg Cells

Although previous studies have demonstrated that 30%–40% of nTreg cells express the IL-27Rα, it was unclear whether this receptor was functional in Foxp3$^+$ CD4$^+$ T cells. In this study, we define nTreg cells as Foxp3$^+$ CD4$^+$ T cells isolated from mice and "inducible" Treg (iTreg) cells as Foxp3$^+$ CD4$^+$ Treg cells generated from Foxp3$^-$ CD25$^-$ precursors in vitro. To address whether nTreg cells respond to IL-27, we incubated naive CD25$^+$ T cells or Foxp3^{GFP+} cells with IL-27. Whereas unstimulated cells had negligible amounts of pSTAT1 or pSTAT3, IL-27 induced pSTAT1 and pSTAT3 in 30%–40% of nTreg cells (Figure 1A). Similarly, IL-27 induced pSTAT1 and pSTAT3 in 50%–70% of iTreg cells (Figure 1B). It is notable that in Treg cells, IFN-γ and IL-10 also activate STAT1 and STAT3, respectively, but this was less than with IL-27 (Figure S1A available online). It is also relevant to note that previous reports have suggested that IL-27 antagonizes iTreg cell development (Huber et al., 2008; Neufert et al., 2007; Stumhofer et al., 2007; Cox et al., 2011), and in our experiments, the frequency of Treg cells were initially reduced in the presence of IL-27, but Treg cells were generated and their numbers increased over time (Figures S1B and S1C). Together, these data suggest that existing and emerging Treg cell responses can be influenced by IL-27 and that IL-27 can actually promote Treg cell expansion.

Because IL-27 induces the expression of T-bet in effector CD4$^+$ T cells, studies were performed to determine whether IL-27 had a similar effect on Treg cells. When naive Foxp3$^-$ CD4$^+$ T cells were used to generate iTreg cells, those cultured in the presence of IL-27 expressed elevated levels of T-bet (Figure S1D). When Treg cells were differentiated in the presence of IL-27 plus α-IL-4 and α-IFN-γ (neutral conditions), it still promoted Treg cell expression of T-bet (Figure 1C). Thus, independent of its ability to promote IFN-γ, IL-27 promotes Treg cell expression of T-bet. It is notable that long-term TCR signaling was associated with the eventual upregulation of T-bet in cultures of Treg cells, although the highest amounts of T-bet were always observed in the presence of IL-27 (Figure S1E).

Previous studies established that activation of T-bet in Treg cells promotes expression of CXCR3 (Koch et al., 2009), a chemokine receptor involved in lymphocyte migration during Th1 cell responses (Lord et al., 2005). When iTreg cells were generated with IL-27, there was a consistent 4- to 5-fold increase in CXCR3 levels and nTreg cells incubated with IL-27 for 24 to 48 hr expressed high levels of T-bet and CXCR3 (Figure 1C). Similarly, although iTreg cells stimulated with PMA and ionomycin did not produce IL-10 or IFN-γ, those generated in the presence of IL-27 expressed IFN-γ and IL-10 (Figure 1D). Together, these data demonstrate that IL-27 promotes cytokine production by Treg cells and influences their proliferation, survival, and chemokine receptor expression.

IL-27-Driven Expansion of CXCR3$^+$ Treg Cells Requires STAT1 and T-bet

Because IL-27 activates STAT1 and STAT3, experiments were performed to determine which pathway contributed to the upregulation of T-bet and CXCR3 in iTreg cells. As noted earlier, addition of IL-27 to the cultures led to the induction of T-bet in 15%–30% of the iTreg cells, and a modest but consistent increase in T-bet was observed in the *Stat3*$^{-/-}$ CD4$^+$ T cells (Figure S1F). However, in the *Stat1*$^{-/-}$ Treg cell cultures, IL-27 did not induce T-bet (Figure 1E). In addition to T-bet, Eomesodermin (Eomes) has been shown to promote CXCR3 expression (Intlekofer et al., 2008), and polyclonal expansion of naive cells CD4$^+$ T cells in the presence of IL-27 led to increased levels of Eomes and T-bet, but in iTreg cells, IL-27 did not promote Eomes (Figure S1G). This finding is consistent with the idea that TGF-β (required for the iTreg cell cultures) suppresses Eomes but not T-bet (Narayanan et al., 2010). Moreover, Treg cells generated with IL-27 from *Eomes*$^{-/-}$ T cells showed upregulation of CXCR3, similar to wild-type (WT) Treg cells (Figure 1F), whereas *Tbx21*$^{-/-}$ iTreg cells had impaired upregulation of CXCR3 (Figure 1F). These data suggest a model in which the ability of IL-27 to activate STAT1 drives T-bet and CXCR3 expression in a subset of iTreg cells.

IL-27 Treatment Increases Treg Cell Expression of T-bet and CXCR3 In Vivo

To assess the effects of IL-27 on the phenotype of Treg cells in vivo, Chen et al. (2005) used hydrodynamic gene delivery to administer DNA plasmid "minicircles" that express IL-27 (IL-27MC). Mice that received IL-27MC or GFP vector-only controls were sacrificed after 4 weeks for analysis of their T cell populations. No pathology was noted in either experimental group (data not shown), and although there was increased cellularity associated with IL-27MC (Figure S2A), there was no decrease in Treg cell frequency (Figure S2B). Although total splenic CD4$^+$ T cells from mice given IL-27MC had higher amounts of T-bet (Figure S2C), further analysis revealed that, consistent with the ability of IL-27 to promote a population of T-bet$^+$ CXCR3$^+$ Treg cells in vitro, only the Foxp3$^+$ Treg cell population had higher T-bet and CXCR3 expression (Figures 2A–2D).

Infection with *T. gondii* Induces an Immunosuppressive T-bet$^+$ CXCR3$^+$ Treg Cell Population

Challenge with *T. gondii* results in the development of CD4$^+$ effector T cells that produce IFN-γ and IL-10 (Jankovic et al., 2007; Stumhofer et al., 2007). Analysis of Treg cells from infected mice after restimulation with PMA and ionomycin and/or directly ex-vivo from Vert-X IL-10-eGFP reporter mice revealed that these cells were also a source of IFN-γ and IL-10 (Figures 3A and 3B). After restimulation, a portion of these *T. gondii*-induced IL-10$^+$ Treg cells coexpressed IFN-γ as well as T-bet and CXCR3 (Figures 3C–3E). Further analysis of BrdU incorporation and Ki-67 expression revealed that infection promoted proliferation of these cells (Figures S3A and S3B). The appearance of CXCR3$^+$ T-bet$^+$ Treg cells coincided with the emergence of a population of cells that produced IL-27p28. Although conventional DCs (CD11c^{hi} Class-II$^+$) produced IL-12p40 in the spleen and the lamina propria (LP) population, IL-27p28 production

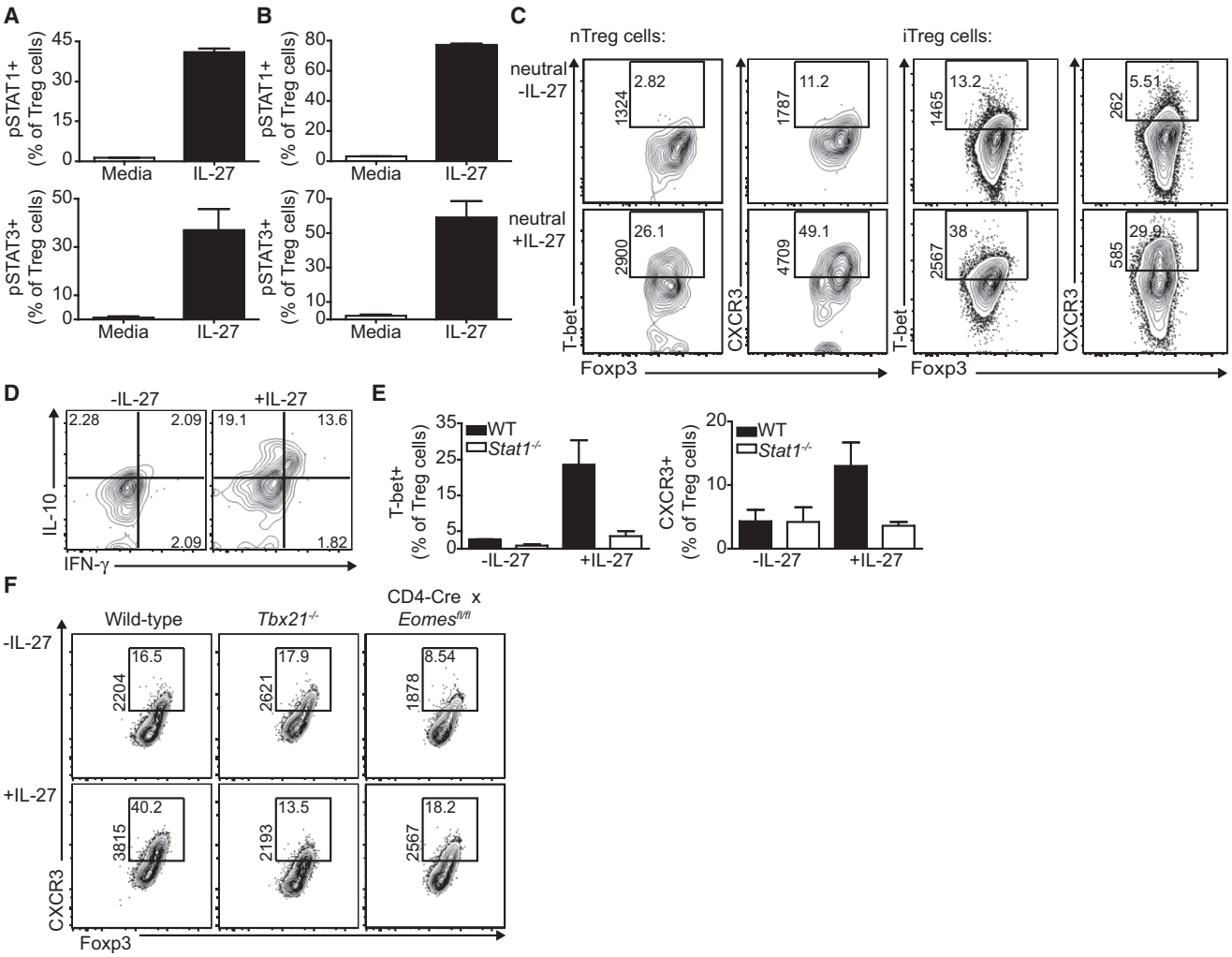

Figure 1. IL-27 Treatment of Treg Cells Induces STAT1 and STAT3 Phosphorylation and the Expansion of a STAT1-Dependent T-bet$^+$ CXCR3$^+$ Population

(A and B) Natural Treg (nTreg) cells harvested from naive mice (A) and inducible Treg (iTreg) cells generated in vitro (as described in the Experimental Procedures) were stimulated with IL-27 or media alone (B), and phosphorylated STAT1 and STAT3 were measured by flow cytometry. Plots depict the mean percentage ± standard error of the mean (SEM) of Treg cells (gated on LIVE/DEAD® Fixable Aqua Dead Cell marker$^-$, CD4$^+$, Foxp3$^+$ cells) expressing pSTAT1 or pSTAT3.

(C) nTreg cells or iTreg cells were cultured in the presence of neutralizing antibodies to IFN-γ and IL-4 (neutral conditions) and in the presence or absence of IL-27. After 48 hr of culture, the expression of T-bet and CXCR3 was measured by flow cytometry. Plots depict the percentage of positive Treg cells (number inside gate) and the geometric mean channel fluorescence (MFI) to the left of the gate.

(D) iTreg cells were cultured in the presence or absence of IL-27 for 72 hr and subsequently restimulated with PMA and ionomycin in the presence of BFA and monensin Golgi inhibitors for 5 hr. The production of IFN-γ and IL-10 by iTreg cells was measured by flow cytometry.

(E) iTreg cells were generated from wild-type (WT) and *Stat1*$^{-/-}$ deficient T cells in the presence or absence of IL-27 for 72 hr. Plots depict the mean percentage ± SEM of Treg cells that expressed T-bet or CXCR3.

(F) iTreg cells were generated from WT, *Tbx21*$^{-/-}$, and CD4-Cre x *Eomes*$^{fl/fl}$ mice in the presence or absence of IL-27 for 48 hr. The percentage (inside plot) and MFI (outside plot) of Treg cells expressing CXCR3 was measured by flow cytometry. All plots are representative of three independent experiments with three replicates per group. See also Figure S1.

was the highest in the LP and was made by monocytes and macrophages (CD11b$^+$ CD11c^{Lo}) (Figure 3F).

To determine whether Treg cells contributed to the control of the Th1 cell response during toxoplasmosis, we used DEREG mice to deplete Treg cells and performed analysis of effector cell proliferation and cytokine production. This treatment resulted in ~70% loss of Treg cells (Figure S3C) and an increased frequency of Ki-67$^+$ effector CD4$^+$ and CD8$^+$ T cells (Figure S3D). This effect was accompanied by increased T-bet and IFN-γ expression in CD4$^+$ and CD8$^+$ T cells in response to soluble *Toxoplasma* antigen (STAg) (Figure 3G; Figure S3E). Thus, the Treg cells present in infected mice limited the Th1 effector cell response to *T. gondii*.

The Role of IL-27 in Regulating Treg Cell Populations during Multiple Th1 Cell Infections

To determine whether IL-27 was involved in generating Treg cell heterogeneity during Th1 responses, we challenged *Il27*$^{-/-}$ mice

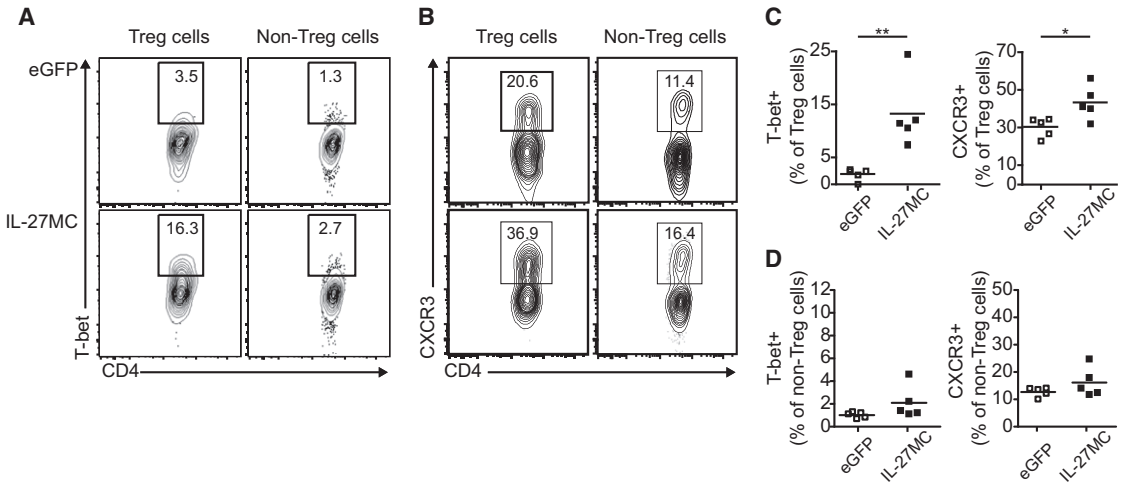

Figure 2. Ectopic Expression of IL-27 Increases Treg Cell Expression of T-bet and CXCR3

(A–D) WT mice were injected by hydrodynamic tail vein delivery control (eGFP) or IL-27 minicircles (IL-27MC) and examined 4 weeks after treatment.

(A and B) T-bet (A) and CXCR3 (B) expression by Treg cells (gated on LIVE/DEAD® Fixable Aqua Dead Cell marker$^-$, CD4$^+$, Foxp3$^+$ cells) and non-Treg T cells (gated on LIVE/DEAD® Fixable Aqua Dead Cell marker$^-$, CD4$^+$, Foxp3$^-$ cells) isolated from the spleen were measured by flow cytometry.

(C and D) The percentage of Treg (C) and non-Treg (D) cell expression of T-bet and CXCR3 from individual mice. Data are representative of two independent experiments with five mice per group. Statistical significance was determined by a two-tailed unpaired Student's t test. Error bars indicate SEM. *$p < 0.05$, **$p < 0.01$. See also Figure S2.

orally with *T. gondii* and analyzed their Treg cell populations. In these experiments, the Foxp3$^-$ CD4$^+$ effector (CD44^{hi}) T cells from WT or *Il27*$^{-/-}$ mice expressed equivalent levels of T-bet or CXCR3 (Figure 4A), but several Treg cell populations from the *Il27*$^{-/-}$ mice had reduced expression of T-bet, CXCR3, and IL-10 (Figures 4B–4D; Figure S3F). Despite the systemic levels of IFN-γ and IL-12 (Figure 4E), a striking deficiency in T-bet$^+$ CXCR3$^+$ Treg cells was observed in the gut-associated lymphoid tissue (GALT). In contrast, at peripheral sites such as the spleen, these populations were not affected, consistent with the increased expression of IL-27 observed in the gut versus the spleen (Figure 3F). To determine whether IL-27 had a similar role in other models, we infected WT or *Ebi3*$^{-/-}$ mice with *Leishmania major* or *Salmonella typhimurium*. These infections also induced T-bet$^+$ CXCR3$^+$ Treg cells and in the absence of IL-27, there was a defect in Treg cell expression of T-bet and CXCR3 (Figures S3G and S3H). These results indicate a dominant role for IL-27 in Treg cell polarization at sites of ongoing Th1 cell responses.

Acute Lethality in *T. gondii*-Infected *Il27*$^{-/-}$ Mice Is Rescued by Transfer of Treg Cells

To test whether the infection-induced CD4$^+$ T cell-mediated pathology in *Il27*$^{-/-}$ mice could be ameliorated by Treg cells, we infected WT and *Il27*$^{-/-}$ mice with *T. gondii,* and at days 4, 7, and 10 postinfection provided mice with iTreg cells. This regimen was chosen because Treg cell homeostasis is altered during *T. gondii* infection as a result of increased Treg cell death (Oldenhove et al., 2009), and repeated transfer of Treg cells alleviates inflammation in several models (Darrasse-Jèze et al., 2009; Grainger et al., 2010; Mor et al., 2007; Zheng et al., 2006). Although infected *Il27*$^{-/-}$ mice developed immune pathology and succumbed to acute infection, those that received IL-27-conditioned Treg cells, or Treg cells generated under neutral conditions, were rescued (Figure 4F). This effect was associated with reduced numbers of effector T cells and decreased serum alanine aminotransferase (Figures S4A–S4C), suggesting that the transferred Treg cells limit effector cell expansion and pathology. Compared with the endogenous Treg cells, the Treg cells transferred into infected *Il27*$^{-/-}$ mice expressed higher levels of IL-10, IFN-γ, T-bet, CXCR3, and CTLA-4 and had increased proliferation as measured by Ki-67 (Figures S4D–S4G). When Treg cells were transferred into naive mice, Foxp3 expression was not retained, whereas in infected mice transferred cells sustained Foxp3 expression at sites of inflammation (Figure S4G), suggesting that environmental cues maintain these cells. Moreover, when adoptive transfer experiments were conducted with Treg cells generated from *Il10*$^{-/-}$ mice, recipient animals succumbed rapidly to infection, indicating that IL-10 is required for Treg cell-mediated rescue of *Il27*$^{-/-}$ mice (Figure 4F). These data establish that Treg cells can ameliorate the pathology observed in the *Il27*$^{-/-}$ mice and are consistent with a model in which *Il27*$^{-/-}$ mice infected with *T. gondii* have an underlying Treg cell defect, which contributes to the development of immune pathology. Nevertheless, these data have to be interpreted cautiously. The ability of neutral Treg cells to rescue these mice may be attributed to their eventual acquisition of T-bet and CXCR3 noted after TCR stimulation (Figure S1E). However, because neutral Treg cells could promote the survival of *Il27*$^{-/-}$ mice, *Il27ra*$^{-/-}$ and *Ifngr1*$^{-/-}$ iTreg cells were generated and transferred into infected *Il27*$^{-/-}$ mice to determine whether expression of these receptors was required for protection (Figure S5A). These studies revealed that both cytokine receptors were required to rescue *Il27*$^{-/-}$ mice and suggest that although IL-27 is required for Treg cell expression of T-bet and CXCR3 at the sites of inflammation,

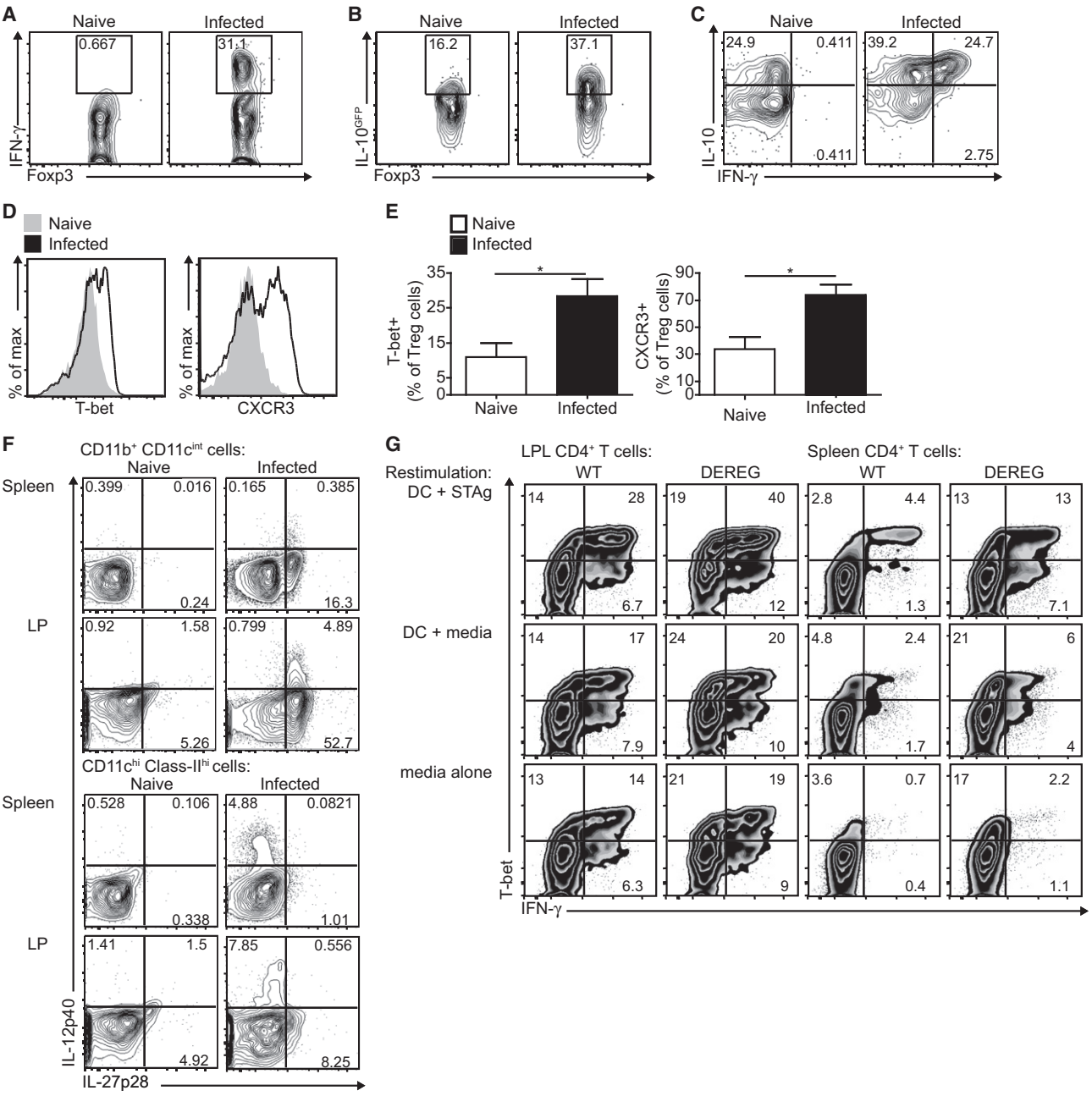

Figure 3. Treg Cells Have a Th1 Cell Phenotype and Suppress Effector T Cell IFN-γ Production during Toxoplasmosis

(A and B) Treg cells (gated on LIVE/DEAD® Fixable Aqua Dead Cell marker$^-$, TCR-β$^+$, CD4$^+$, Foxp3$^+$) were isolated from the lamina propria lymphocytes (LPLs) of naive or *T. gondii*-infected WT mice and analyzed for their expression of IFN-γ after restimulation with PMA and ionomycin in the presence of Golgi inhibitors (A) or directly ex vivo without restimulation from Vert-X IL-10-eGFP reporter mice (B).

(C) LPL Treg cells from WT *T. gondii* infected mice co-express IFN-γ and IL-10 after restimulation with PMA and ionomycin in the presence of Golgi inhibitors.

(D) T-bet and CXCR3 expression by Treg cells isolated from the mesenteric lymph nodes (mLN) of naive (shaded histogram) or *T. gondii*-infected mice (solid line).

(E) The mean percentage ± SEM of T-bet and CXCR3 expressing Treg cells in the mLN of naive and infected mice are depicted. Data are representative of >5 experiments; n = 5. Statistical significance was determined by a two-tailed unpaired Student's t test. *$p < 0.05$.

(F) Cells were isolated from the spleen and LP of naive and infected mice and incubated with Golgi inhibitors for 6–8 hr. IL-12p40 and IL-27p28 expression was measured in LIVE/DEAD® Fixable Aqua Dead Cell marker$^-$, CD19$^-$, B220$^-$, NK1.1$^-$, and CD3$^-$ cells that are CD11b$^+$ CD11c^{int} (top panel) or CD11c^{hi} MHC Class-II^{hi} (bottom panel).

(G) WT and DEREG mice were infected with *T. gondii* and treated with diphtheria toxin on days 2–9 of infection. On day 9 postinfection, T cells were isolated from the lamina propria and spleen by CD90.2 expression and cultured with media alone, dendritic cells, or dendritic cells pulsed with soluble *Toxoplasma* antigen (STAg). Treg cells (gated on LIVE/DEAD® Fixable Aqua Dead Cell marker$^-$, TCR-β$^+$, CD4$^+$, and Foxp3$^+$ cells) were stained for T-bet and IFN-γ expression. These data are representative of three experiments, n ≥ 3 per experiment. See also Figure S3.

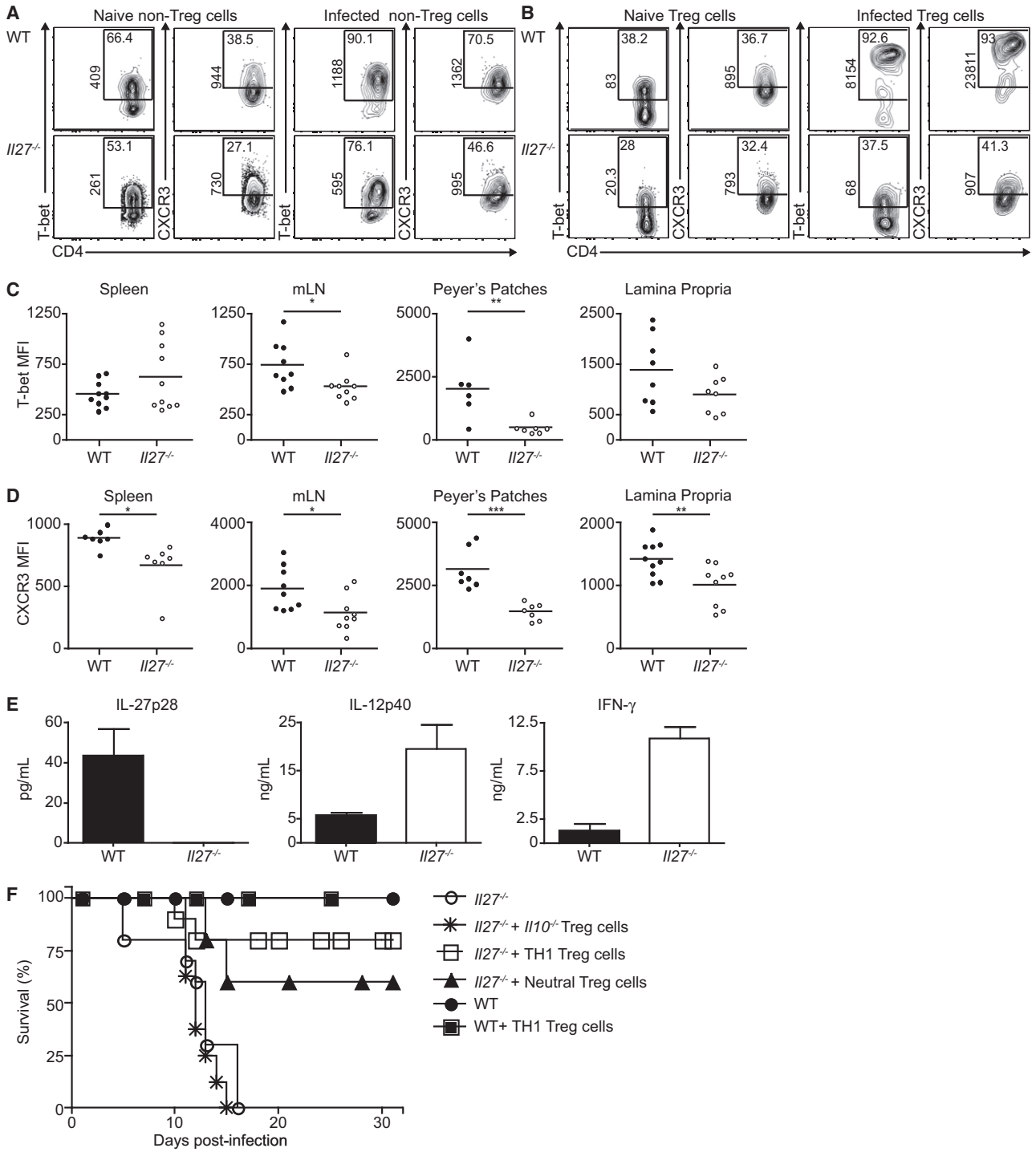

Figure 4. Th1 Treg Cell Development during *T. gondii* Infection Is IL-27-Dependent and iTreg Cells Can Rescue Acute Pathology in *Il27*$^{-/-}$ Mice

(A–D) Cells were isolated from the spleen, mLN, Peyer's patches, and LPL of naive and *T. gondii*-infected WT and *Il27*$^{-/-}$ mice. T-bet and CXCR3 expression were measured on non-Treg CD4$^+$ T cells (gated on LIVE/DEAD® Fixable Aqua Dead Cell marker$^-$, TCR-β$^+$, CD4$^+$, and Foxp3$^-$ cells; A) and Treg cells (gated on LIVE/DEAD® Fixable Aqua Dead Cell marker$^-$, TCR-β$^+$, CD4$^+$, and Foxp3$^+$ cells; B); Peyer's patches are shown. The MFI of Treg cell T-bet (C) and CXCR3 (D) expression from individual mice are shown for all tissues. Data are representative of >5 experiments. Statistical significance was determined by a two-tailed unpaired Student's *t* test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

(E) Levels of IL-27p28, IL-12p40, and IFN-γ were measured in the serum of WT and *Il27*$^{-/-}$ mice on day 9 postinfection by ELISA. Error bars indicate SEM.

(F) WT and *Il27*$^{-/-}$ mice were infected intraperitoneally with *T. gondii* and received PBS or 2 to 4 × 10^6 WT Treg cells cultured in neutral conditions (two experiments, n = 5), WT Treg cells cultured with IL-27 (Th1 Treg cells) (four experiments, n = 10), or *Il10*$^{-/-}$ Treg cells cultured with IL-27 (two experiments, n = 8). Mice received Treg cells on day 4, 7, and 10 postinfection. Percent survival is represented with Kaplan-Meier analysis. See also Figures S3 and S4.

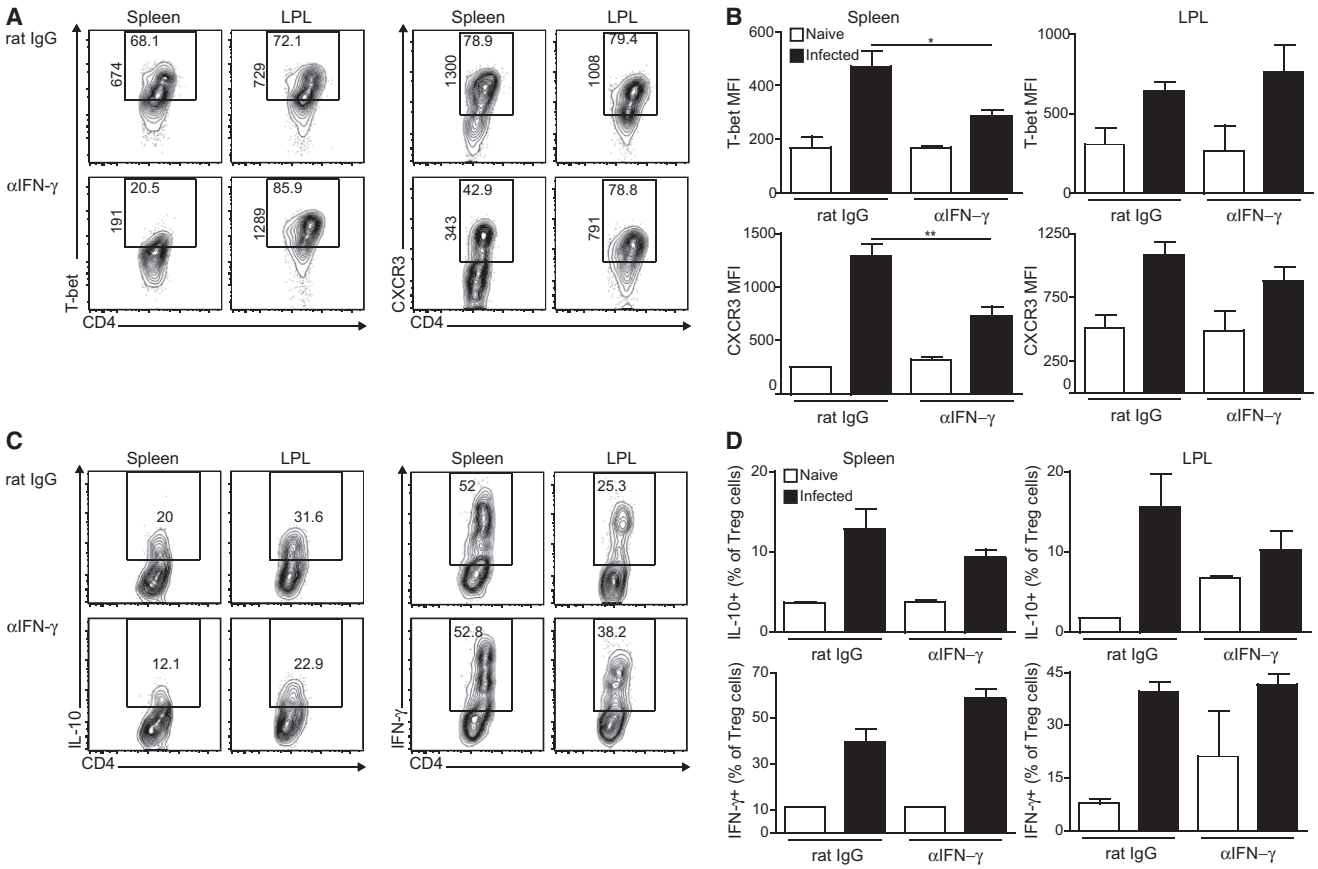

Figure 5. IFN-γ Promotes the Th1 Treg Cell Phenotype in the Periphery, but Not Sites of Inflammation

(A–D) Mice were infected with *T. gondii* and treated with anti-IFN-γ or control antibodies (rat IgG) on day 3, 5, 7, and 9 postinfection.

(A) On day 10, cells were isolated from the spleen and LPL of naive and infected mice and T-bet and CXCR3 expression by Treg cells were measured by flow cytometry (gated on LIVE/DEAD® Fixable Aqua Dead Cell marker$^-$, TCR-β$^+$, CD4$^+$, and Foxp3$^+$ cells).

(B) Plots depict the MFI of T-bet and CXCR3 expression by Treg cells. Statistical significance was determined by a two-tailed unpaired Student's t test. Error bars indicate SEM. *$p < 0.05$, **$p < 0.01$.

(C) Spleen and LPL cells were restimulated with PMA and ionomycin in the presence of Golgi inhibitors and analyzed for IL-10 and IFN-γ expression within Treg cells.

(D) Plots depict the frequency of Treg cells expressing IL-10 and IFN-γ after restimulation. Plots are representative of two experiments, n = 5. See also Figure S5.

signals through IFN-γR1 also contribute to the rescue of these mice.

Differential Effects of IFN-γ and IL-27 on Treg Cells

Although our studies have focused on the effects of IL-27 on Treg cells, our own data (Figure S5A) indicate that IFN-γ also has a role in these events, and Treg cells from the spleens (but not the gut) of infected mice have high expression of IFN-γR1 and IFN-γR2 (Figure S5B). To directly address the role of IFN-γ in generating Treg cell diversity during toxoplasmosis, we treated infected mice with α-IFN-γ starting 3 days after infection. Neutralization of IFN-γ did not alter basal expression of T-bet or CXCR3 in Treg cells from naive mice, but in infected mice there was a significant decrease in levels of T-bet and CXCR3 in the spleen (Figures 5A and 5B). In contrast, there was no effect of IFN-γ depletion on Treg cell expression of T-bet and CXCR3 in the GALT of infected mice (Figures 5A and 5B). Furthermore, after restimulation, Treg cell production of IL-10 and IFN-γ was not lower in the spleen or the LPL in the absence of IFN-γ, demonstrating that IFN-γ is not critical for Treg cell production of these cytokines (Figures 5C and 5D). These results suggest that the effects of IFN-γ are prominent at peripheral sites, whereas the effects of IL-27 are most apparent at the local sites of inflammation.

To better understand the differential effects of IFN-γ and IL-27 on Treg cells, we performed a series of studies to directly compare their signaling and effects on Treg cell differentiation and transcriptional responses. When Treg cells were stimulated with IFN-γ or IL-27, both cytokines induced pSTAT1 but only IL-27 activated STAT3 and STAT5 (Figures 6A and 6B). However, although the kinetics of pSTAT1 signaling was similar, IL-27 induced markedly elevated amounts of pSTAT1 (Figures 6A and 6B). Next, the effects of IFN-γ and IL-27 on Treg cells were compared in vitro. By day 5 of iTreg cell culture, IFN-γ and IL-27 treatment resulted in increased T-bet and CXCR3 expression but IL-27 had a more profound effect (Figures 6C–6E). In nTreg cell cultures, treatment with IL-27 resulted in higher T-bet expression compared to IFN-γ treatment, although the

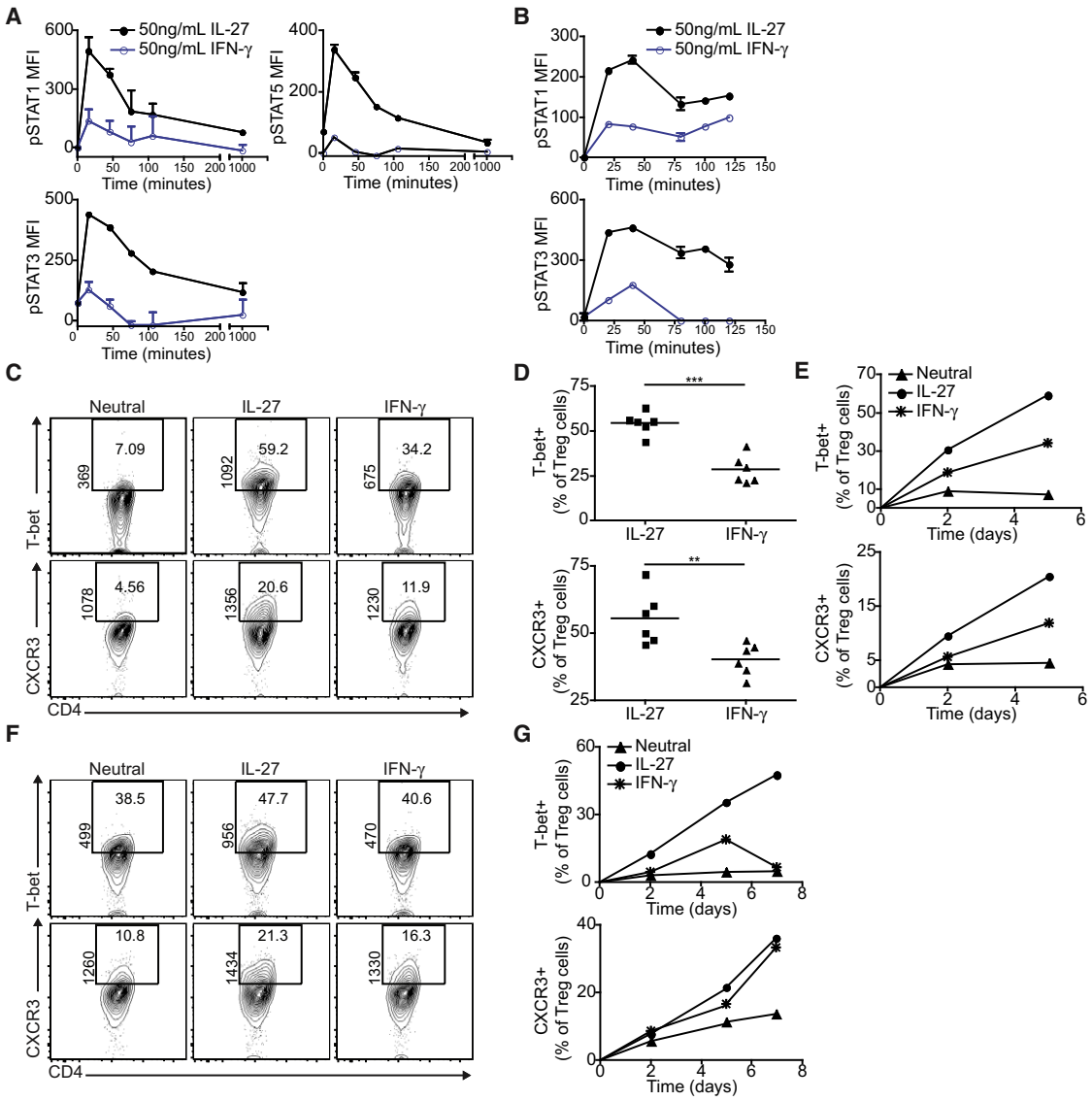

Figure 6. IFN-γ and IL-27 Have Distinct Effects on Treg Cells

(A) nTreg cells were isolated from naive mice and treated with media alone, IFN-γ, or IL-27. Levels of pSTAT1, pSTAT3, and pSTAT5 were measured over time by flow cytometry. Results were normalized to media control. Plots are representative of two experiments, n = 2.

(B) iTreg cells were generated under neutral conditions for 7 days and stimulated as in (A).

(C–E) iTreg cells were generated under neutral conditions or with IL-27 or IFN-γ (without blocking antibodies to IFN-γ and IL-4) and T-bet and CXCR3 expression was measured on day 5 (C) in replicate cultures (D) and over time (E). Plots are representative of three experiments. Statistical significance was determined by a two-tailed unpaired Student's t test. Error bars indicate SEM. $^{**}p < 0.01$, $^{***}p < 0.001$.

(F and G) NTreg cells were isolated and cultured under neutral conditions or treated with IFN-γ or IL-27 and monitored for T-bet and CXCR3 expression on day 5 of culture (F) and over time (G). Plots are representative of three independent experiments. See also Figure S6.

cytokine-induced expression of CXCR3 was similar (Figure 6G). Perhaps the most notable difference was that although both IL-27 and IFN-γ-conditioned Treg cells produced IFN-γ, only the IL-27-conditioned Treg cells made IL-10 (Figure S6A).

Finally, expression profiling of Treg cells generated during IL-27 and IFN-γ treatment was performed. To obtain sufficient cells for analysis and ensure a uniform starting population, we generated iTreg cells under neutral conditions and then cultured them in neutral, IFN-γ, or IL-27 conditions for 10 and 48 hr. Microarray analysis identified 185 genes that were differentially expressed compared to neutral controls, either early (10 hr) or late (48 hr) after treatment ($p \leq 0.05$). Hierarchical clustering identified groups of genes that showed similar expression patterns across the three treatments and two time points and revealed three distinct clusters that are regulated in different ways by IL-27 compared to IFN-γ (Figure 7; Figure S6B). Cluster 1 (23 genes) was enriched for metabolic functional categories and is suppressed by IL-27 while remaining relatively unchanged in

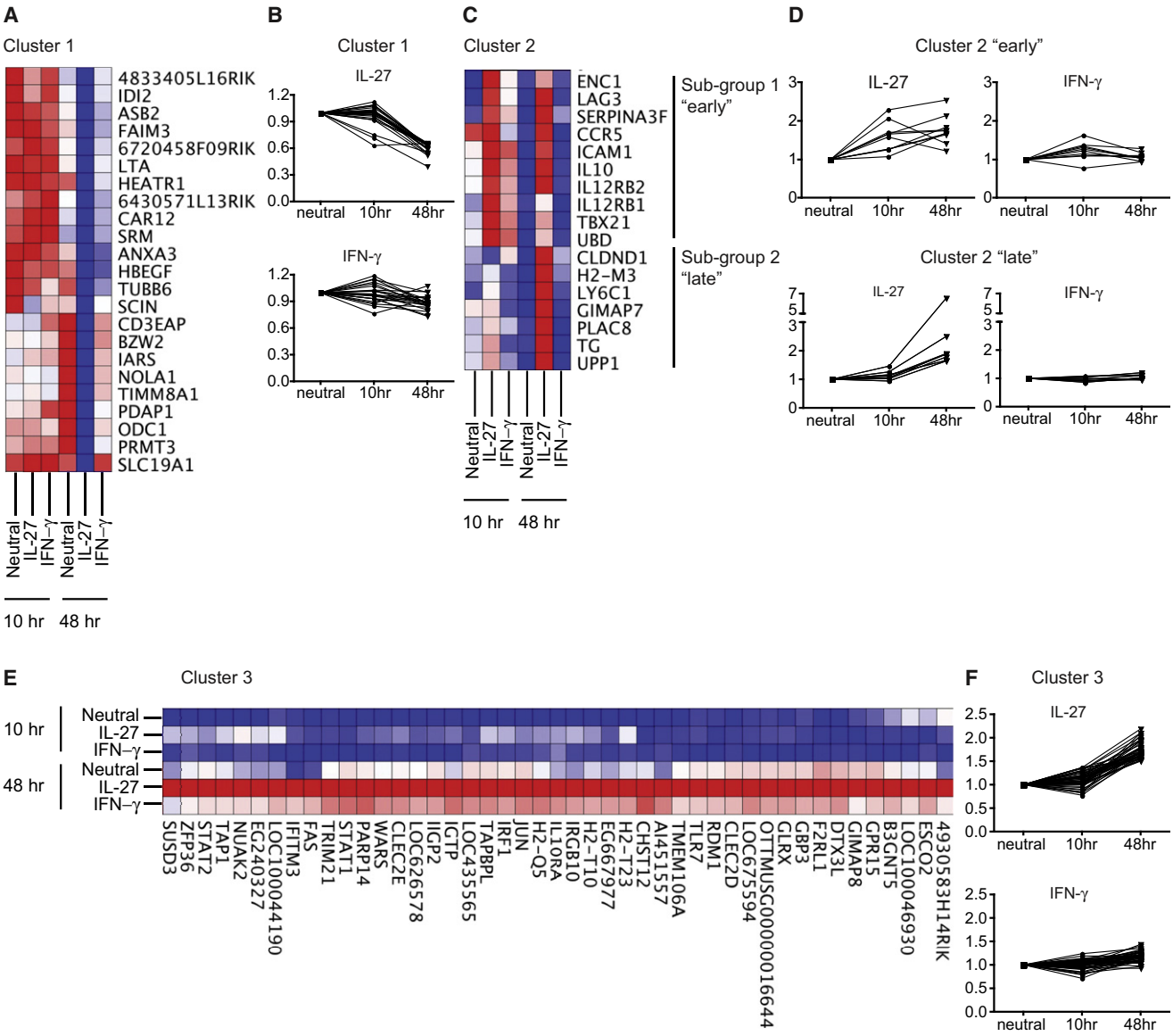

Figure 7. IL-27 Induces a Gene Expression Profile in Treg Cells that Is Distinct from the Profile induced by IFN-γ

(A–F) iTreg cells were cultured under neutral conditions for 7 days, harvested, and then exposed to neutral, IL-27, or IFN-γ Treg cell culture conditions for 10 or 48 hr. mRNA was isolated and microarray analysis was performed as described in the Experimental Procedures. Hierarchical clustering analysis was performed on genes that were differentially regulated 1.5-fold or greater, compared to neutral conditions, by either IL27 or IFN-γ after 10 hr or 48 hr culture, $p < 0.05$. Three clusters of genes are shown as heatmaps. The following are shown in these panels: (A) genes downregulated most strongly by IL-27; (C) genes upregulated by IL-27 but not IFN-γ; and (E) genes induced by both cytokines, but to a greater degree by IL-27. The heatmap color indicates log2 expression value. Clusters are also represented by line graphs showing the fold changes in gene expression (B, D, and F). Each line shows gene expression changes for a single gene. Several genes had more dramatic changes in expression at either the 10 hr or 48 hr time points and thus were grouped as subgroup 1 "early" and subgroup 2 "late," respectively. See also Figure S6.

the presence of IFN-γ (Figures 7A and 7B). In contrast, cluster 2 (17 genes) is induced by IL-27, but not IFN-γ (Figures 7C and 7D). This cluster contains two subgroups: the first includes ten genes induced early by IL-27 but were either not induced by IFN-γ or weakly induced at 10 hr and were not sustained to 48 hr (Figure 7D). This subgroup includes *Tbx21*, *Il10*, *Lag3*, *Ccr5*, *Il12rb1*, and *Il12rb2*, highlighting a group of genes involved in T cell activation that are coordinately regulated by IL-27. The second subgroup includes seven genes that are not affected early by either cytokine but are induced at 48 hr by IL-27 (Figure 7D). Finally, a third cluster of 45 genes was identified that is enhanced by IL-27 and IFN-γ, but for which IL-27 was a more potent inducer (Figures 7E and 7F). This cluster includes many canonical STAT1 target genes, further demonstrating the ability of IL-27 to act as an activator of STAT1-dependent gene transcription in Treg cells. Thus, although IFN-γ and

IL-27 can give rise to a phenotypically similar population of Treg cells, these two cytokines have distinct transcriptional effects on Treg cells that are indicative of a more complex biology and may reflect the regional effects observed in vivo.

DISCUSSION

In the last decade, there has been a growing appreciation of the inhibitory properties of IL-27 in the setting of autoimmunity and inflammation (Stumhofer and Hunter, 2008; Yoshida and Miyazaki, 2008). Indeed, studies with *Il27ra*$^{-/-}$ mice infected with *T. gondii* have identified a role for IL-27 in limiting the production of IL-2, antagonizing Th17 cells and promoting effector cell production of IL-10 (reviewed in Hall et al., 2012). In early studies, it did not appear that the enhanced inflammation observed in these mice was a consequence of a defective Treg cell response, given that IL-27 did not alter Treg cell activity in suppressor assays, nor are there any overt differences in the frequency of Treg cell populations in *Il27ra*$^{-/-}$ mice (Villarino et al., 2003 and this work). The data presented here highlight that the ability of IL-27 to promote a specialized population of Treg cells contributes to its suppressive activities in multiple experimental models and indicate that this is one of the many pathways that limit overt T cell-mediated inflammation in this model (Aliberti et al., 2002; Bhadra et al., 2011; Buzoni-Gatel et al., 2001; Gazzinelli et al., 1996).

It has been suggested that Treg cells have a limited role during toxoplasmosis (Couper et al., 2009; Jankovic et al., 2007), but the Treg cell depletion studies presented here indicate that these cells are relevant. The finding that Treg cell transfers can reverse the infection-induced pathology observed in *Il27*$^{-/-}$ mice provides additional support for the idea that Treg cells are operational during toxoplasmosis. However, these transfer experiments have to be interpreted carefully, and they illustrate how understanding the mechanisms by which adoptively transferred Treg cells suppress in vivo remains elusive. Although our system clearly shows that Treg cells need to express *Il27ra*, *Ifngr1*, and *Il10* to rescue *Il27*$^{-/-}$ mice, it is unclear how these signals are integrated. Because the transfer of activated Treg cells can rescue the *Il27*$^{-/-}$ mice, it implied that signals other than IL-27 (such as IFN-γ or TCR stimulus) could be important. However, given these data, if IFN-γ or TCR were sufficient to contribute to Treg cell function in vivo, we would expect that it could contribute to protection upon adoptive transfer of *Il27ra*$^{-/-}$ Treg cells, which is not the case. We cannot rule out a role for IL-27Rα independently of IL-27 and/or gp130 given that the IL-27Rα has the capacity to signal via JAK1-STAT1 independently of gp130 (Pradhan et al., 2007; Takeda et al., 2003). Moreover, the IL-27Rα is also a component of the receptors for cytokine-like factor-1 (CLF-1) and humanin, and there is an alternatively spliced form of IL-27Rα that may be involved in *trans*-signaling (reviewed in Hall et al., 2012). Thus, it remains to be determined whether the IL-27-independent properties of IL-27Rα play a role in Treg cell function during infection. Alternatively, our mRNA expression data (Figure S6C) suggest the possibility that Treg cells may also produce IL-27, implying that autocrine signaling through IL-27Rα may be important for the function of Treg cells. Nevertheless, these observations raise fundamental questions about whether this Th1 cell-like Treg cell population is derived from nTreg cells or iTreg cells, whether these cells are specific for *Toxoplasma*, and how IFN-γR1, IL-27Rα and IL-10 contribute to their function in vivo.

Although the emphasis of these studies has been on the role of IL-27 during infection, our findings imply that IL-27 and IFN-γ act in distinct sites during infection, perhaps a consequence of local differences in the cytokine environment. However, despite the systemic elevation of IFN-γ present in infected *Il27*$^{-/-}$ mice, there is still a defect in the generation of T-bet^{+} Treg cells at sites of inflammation, indicating a critical role for IL-27 in mediating Treg cell expression of T-bet and CXCR3. Additionally, the ability of IL-27, but not IFN-γ, to promote the expression of IL-10 in Treg cells highlights distinct functions of these cytokines.

The observation that in naive mice, only a subset of Treg cells expresses the IL-27 and IFN-γ receptors raises the question of whether these Treg cells subsets are ''hard-wired'' to deal with specific types of inflammation. Indeed, little is known about the heterogeneity in Treg cell expression of cytokine receptors and whether this predicts their capacity to influence distinct types of inflammation. In the context of trying to understand Treg cell heterogeneity, microarray analysis of Treg cells isolated from different anatomical sites revealed that distinct subsets of Treg cells exist with nonoverlapping transcriptional profiles (Feuerer et al., 2009; Feuerer et al., 2010). Similarly, when we compared the transcriptional profiles of Treg cells treated with IFN-γ and IL-27, these data revealed at least three clusters of genes that are differentially regulated. The largest cluster consists of many known STAT1 target genes, reinforcing the notion that IL-27 is a potent inducer of STAT1-mediated transcription. It is unclear how the specific STAT1 genes that we have identified as IL-27 targets might contribute to Treg cell activity, but these data will aid in the selection of candidates for future studies of Treg cell function. In addition, we have identified a subset of genes that are specifically induced by IL27 but not IFN-γ, providing candidate mediators for IL-27-specific Treg cell function. For instance, we found that *Ly6c1* is strongly induced by IL-27. Although its role in Treg cell biology has not been addressed, it has been implicated in the function and homing of effector T cells (Jaakkola et al., 2003; Marshall et al., 2011). These data also highlight that examining a select few phenotypic markers (such as T-bet and CXCR3), may oversimplify the complex heterogeneity that exists in Treg cells during infection.

Many studies have defined how factors such as IL-2, TGF-β, and Foxp3 have a prominent role in the homeostasis and function of Treg cells (Apostolou et al., 2008; Bayer et al., 2007; Fontenot et al., 2005). Although there is good evidence that an ongoing immune response can limit Treg cell function and differentiation (Caretto et al., 2010; Mantel et al., 2007; Pasare and Medzhitov, 2003; Wei et al., 2007), recent reports indicate that by utilizing the same transcription factors as their effector counterparts, Treg cells may become specialized to operate in distinct inflammatory environments (Chaudhry et al., 2009; Koch et al., 2009; Zheng et al., 2009; Lu et al., 2010). These findings have led to models in which environmental cues promote the development of specialized Treg cell subsets. The finding that IL-27 promotes a specialized subset of Treg cells delineates a unique pathway by which they are influenced by the inflammatory environment. These findings may be directly relevant to human disease, and it is notable that a loss of Treg cells has

been observed in human patients with inflammatory bowel disease (IBD) (Eastaff-Leung et al., 2010). A recent report linked a polymorphism in the IL-27p28 loci, associated with reduced production of *IL27* transcripts, to increased susceptibility to IBD (Imielinski et al., 2009). The finding that IL-27 can profoundly influence Treg cell populations in the gut may offer a partial explanation for the susceptibility of these particular patients to IBD.

EXPERIMENTAL PROCEDURES

Mice and Infection

Il27$^{-/-}$ mice were generated by Lexicon Pharmaceuticals, Inc. Wild-type C57BL/6J (WT), *Il10*$^{-/-}$, Swiss Webster and CBA/CaJ mice were purchased from Jackson laboratory. *Tbx21*$^{-/-}$ and CD4-Cre × *Eomes*$^{fl/fl}$ mice were provided by S. Reiner. *Stat1*$^{-/-}$ mice were purchased from Taconic labs. Foxp3^{GFP} reporter mice were obtained from V. Kuchroo, and Vert-X IL-10-eGFP (C57BL/6- *Il10tm1.1Karp*) reporter mice (Madan et al., 2009) were provided by C. Karp and bred in our facility. All mice were housed in a specific-pathogen free environment at the University of Pennsylvania School of Veterinary Medicine in accordance with federal guidelines and with approval of the Institutional Animal Care and Use Committee. The ME49 strain of *T. gondii* was maintained in Swiss Webster and CBA/CaJ mice and used as a source of tissue cysts for oral (100 cysts) or i.p. (20 cysts) infections. For Treg cell depletion experiments, WT or DEREG mice (Lahl et al., 2007) were infected orally with 10 cysts of ME49. Two days after infection, mice were injected with diphtheria toxin (Calbiochem) in endotoxin-free PBS. One microgram of toxin was injected i.p. for 7 consecutive days. For the depletion of IFN-γ, WT mice were infected orally and starting at day 3 postinfection treated with 2 mg anti-mouse IFN-γ (clone XMG1.2, BioXcell) or ratIgG control (Sigma) every 2 days.

Regulatory T Cell Differentiation

For iTreg cells, CD4$^+$ CD25$^-$ or Foxp3^{GFP-} cells were separated by FACS or MACs sorting (Miltenyi). Cells were rested for 30 min in complete RPMI (cRPMI) media (1% penicillin/streptomycin, 2 mM L-glutamine, 10% fetal bovine serum, 0.1% beta-mercaptoethanol, 1% nonessential amino acids, 1 mM sodium pyruvate, and 20 mM HEPES) (GIBCO). Cells were cultured at 1 × 10^6 cells/mL in αCD3-coated (1 μg/mL; clone 145-2C11; eBioscience) 96-well U-bottom plates (Costar) in cRPMI containing αCD28 (1 μg/mL; clone 37.51; eBioscience), recombinant human (rHu) TGF-β2 (5 ng/mL, eBioscience), with or without rHu IL-2 (100U/mL; Proleukin), anti-IFN-γ (10 μg/mL; clone XMG1.2), anti-IL-4 (10 μg/mL; clone 11B11) blocking antibodies, and recombinant mouse (rMu) IL-27 (50ng/mL; Amgen), with or without rMu IFN-γ (50 ng/mL; R&D). Media was added every 2 days with initial cytokines and neutralizing antibodies. For nTreg cells, sorted Foxp3$^+$ cells were cultured on plate-bound αCD3 in media containing αCD28, 100 U/mL rHu IL-2, 5 ng/mL rHu TGF-β2, with or without neutralizing antibodies to IFN-γ, or IL-4, with rMu IL-27 or rMu IFN-γ.

Isolation and Flow Cytometric Analysis of Immune Populations

Single-cell suspensions from the spleens, mesenteric lymph nodes (mLNs), and Payer's patches were prepared with standard methods. For the analysis of lamina propria lymphocytes (LPLs), small intestines were collected in PBS at 4°C and cut longitudinally, and fecal contents were removed in PBS. Epithelial cells were stripped (5 mM EDTA and 1 mM DTT) in cRPMI, digested (0.16U/mL Liberase TL) (Roche) for 30 min at 37°C, and processed for lymphocytes.

Cells were stained in FACS buffer (0.5% BSA, 2mM EDTA in PBS) with Fc block (2.4g2, BD) containing LIVE/DEAD® Fixable Aqua Dead Cell marker (Invitrogen), with the following surface antibodies: CD4 Percp-Cy5.5 (RM4-5, eBioscience), CD8a PE-Texas Red (53-6.7, Abcam), CD44 PE-Cy7 (IM7, eBioscience), CD62L APC-eFluor780 (MEL-14, eBioscience), TCR-β Alexa Fluor700 (H57597, BioLegend), CD3 eFluor450 (17A2, eBioscience), CD25 APC-eFluor780 (PC61.5, eBioscience), CXCR3 PE and APC (220803, R&D), and PE-Cy7 (CXCR3-173, BioLegend). All intracellular staining was done with the Foxp3/transcription factor staining buffer set (eBioscience) for Foxp3 Alexa Fluor488 and eFluor450 (FJK-16 s, eBioscience), T-bet eFluor660, and FITC (4B10, eBioscience and BioLegend, respectively), Ki-67 Alexa Fluor488 and Alexa Fluor647 (B56, BD), CTLA-4 PE (UC10-4F10-11, BD), and EOMES PE (Dan11mag, eBioscience).

For detecting cytokine production, isolated cells were cultured in cRPMI in six replicates at 1 × 10^6 cells/mL in a 96-well U-bottom plate, with PMA and ionomycin for 5 hr with Brefeldin A (Sigma) and monensin (BD) Golgi inhibitors. Cells were rinsed, stained for surface markers at 4°C, and fixed with 4% PFA in PBS for 10 min at RT. Intracellular cytokines were detected by staining in FACs buffer containing 0.5% saponin (Sigma), IL-10 APC (JES5-16E3, eBioscience), IFN-γ PE-Cy7 or Percp-Cy5.5 (XMG1.2, eBioscience), and Foxp3. Intracellular IL-10 was detected ex vivo with Vert-X IL-10-eGFP reporters and antibodies for GFP (polyclonal rabbit anti-GFP, eBioscience and FITC-conjugated rat anti-rabbit Jackson Immunoresearch) with staining for Foxp3.

STAT Phosphorylation Assays

nTreg cells were isolated ex vivo, and iTreg cells were generated in vitro. Before stimulation, cells were washed in 0.5% BSA RPMI and rested at 4°C for 20 min. WT and *Il27ra*$^{-/-}$ Treg cells were incubated with 50 ng/mL rMu IL-27, 50 ng/mL rMu IL-10 (R&D), and 50 ng/mL rMu IFN-γ for 20 min or over various time points at 37°C in the presence of LIVE/DEAD® Fixable Aqua Dead Cell marker and then immediately fixed on ice in 4% PFA for 20 min. After PBS rinse, cells were permeabilized in 90% methanol on ice for 1 hr or stored at −20°C overnight. Staining was performed in Fc block with BD PhosFlow antibodies to pSTAT1 (pY701) PE, pSTAT3 (pY705) Alexa Fluor488, and pSTAT5 (pY694) Alexa Fluor647 with antibodies to T-bet, Foxp3, TCR-β, CD4, and CD25.

Production and Delivery of IL-27 Minicircle DNA

The p2øC31.RSV.hAAT.bpA plasmid was provided by Z.-Y. Chen (Stanford University, Stanford, CA), and the vector was modified to include unique 5′ PmeI and 3′ PacI restriction sites flanking hAAT for directional cloning of cDNAs. PCR amplification was used for placing 5′ PmeI and 3′ PacI cloning sites on the linked mIL-27 cDNAs, which were ligated with the modified minicircle plasmid. Minicircle DNA was produced as described (Chen et al., 2005) with minor modifications for overnight cultures. Terrific broth containing 100 ug/ml ampicillin was inoculated and incubated for 18 hr and shaken at 270 rpm. Endotoxin free QIAGEN megaprep kits were used for DNA purification, which was resuspended in endotoxin-free Tris EDTA. Minicircle DNA was dialyzed in Midi MWCO 3.5 kDa tubes overnight against Tris EDTA and minicircle DNA was verified by restriction digestion and sequencing. For a hydrodynamics-based transfection procedure (Liu et al., 1999), 20 mg of minicircle DNA in 2 ml of Ringer's solution was administered via tail vein injection within 5–8 s.

ELISAs and LP Dendritic Cell Intracellular Cytokine Detection

IL-27p28 (DuoSet, R&D), IL-12p40 (C17.8 and C15.6 biotin), and IFN-γ (AN18 and R4-6A2 biotin, eBioscience) were measured by ELISA. For detecting intracellular IL-27p28 and IL-12p40, LP dendritic cells (LpDCs) were enriched with the 1-Step 1.077/265 gradient (Accurate Chemical & Scientific Corp.), incubated for 6–8 hr at 37°C in the presence of Brefeldin A and Monensin, surface stained in Fc block including LIVE/DEAD® Fixable Aqua Dead Cell marker for MHC Class II I-A I-E Alexa Fluor 700 (M5/114.15.2, BioLegend), CD11c PE-Cy7 (N418, eBioscience), CD11b PerCP-Cy5.5 (M1/70, eBioscience), NK1.1 Pacific Blue (PK136, BioLegend), CD3 eFluor450, and CD19 eFluor450 (1D3, eBioscience), and fixed with 4% PFA. Cytokines were detected by staining for IL-12p40 PE (C15.6, BD) and IL-27p28 Alexa Fluor 647 (clone MM27-7B1, BioLegend) for 30 min in 0.5% Saponin.

Adoptive Transfer Studies

WT or *Il27*$^{-/-}$ animals were infected intraperitoneally with 20 cysts ME49 *T. gondii* and monitored for morbidity. Neutral Treg cells were generated from naive WT CD25$^-$ CD4$^+$ T cells or Th1 Treg cells were generated from naive WT or *Il10*$^{-/-}$ CD4$^+$ CD25$^-$ T cells. At day 4 postinfection, mice received either i.v. PBS or 2 to 4 × 10^6 Treg cells, followed injections of Treg cells on day 7 and 10.

Gene Expression Profiling, Hierarchical Clustering and Ontology Enrichment Analysis

For whole-genome-expression microarray, iTreg cells were generated under neutral conditions for 7 days to ensure a homogeneous starting population of Treg cells. Cells were harvested and then exposed to neutral, IL-27, or IFN-γ Treg cell culture conditions for 10 or 48 hr. RNA was isolated with RNeasy Plus (QIAGEN) and quality was assessed by Bioanalyzer (Agilent). Biotin-labeled complementary RNA (cRNA) was made with the Illumina TotalPrep RNA amplification kit. An Illumina MouseWG-6 version 2 expression beadchip was hybridized with cRNA from three biological replicates and scanned on a beadscan unit. Data were quantile normalized and differential expression analysis was carried out with GenomeStudio v1.8 software (Illumina). Genes were considered differentially regulated by IL-27 or IFN-γ if expression level changed $\geq$1.5-fold compared to neutral controls with a corresponding diff-score $\geq$13 or ≤ -13 (equivalent to $p \leq 0.05$). Hierarchical clustering (Eisen et al., 1998) and heat map tools available on GenePattern (Reich et al., 2006) were used for analyzing and displaying microarray data. Gene ontology (GO) enrichment analysis was done with the Database for Visualization and Integrative Discovery (DAVID) (Dennis et al., 2003) with enrichment defined relative to entire microarray. Only GO terms shared by three or more genes and that had an enrichment p value of < 0.05 were considered for analysis.

Statistical Analysis

Statistical significance was determined by a two-tailed unpaired Student's t test. Error bars indicate the standard deviation of the mean; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Percent survival is represented with Kaplan-Meier analysis.

ACCESSION NUMBERS

The microarray data are available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/gds) under the accession number GSE38686.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.06.014.

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