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Regulation of Proinflammatory Th17 Responses during *Trypanosoma cruzi* Infection by IL-12 Family Cytokines

Dustin Cobb and Ronald B. Smeltz

Previously, we reported that the transcription factor T-bet (*Tbx21*) regulates Th17 responses to *Trypanosoma cruzi* infection in an IFN-γ-independent manner. In an effort to further understand this regulation, we examined the development and plasticity of Th17 cells during *T. cruzi* infection. Th17 cells recovered from infected *Tbx21*−/− mice were amenable to the inhibitory effects of T-bet, as ectopic expression of T-bet reduced IL-17 expression. We subsequently addressed the role of IL-12 family cytokines IL-12 and IL-27 and report that IL-12p35−/− mice infected with *T. cruzi* exhibited a significant increase in Th17 cells and Th17-associated inflammation. Ex vivo culture of these cells with IL-12 led to a dramatic reduction in IL-17 production and concomitant decrease in IFN-γ. Importantly, the ability of IL-12 to suppress IL-17 was independent of IFN-γ. Surprisingly, and contrary to results reported for other pathogens, IL-27 had no inhibitory effect on Th17 development, as Ebi-3−/− mice failed to show any increase in their *T. cruzi*-specific Th17 response. Furthermore, IL-27 could not compensate or synergize with IL-12 to suppress IL-17 production ex vivo. Thus, we have established that IL-12, not IL-27, is critical for regulating Th17 responses to *T. cruzi*. The Journal of Immunology, 2012, 188: 3766–3773.

*Trypanosoma cruzi* is a protozoan parasite and human pathogen, responsible for causing potentially life-threatening infections as well as chronic Chagas disease in millions of people worldwide. *T. cruzi* infection induces a potent proinflammatory cytokine response that is necessary for acute resistance. Indeed, effector cytokines such as IFN-γ, TNF-α, and IL-17 play a key role in this process (1–3). These cytokines can be produced by a variety of cells, including T cells, which are necessary for long-term (including vaccine-mediated) immunity against *T. cruzi*. Although the importance of cytokine production is evident, its regulation is equally as important to prevent host tissue damage. For example, experiments using in vivo neutralization of IL-17 or IL-17–deficient mice have demonstrated that IL-17 is necessary for acute resistance to *T. cruzi* (3, 4). Indeed, T cells from wild-type mice produce IL-17 early during *T. cruzi* infection. However, over time the cytokine balance appears to shift and favor IFN-γ as T cells recovered from infected mice produce copious amounts of IFN-γ in response to *T. cruzi* Ags. Indeed, studies in humans have confirmed that IFN-γ is an important determinant in control of Chagas disease (5). In contrast, chronic *IL-17–deficient mice* are produced mainly by APCs and have a significant role in shaping the development of CD4+ effector T cell subsets in vivo. For example, IL-12 (a heterodimer of p35 and p40 subunits) is a potent inducer of Th1 responses and in some instances is required for their maintenance (10, 11). Similarly, IL-27 (Ebi-3 and p28 subunits) has been reported to influence Th1 and Th17 development. In addition, IL-12 and IL-27 are capable of activating T-bet, either directly or indirectly, further promoting the stability of Th1 phenotype. Importantly, both IL-12 and IL-27 are expressed during the course of *T. cruzi* infection and play important roles in *T. cruzi* resistance. However, the underlying causes of increased susceptibility in IL-12 and IL-27R–deficient mice are not completely understood. In contrast to IL-12 and IL-27, IL-23 (IL-12p40 and IL-23p19) has an important role in promoting Th17 differentiation and IL-17 production. Thus, despite shared usage of cytokine subunits, the IL-12 family of cytokines can have divergent roles in promoting Th cell responses to infection. To address the important issue of development of effector T cell responses to *T. cruzi*, we performed studies in which IL-12 or IL-27–deficient mice were infected with *T. cruzi* and subsequently analyzed for development of Th17 responses and neutrophilia, the cardinal feature associated with Th17 inflammation. T cells from infected mice were then analyzed ex vivo for the stability of the cytokine-producing phenotype. Specifically, we wished to deter-

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Abbreviations used in this article: −/−, deficient; LEAF, low-endotoxin/azide-free; p.i., postinfection; RV, retroviral; *Tbx21*−/−, T-bet-deficient mice.

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mine the importance of IL-12 and IL-27 in regulating Th17 responses to T. cruzi, as well as the relative hierarchy between IL-12, IL-27, and IL-23 in their abilities to influence IL-17 responses to T. cruzi. We report that IL-12 is critical for regulating Th17 responses to T. cruzi, including the ability to promote terminal differentiation of Th17 cells into Th1 cells in an IFN-γ-independent manner. In sharp contrast, and contrary to our expectations, IL-27 had no significant impact on Th17 responses to T. cruzi. Further, IL-27 had no effect, either alone or in combination with IL-12, to promote the conversion of Th17 cells into Th1 cells. These results shed new light on the regulation of T. cruzi-specific T cells during the course of infection.

Materials and Methods

Mice and parasite infections

Age- and sex-matched C57BL/6, Tbx21<sup>-/-</sup>, Il12p35<sup>-/-</sup>, Il12p40<sup>-/-</sup>, and Ebi-3<sup>-/-</sup> mice were obtained from The Jackson Laboratory and used between 6 and 8 wk of age. Mice were housed in an Association for Assessment and Accreditation of Laboratory Animal care-accredited facility under pathogen-free conditions and used in accordance with an Institutional Animal Care and Use Committee-approved protocol. For infections, mice were infected i.p. with 1 × 10<sup>6</sup> T. cruzi promastigotes (CL strain).

CD4<sup>+</sup> T cell isolation and retroviral transduction

CD4<sup>+</sup> T cells were purified from the spleens of T. cruzi-infected mice on day 9 postinfection (p.i.) by positive selection using CD4<sup>+</sup> microbeads (Miltenyi Biotec). A total of 0.5 × 10<sup>6</sup> CD4<sup>+</sup> T cells was cultured with 2 × 10<sup>6</sup> irradiated splenocytes and 0.5 μg/ml low-endotoxin/azide-free (LEAF) anti-CD3 (clone 145-2C11; BioLegend) in 24-well plates. CD4<sup>+</sup> T cells from T. cruzi-infected mice were supplemented with rIL-23 (20 ng/ml) and recombinant human IFN-γ (10 ng/ml). CD4<sup>+</sup> T cells were stimulated for 24 h and then transduced with retrovirus supernatant by centrifugation at 1000 × g for 1 h at room temperature in the presence of 10 μg/ml polybrene. Viral supernatants were removed after 24 h and replaced with fresh media. After 4 to 5 d of culture, T cells were re-stimulated with plate-bound anti-CD3 (5 μg/ml) for 6 h with the addition of the Golgi inhibitor monensin during the final 4 h. Following, restimulation GFP<sup>+</sup> cells were FACS sorted (FACS Aria; BD Bioscience) for intracellular cytokine staining. Retroviral plasmids encoding empty-GFP and T-bet-GFP were a generous gift of Dr. Eun Hwang (Ewha Womans University, Seoul, Korea) and have been described previously (12).

Ex vivo cell culture

A total of 2 × 10<sup>6</sup> splenocytes from T. cruzi-infected mice were cultured in complete RPMI 1640 (10% FBS, 1% HEPES, 1% penicillin/streptomycin, 1% L-glutamine, and 2-ME). Splenocytes were restimulated with 0.5 μg/ml LEAF-purified anti-CD3 or T. cruzi lysate (10 μg/ml) for Ag-specific responses for 72 h. For the neutralization of IFN-γ, LEAF-purified anti-IFN-γ (clone XMG1.2; BioLegend) Abs were administered to ex vivo cell cultures at a concentration of 5 μg/ml.

Recombinant cytokines

For Th17 differentiation, cells were stimulated in the presence of rIL-6 (20 ng/ml) and TGF-β (10 ng/ml). For ex vivo culture experiments, recombinant mouse IL-12 (p70), IL-27 (100 ng/ml), and IL-23 (20 ng/ml) were used. All cytokines were purchased from BioLegend.

Fluorescent Abs and flow cytometry

Peripheral blood leukocytes and splenocytes were incubated with anti-CD16/32 and then stained with anti-Gr-1 (PE) fluorescent Abs. Likewise, splenocytes were stained with anti-CD4 (FITC) and anti-CCXCR3 (PE) Abs. For intracellular cytokine staining, cells were stained with anti-CD4 (allophycocyanin/Cy7) and fixed with 4% paraformaldehyde. After fixation, cells were permeabilized using Permabilization Wash Buffer (BioLegend) and subsequently stained with anti–IFN-γ (APC) and anti–IL-17 (PE) Abs. All fluorescent Abs were obtained from BioLegend. Cells were analyzed by flow cytometry using an FC500 instrument (Beckman Coulter).

Intracellular T-bet staining

First, cell-surface staining for CD4 was performed. Cells were then fixed with Foxp3 Fix/Perm (BioLegend) for 20 min. Fixed cells were then permeabilized with Foxp3 Perm Buffer (BioLegend) for 15 min. Following permeabilization, cells were stained with anti–T-bet (PE) and then analyzed by flow cytometry.

Measurement of cytokines

Cell-culture supernatants were collected after 72 h and analyzed for the presence of IFN-γ and IL-17 using BioLegend’s ELISA MAX Standard Sets according to the manufacturer’s recommendations.

Statistical analyses

Data were analyzed using student t test or Mann–Whitney U test as appropriate (SigmaPlot 12.0; Systat Software). A p value < 0.05 was considered significant. Data are represented as means ± SD of experimental groups.

Results

Ectopic expression of T-bet reverses the IL-17–producing phenotype of primed T cells recovered from Tbx21<sup>-/-</sup> mice infected with T. cruzi

We recently reported that T-bet was dispensable for the development of protective Th1 responses against T. cruzi (8), a protozoan parasite and causative agent of Chagas disease in humans. However, T-bet was required to repress the development of T. cruzi-specific Th17 cells and Th17-associated inflammation (i.e., neuropathia) and did so in a manner independent of IFN-γ (8, 9). To determine if Th17 cells from infected Tbx21<sup>-/-</sup> mice represented a stable phenotype or possessed developmental plasticity, we used retroviral (RV)-mediated gene delivery to introduce T-bet into CD4<sup>+</sup> T cells recovered from Tbx21<sup>-/-</sup> mice infected with T. cruzi (Fig. 1A). As shown in Fig. 1B, and consistent with our previous studies, ∼50% of cytokine-producing CD4<sup>+</sup> T cells from T. cruzi-infected Tbx21<sup>-/-</sup> mice produced IL-17 only, whereas ∼20% produced IFN-γ only. We previously confirmed that IL-17 production in infected Tbx21<sup>-/-</sup> mice is T. cruzi specific (8). Complementation of Tbx21<sup>-/-</sup> cells from infected Tbx21<sup>-/-</sup> mice with T-bet RV led to a dramatic reduction in the number of IL-17–producing cells (Fig. 1C, 50–100%), with a concomitant increase in IFN-γ–producing cells (Fig. 1C, 20–75%). Importantly, addition of anti–IFN-γ to RV cultures failed to reverse the inhibitory effects of ectopic T-bet expression. Thus, Th17 cells generated in Tbx21<sup>-/-</sup> mice infected with T. cruzi possess developmental plasticity and readily convert into Th1 cells in a T-bet–dependent, IFN-γ–independent manner.

Differential effects of IL-12 family cytokines IL-12, IL-27, and IL-23 on IL-17 production by T cells from wild-type mice infected with T. cruzi

The IL-12-family cytokines, which consist of IL-12, IL-27, and IL-23, are produced by APCs and essential for regulating Th cell differentiation (13). First, we wanted to determine what effect these cytokines had on IL-17 production in T cells from wild-type mice infected with T. cruzi. Splenocytes from wild-type T. cruzi-infected mice were restimulated on day 9 p.i. with anti-CD3 in the presence of IL-12, IL-27, or IL-23. The addition of IL-12 resulted in an ∼60% decrease in IL-17 production as determined by ELISA (Fig. 2A). Surprisingly, IL-27 had no apparent effect on IL-17 production by primed T cells recovered from T. cruzi-infected mice. This is in contrast to the effects of IL-27 on naive T cells in vitro, as IL-27 was capable of suppressing TGF-β plus IL-6–mediated Th17 differentiation (Fig. 2B). Whereas IL-12 exhibited suppressive effects on the ability of T cells to produce IL-17, IL-23 enhanced IL-17 production by ∼5-fold (Fig. 2A). These results demonstrate that T cells primed during T. cruzi infection are responsive to both IL-12 and IL-23 and that these cytokines have the ability to differentially regulate IL-17 production.
Increased development of Th17 responses to T. cruzi infection in the absence of IL-12p35

Because IL-12 had a potent inhibitory effect on IL-17 production by primed T cells obtained from T. cruzi-infected mice, we next sought to determine the role of IL-12 in regulating the development of Th17 responses to T. cruzi infection in vivo. To do this, C57BL/6, IL-12p35−/− deficient (IL-12p35−/−), IL-12p40−/− deficient (IL-12p40−/−), and Tbx21−/− mice were infected with T. cruzi. On day 9 p.i., infected mice were euthanized, and T cell responses were analyzed by culturing splenocytes with T. cruzi lysate. T cells from infected C57BL/6 mice had a Th1/IFN-γ response that predominated over that of IL-17 (Fig. 3A). As expected, infection of IL-12−/− deficient mice with T. cruzi led to impaired Th1 development (14, 1). Despite the lack of IFN-γ, we observed a significant increase in T. cruzi-specific IL-17 production (Fig. 3A). The increase in IL-17 in infected IL-12p35−/− mice was not apparent in IL-12p40−/− mice, however, which lack both IL-12 and IL-23 (p40+p19 subunits). This supports the notion that Th17 development and IL-17 production in IL-12p35−/− mice infected with T. cruzi is mediated by IL-23. To evaluate the consequences of increased Th17 development and IL-17 production in IL-12p35−/− mice infected with T. cruzi, peripheral blood was analyzed for Th17-associated inflammation. On day 9 p.i., blood was collected, and cells were stained for the neutrophil marker Gr-1 (15). We observed a significant increase in the percentage of Gr-1+ cells in the blood of infected IL-12p35−/− mice compared with infected wild-type mice (Fig. 3B, 3C). These results demonstrate that IL-12 regulates the development of T. cruzi-specific Th17 responses to T. cruzi infection in vivo.

Increased Th17 responses in IL-12p35−/− mice are associated with a significant reduction in the expression and activity of T-bet

We previously demonstrated that T cell-specific expression of T-bet is critical for regulating Th17 responses to T. cruzi (8) (Fig. 1B, 1C). Because IL-12p35−/− mice developed a significant Th17 response to T. cruzi, we next wanted to determine how IL-12 deficiency correlated with reduced levels of T-bet expression in CD4+ T cells recovered from infected mice. Thus, CD4+ T cells were analyzed by intracellular FACS. As expected, there was a significant increase in the percentage of CD4+ T cells that expressed T-bet in C57BL/6 mice infected with T. cruzi.

FIGURE 2. Differential effects of IL-12 family cytokines IL-12, IL-23, and IL-27 on IL-17 production by T cells from wild-type mice infected with T. cruzi. A) Splenocytes from wild-type mice infected with T. cruzi were harvested on day 9 p.i. Cells were stimulated with anti-CD3 Ab and cultured in the presence of rIL-12, rIL-27, or rIL-23. Culture supernatants were collected after 72 h and tested for IL-17 by ELISA. B) Naive CD4+ T cells were cultured under Th17-skewing conditions (IL-6, TGF-β, and anti–IFN-γ) with or without IL-27. Data are shown as a percentage of IL-17 production with anti-CD3 stimulation only. Results are representative of at least three independent experiments consisting of two mice per group and expressed as means ± SD.
cruzi compared with naive uninfected control mice (Fig. 3E). In stark contrast, the percentage of CD4+ T cells from infected IL-12p35−/− mice that expressed T-bet was significantly lower than in infected wild-type mice (Fig. 3E). Additionally, T-bet–dependent CXCR3 activity was also significantly reduced in IL-12p35−/− CD4+ T cells compared with wild-type CD4+ T cells (Fig. 3F). Therefore, under conditions of IL-12 deficiency, CD4+ T cells from T. cruzi–infected mice have reduced levels of T-bet expression and activity, and this reduction is associated with a significant increase in Th17 responses.

IL-27 is dispensable for regulating Th17 responses to T. cruzi infection

As shown in Fig. 2A, we observed that IL-27 had no observable effect on IL-17 production by primed/activated T cells ex vivo. However, we could not exclude the possibility that the effects of IL-27 were limited to the initial priming/differentiation of T cells. For example, IL-27 has been shown to regulate Th17 responses during Toxoplasma gondii infection (16). To determine the importance of IL-27 in regulating Th17 development during T. cruzi infection, we infected Ebi-3−/− mice with T. cruzi. Ebi-3, together with the subunit p28, forms the heterodimeric cytokine IL-27 (17). On day 9 p.i., splenocytes from C57BL/6, Ebi-3−/−, and Tbx21−/− mice were cultured ex vivo with T. cruzi lysate and cytokine responses analyzed by ELISA. Contrary to our predictions, Ebi-3−/− mice did not exhibit any significant increase in T. cruzi–specific IL-17 production (Fig. 4A). Because of this unexpected result, we considered the possibility that restimulation with T. cruzi lysate masked any potential inhibitory effects of IL-27. Thus, we performed restimulation assays using anti-CD3. However, even with anti-CD3 restimulation, IL-27 failed to show any significant inhibitory effect on IL-17 production (Fig. 4A). Consistent with these data, infected Ebi-3−/− mice also did not show any signs of increased neutrophilia compared with wild-type mice (Fig. 4B, 4C), further confirming the lack of any significant increase in Th17 response/inflammation. Finally, as an additional measure for detection of IL-17–producing T cells, we performed intracellular FACS analysis of T cells recovered from infected Ebi-3−/− mice. However, we again failed to detect any increase in IL-17–producing T cells in infected Ebi-3−/− mice.

**FIGURE 3.** Increased development of Th17 responses to T. cruzi infection in the absence of IL-12p35. (A) Splenocytes from C57BL/6, IL-12p35−/−, IL-12p40−/−, and Tbx21−/− mice infected with T. cruzi were harvested on day 9 p.i. Cells were restimulated with T. cruzi lysate. Culture supernatants were collected after 72 h and tested for IL-17 and IFN-γ by ELISA. (B) Neutrophilia was determined by analyzing peripheral blood leukocytes on day 9 p.i. for Gr-1 expression by flow cytometry. Histograms from one representative experiment are shown. (C) The average percent of splenocytes that are Gr-1−. (D) The average percent of spleen cells that are Gr-1−. (E) Splenocytes from naive uninfected mice or T. cruzi–infected C57BL/6 and IL-12p35−/− mice were analyzed by FACS on day 9 p.i. for T-bet expression by gating on CD4+ T cells. Percentage of CD4+ T cells expressing T-bet are shown in the bar graph (right panel). (F) T-bet activity was determined by analyzing splenocytes for CXCR3 expression. Percentage of CD4+ T cells expressing CXCR3 is shown in the bar graph (right panel). Histograms are representative of one individual experiment. Results are representative of at least five independent experiments consisting of two mice per group and expressed as means ± SD.
IL-27 is dispensable for regulating Th17 responses to T. cruzi infection. (A) Splenocytes from C57BL/6, Ebi-3−/−, and Tbx21−/− mice infected with T. cruzi were harvested on day 9 p.i. Cells were restimulated with T. cruzi lysate or anti-CD3. Culture supernatants were collected after 72 h and tested for IL-17 and IFN-γ by ELISA. (B) Neutrophilia was determined by analyzing peripheral blood leukocytes on day 9 p.i. for Gr-1 expression by flow cytometry. Histograms from one representative experiment are shown. (C) The average percent of PBL that are Gr-1−. (D) The average percent of spleen cells that are Gr-1−. (E) Splenocytes from infected C57BL/6 and Ebi-3−/− mice were restimulated on day 9 p.i. with anti-CD3 and stained for intracellular IL-17 and IFN-γ. Cells are gated on CD4+ events. Results are representative of three independent experiments consisting of two mice per group and expressed as means ± SD.

FIGURE 4.

Normal induction of T-bet and T-bet–dependent CXCR3 expression in IL-27–deficient mice infected with T. cruzi

Because IL-27 had no apparent role in regulating Th17 responses to T. cruzi, we hypothesized that in Ebi-3−/− mice, other cytokines (i.e., IL-12) were sufficient for induction of T-bet and thus T-bet–dependent control of Th17 responses to T. cruzi. To test this assertion, T-bet expression in CD4+ T cells recovered from Ebi-3−/− mice was analyzed at day 9 p.i. Indeed, there was a significant increase in the percentage of CD4+ T cells that expressed T-bet in both C57BL/6 and Ebi-3−/− mice infected with T. cruzi (Fig. 5A). Furthermore, T-bet–dependent induction of CXCR3 was normal in CD4+ T cells obtained from infected Ebi-3−/− mice (Fig. 5B). These results demonstrate that during T. cruzi infection, IL-27 is dispensable for induction of T-bet, T-bet–dependent induction of CXCR3, and regulation of Th17 responses.

Relative stability and plasticity of IL-17–producing T cells from T. cruzi–infected mice

The propensity of Th17 cells to undergo a conversion in their cytokine expression profile has been documented. For example, transfer of in vitro–polarized Th17 cells into lymphopenic recipients results in their conversion into mainly Th1 cells (19). Furthermore, Th17 cells generated in vivo could be converted into Th1/Th17 cells ex vivo in a manner that was dependent upon both IL-12 and IFN-γ (20). To apply these previous findings to our model of T. cruzi infection, we wanted to address the relative plasticity of Th17 cells generated in vivo in response to T. cruzi infection. Thus, splenocytes were harvested from T. cruzi–infected IL-12p35−/− mice (day 9 p.i.) and subsequently restimulated in the presence of various cytokines ex vivo. The addition of IL-12 led to a significant reduction in IL-17 production (Fig. 6A), as well as an increase in IFN-γ (not shown). To determine if IL-γ was necessary for IL-12–dependent inhibition of IL-17 production by T cells from T. cruzi–infected mice, we neutralized IFN-γ during restimulation. Surprisingly, and in contrast to the aforementioned study (20), neutralization of IFN-γ had no effect on the ability of IL-12 to inhibit IL-17 (Fig. 6A), demonstrating that the effects of IL-12 are independent of IFN-γ. To confirm these data using Ag-specific stimulation, splenocytes from infected IL-12p35−/− mice were restimulated with T. cruzi lysate. Similar to results obtained with polyclonal stimulation, IL-12 suppressed T. cruzi–specific IL-17 production in an IFN-γ–independent manner (Fig. 6B). Similarly, to determine if IL-27 could compensate for IL-12 deficiency and promote the conversion of Th17 cells, T cells from infected IL-12p35−/− mice were cultured as above in the presence of IL-27. We observed that addition of IL-27 had no effect on the cytokine-producing potential of T cells stimulated with either anti-CD3 or T. cruzi lysate (Fig. 6). Despite the lack of any noticeable effect of IL-27 on IL-17, we reasoned that IL-27 may influence IL-12–dependent inhibition of IL-17 by synergizing with IL-12 to suppress the production of IL-17. For example, IL-27 has been shown to enhance the responsiveness of T cells to IL-12 by causing an increase in the expression of IL-12R β2 (21). However, when T cells were cultured in the presence of both IL-12 and IL-
there was no further increase in IL-17 suppression (Fig. 6). Thus, despite our prediction of a hierarchy and potential synergy in the abilities of IL-12 family cytokines to regulate Th17 responses to *T. cruzi*, we report that IL-12, not IL-27, is critical for regulating their development and subsequent conversion from Th17 cells into Th1 cells.

**Discussion**

IL-17 has been shown to play an important role in acute resistance to *T. cruzi*. In contrast, chronic IL-17 production is associated with increased morbidity and mortality from *T. cruzi* infection (7), as well as autoimmune diseases (6). These studies suggest that proper regulation of Th17 responses is required for successful control of *T. cruzi* infection and preventing immunopathology. We previously reported that the T-box transcription factor T-bet was critical for regulating proinflammatory Th17 responses to *T. cruzi* (8). Despite an otherwise normal Th1 response, T-bet-deficient mice developed increased numbers of Th17 cells, chronic neutrophilia, and increased morbidity and mortality after *T. cruzi* infection (8, 22). Surprisingly, IFN-γ was dispensable for this regulation, as T cells from T-bet-deficient mice produced IFN-γ, and neutralization of IFN-γ had no additional effect on IL-17 production. Consistent with these data, T cells from IFN-γ²/² mice expressed normal levels of T-bet as well as T-bet–dependent activity (i.e., CXCR3 expression).

To conclusively show the importance of T-bet in regulating *T. cruzi*-specific Th17 cells, we demonstrate that ectopic expression of T-bet was necessary and sufficient to inhibit IL-17 expression in T cells recovered from *Tbx21*²/² mice infected with *T. cruzi*, as there was a dramatic decrease in the number of IL-17⁺ T cells with a concomitant increase in IFN-γ⁺ T cells. When we examined the relative number of IL-17⁺IFN-γ⁺ double-positive T cells, there was a slight decrease in this population, but no statistically significant difference was seen between empty and T-bet RV. However, because any T cell undergoing a conversion in its cytokine-producing phenotype could pass through this intermediate stage, this result was not unexpected. Nevertheless, it remains possible that some of these cells are resistant to the effects of ectopic T-bet. Experiments are in progress to address this possibility. There was

27, there was no further increase in IL-17 suppression (Fig. 6). Thus, despite our prediction of a hierarchy and potential synergy in the abilities of IL-12 family cytokines to regulate Th17 responses to *T. cruzi*, we report that IL-12, not IL-27, is critical for regulating their development and subsequent conversion from Th17 cells into Th1 cells.
also the possibility of cytokine-dependent cross-regulation of Th17 cells, and we reasoned that cross-inhibition would be via IFN-γ-mediated inhibition of the Th17 cells. However, anti–IFN-γ neutralizing Ab was used in all RV experiments, and despite the absence of IFN-γ, T-bet RV was still able to reverse the phenotype. These data are consistent with our previous report showing that T. cruzi infection of IFN-γ–deficient mice does not lead to significant Th17 development. It is also supported by our previous findings (8, 9). These data, obtained with Th17 cells primed in vivo in response to a parasitic infection, are in agreement with the reported effects of T-bet on precommitted, in vitro-differentiated Th17 cells (23). Because T-bet (as well as Stat-1) is the critical T cell-intrinsic factor that regulates T. cruzi-specific Th17 responses (8, 9), next sought to identify the T cell-extrinsic factors (e.g., IL-12 family cytokines) that might regulate Th17 responses to T. cruzi. Specifically, our goal was to establish a hierarchy between IL-12 (p35+p40) and IL-27 (Ebi-3+) in their abilities to inhibit Th17 responses to T. cruzi. We observed that IL-12p35 deficiency led to a significant increase in T. cruzi-specific Th17 responses, as well as Th17-associated inflammation (i.e., peripheral blood neutrophilia). As expected, the increase in Th17 cells was paralleled by a significant decrease in T-bet expression and T-bet–dependent activity (CXCR3 expression). In terms of the Th17 response that develops in infected IL-12p35−/− mice, we suspect that IL-23 (p35+p19) is important because infection of IL-12p40–deficient mice with T. cruzi resulted in a significant reduction in IL-17 production as well as peripheral blood neutrophilia. In sharp contrast to the regulatory effects of IL-12, and contrary to our own predictions based on previous studies, IL-27 did not affect IL-17 production by primed T cells obtained from T. cruzi–infected mice, nor did IL-27 (Ebi-3) deficiency have any detectable effect on the development of Th17 responses to T. cruzi. This was surprising, as IL-27 was potent at inhibiting Th17 differentiation mediated by TGF-β plus IL-6 in vitro. However, it should be noted that in these experiments, Th17 cells were the product of two different pathways. In contrast to TGF-β plus IL-6 differentiation in vitro, Th17 cells generated in vivo were a consequence of T. cruzi infection, in which there is an assortment of proinflammatory cytokines produced and the conditions not so well defined. A second possibility for the lack of an assortment of proinflammatory cytokines produced and the in vivo were a consequence of extrinsic factors (e.g., IL-12 family cytokines) that might regulate Th17 responses in Th17 cells generated in vivo. Regardless of whether T cells were restimulated with T. cruzi lysate or with anti-CD3, we failed to detect any effect of IL-27 on IL-17 responses from infected mice. These data, obtained using our model of T. cruzi infection, are in contrast to previously published studies. For example, infection of IL-27−/− mice with Leishmania major or T. gondii resulted in increased IL-17 production (16, 24). In the case of Leishmania infection, there was a concomitant decrease in Th1 development. The defect in Th1 development was not apparent, however, when Ebi-3−/− mice were infected with Leishmania. Ebi-3−/− mice infected with Listeria also produced more IL-17, but again there was no effect on Th1 development (25). Finally, immunization of Ebi-3−/− mice led to increased IL-17 without any effect on Th1 cells (26). Despite the discrepant effects of IL-27/IL-27R deficiency on Th1 development in the aforementioned studies, as well as the general increase in IL-17, increased IL-17 production was not always associated with increased neutrophilia (24). Although we do not know the reasons for these discrepancies, we suspect that differences in the pathogen used and methods used to evaluate recall responses of T cells may be contributing factors. For example, introduction of Ag via immunization or infection may affect the magnitude of IL-17 production in Ebi-3−/− mice. These effects may be attributed to differences in pathogen-specific induction of IL-12 and/or induction of the IL-12 receptor, which could supersede the requirement for IL-27 to control/limit Th17 responses to T. cruzi. As an example, experiments performed using Ebi-3−/− mice on the BALB/c background (which is more susceptible to T. cruzi and produces more IL-4 than IL-12) suggest higher IL-17 production. Of note, however, despite no detectable effect on T. cruzi–specific T cell cytokine production, a significant increase in IFN-γ was observed in sera of infected Ebi-3−/− mice (not shown), consistent with a previous report for IL-27R−/− mice infected with T. cruzi (18). These data suggest that IL-27 may regulate IFN-γ production by non-T cells.

In contrast to the regulatory effects of IL-12, we wondered if the lack of a detectable increase in IL-17 production in IL-27−/− mice was related to IL-10 production. For example, it has been shown that T cells from IL-27R–deficient mice produce less IL-10 and more IL-17 (24). In contrast, infection of Ebi-3−/− mice with L. major revealed that T cell production of IL-10 was actually increased (27). Thus, it was possible that in Ebi-3−/− mice infected with T. cruzi, increased IL-10 prevented aberrant Th17 responses. However, we observed that T cells from Ebi-3−/− mice infected with T. cruzi produced much less IL-10 than their wild-type counterparts (data not shown), and this was observed with T. cruzi–specific stimulation as well as polyclonal activation. Thus, if IL-10 has a regulatory role in IL-17 production, it does not appear to do so in our T. cruzi infection model because the decrease in IL-10 did not result in any increase in IL-17.

Our results show that depending on the local production of IL-12 family cytokines by APC, there is a dramatic effect on the development of T. cruzi-specific T cell responses and the associated inflammation. However, we reasoned that T cells maintain their responsiveness to these cytokines and hence may be subjected to their influence at later times during the course of infection, which would be an important regulatory mechanism to prevent the chronic effects of IL-17. Hence, we wanted to determine if Th17 cells generated during the course of T. cruzi infection demonstrated plasticity and were amenable to the effects of IL-12 and IL-27. We also wanted to test the hierarchical importance of IL-12 and IL-27, including the possibility that IL-27 might compensate for IL-12 deficiency in promoting terminal Th1 differentiation. We report that exogenous IL-12 treatment inhibited IL-17 production by Th17 cells recovered from infected IL-12p35−/− mice, and this effect was observed using both polyclonal as well as Ag-specific restimulation. Inhibition of IL-17 was paralleled by an increase in IFN-γ production. One of the most surprising results was that the ability of IL-12 to inhibit IL-17 was independent of IFN-γ, as neutralization of IFN-γ had no effect. These data support our previous findings that IFN-γ is mostly dispensable for regulation of T. cruzi–specific Th17 responses (9) and are in contrast to a recent report showing that both IL-12 and IFN-γ were required for converting Th17 cells into Th1 cells (20). Finally, and consistent with the results obtained with Ebi-3−/− mice, addition of rIL-27 to cultures from IL-12p35−/− mice had little effect on T. cruzi–specific IL-17 production. Thus, we conclude that IL-12 is not only important for promoting Th1 responses to T. cruzi, but is also critical for regulating Th17 responses to T. cruzi infection in an
IFN-γ-independent manner. Finally, and in contrast to the effects of IL-27 reported for other disease models, we report that IL-27 does not play any significant role in regulating Th17 responses to *T. cruzi*. These data shed new light on the regulation of proinflammatory T cell subsets during the course of *T. cruzi* infection, which is an important aspect of immunity to *T. cruzi* and necessary for preventing the chronic effects of IL-17 on the host.

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Disclosures

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