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IL-17 Is Necessary for Host Protection against Acute-Phase *Trypanosoma cruzi* Infection

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IL-17A is a key cytokine that induces inflammatory responses through the organized production of inflammatory cytokines, such as IL-6, TNF-α, and GM-CSF, and induces neutrophil migration. The roles of IL-17A in infection of intracellular protozoan parasites have not been elucidated, although augmented immune responses by IL-17A are important for the resolution of some bacterial and fungal infections. Therefore, we experimentally infected IL-17A–deficient (*IL-17A*$^{-/-}$) mice with *Trypanosoma cruzi*. *IL-17A*$^{-/-}$ mice had a lower survival rate and prolonged worse parasitemia compared with control C57BL/6 wild-type (WT) mice postinfection. In the infected *IL-17A*$^{-/-}$ mice, multiple organ failure was observed compared with WT mice, as reflected by the marked increase in serologic markers of tissue injury, such as aspartate aminotransferase, which resulted in increased mortality of *IL-17A*$^{-/-}$ mice. Expression of cytokines, such as IFN-γ, IL-6, and TNF-α, was lower in liver-infiltrating cells from the *IL-17A*$^{-/-}$ mice compared with WT mice. A similar defect was observed in the expression of neutrophil enzymes, such as myeloperoxidase and lipoxygenase, whereas cellular infiltration into the infected tissues was not affected by IL-17A deficiency. These results suggested that the efficient activation of immune-related cells critical for the killing of *T. cruzi* was impaired in the absence of IL-17A, resulting in the greater susceptibility of those mice to *T. cruzi* infection. From these results, we conclude that IL-17A is important for the resolution of *T. cruzi* infection. *The Journal of Immunology*, 2010, 185: 1150–1157.

Interleukin-17A is a proinflammatory cytokine, largely produced by activated T lymphocytes, and was originally called CTL-associated Ag-8 (1). The mouse IL-17 family consists of six members, including IL-17A (called IL-17), IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25), and IL-17F, all of which share 16–45% homology with IL-17A (2). Among them, IL-17A and IL-17F are mainly produced by activated memory CD4$^+$ T cells, which are now classified as Th17 cells (3, 4). However, the production of IL-17A and IL-17F is not limited to Th17 cells; CD8$^+$ T cells, γδT cells, NK cells, and neutrophils have also been identified as sources of these cytokines (5–7). The receptor for IL-17A is expressed in various tissues, such as lung, kidney, liver, and spleen, and by various types of cells, including fibroblasts, epithelial cells, endothelial cells, monocytes/macrophages, lymphocytes, and marrow stromal cells. These cells produce diverse proinflammatory cytokines and chemokines in response to IL-17A stimulation (3). Yao et al. (8) reported IL-17A to be a potent inducer of IL-6 and IL-8 (CXCL8) by human fibroblasts. In subsequent experiments, it was shown that IL-17A could stimulate the expression of CSFs (G-CSF and GM-CSF), chemokines (CXCL1, CXCL10, CCL2, CCL7, and CCL20), and matrix metalloproteinase-3 and -13. These cytokines and chemokines augment local inflammations by inducing the recruitment of neutrophils and leukocytes. IL-17A also induces the production of IL-1β and TNF-α from macrophages (9), and these cytokines cooperate with each other to induce the production of IL-6 and chemokines (10, 11) and to augment inflammatory reactions (12).

Trypanosoma cruzi, an intracellular protozoan parasite, is the etiologic agent of American trypanosomiasis or Chagas' disease and affects ~16–18 million people in Central and South America (13). Innate and acquired cell-mediated immune responses are induced after experimentally induced acute *T. cruzi* infection, and combined mobilization of NK cells, CD4$^+$ T cells, CD8$^+$ T cells, γδT cells, and Ab-producing B cells are required for establishing host resistance (14, 15). Production of IL-12 by macrophages is triggered by the invasion of blood trypomastigotes of *T. cruzi* early postinfection, and IL-12 induces Th1 differentiation and subsequent IFN-γ production (16, 17). IFN-γ is a critical cytokine in host resistance to *T. cruzi* infection (18, 19); it is produced by NK cells at the early phase of infection and by CD4$^+$ and CD8$^+$ T cells later during the infection (20, 21). IFN-γ, synergistically with TNF-α, induces NO synthesis by macrophages, a critical mediator for killing of causal organisms during the acute phase of infection (22, 23). Furthermore, it is known that *T. cruzi* infection stimulates production of proinflammatory cytokines, such as IL-1α/β, IL-6, and TNF-α (24, 25). Infection-induced inflammatory reactions mediated by these cytokines against *T. cruzi* result in effective expulsion of the

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Abbreviations used in this paper: 5-LOX, 5-lipoxygenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CK, creatine kinase; MLN, mesenteric lymph node; MNC, mononuclear cell; MPO, myeloperoxidase; ROS, reactive oxygen species; STA, soluble *Trypanosoma. cruzi* Ag; WT, wild-type.

parasites, although immunopathology from excessive inflammation also has a significant impact on the pathogenesis of experimental Chagas' disease (26, 27).

The roles of IL-17A in host defense against intracellular protozoan parasites remain to be fully elucidated, although IL-17A has a role in the host's protection against fungal and bacterial infection (28–31). Therefore, we investigated the role of IL-17A in *T. cruzi* infection. Infection by *T. cruzi* led to increased IL-17A production by $CD4^+$ T, $CD8^+$ T, NKT, and γδT lymphocytes. IL-17A–deficient (*IL-17A*$^{-/-}$) mice infected with *T. cruzi* exhibited more severe parasitemia and mortality accompanied by the attenuated production of antiparasitic cytokines, including IFN-γ, IL-6, and TNF-α, compared with wild-type (WT) mice. These results clearly indicated that IL-17A is necessary for the host's protection during acute-phase *T. cruzi* infection.

Materials and Methods

Animals

The generation of *IL-17A*$^{-/-}$ mice on the C57BL/6 background was described previously (32). Briefly, a targeting vector for deleting exons 1 and 2 of the *IL-17A* gene was electroporated into ES (E14.1) cells and selected in the presence of G418. Targeted clones, screened by Southern blot-hybridization analysis, were treated with adenovirus carrying the *cre* gene to delete the *neo* gene. Chimera mice were generated by the aggregation method, using C57BL/6 blastocysts as the recipients, and were mated with C57BL/6 female mice for germline transmission. Mutant mice were backcrossed onto the C57BL/6 background more than eight times (continual backcross) before use in experiments. Mice were housed in microisolator cages and were used between 8 and 12 wk of age. Age- and sex-matched WT C57BL/6 mice (Kyudo, Saga, Japan) were used as controls. All experiments were approved by the institutional animal research committee of Saga University and conformed to the animal care guidelines of the American Physiological Society.

Parasites

T. cruzi (Tulahuén strain) was maintained in vivo in *IFN-γR*$^{-/-}$ mice by every 2 wk passages. For the experiments, *IL-17A*$^{-/-}$ and WT mice were injected i.p. with the plasma containing trypomastigotes. Mice were infected with 2000 trypomastigotes. The number of parasites in the blood was counted for each animal using 4 μl venous blood. For measuring parasitism, DNA was purified from 50 mg tissue specimen with a Maxwell 16 Tissue DNA Purification Kit (Promega, Madison, WI). The content of infected parasites was measured with a PCR using *T. cruzi* 195-bp repeat DNA-specific primers TCZ-F: 5′-GCT CTT GCC CAC AAG GGT GC-3′ and TCZ-R: 5′-CCA AGC AGC GGA TAG TTC AGG-3′ (33). The PCR reaction was performed using SYBR Premix Ex Taq II (Perfect Real Time) (Takara, Shinga, Japan), according to the manufacturer's instructions (denaturation at 95°C for 30 s, followed by 40 cycles of the two-step amplification phase: 95°C for 5 s and then 60°C for 15-s hold). At the same time, we measured the level of murine *β2-microglobulin* gene as an internal control for DNA input using primers Beta2-F: 5′-TGG GAA GCC GAA CAT ACT G-3′ and Beta2-R: 5′-GCA GGC GTA TGT ATC AGT CTC A-3′.

Assay for serum chemistry

Blood was collected at indicated days postinfection, and serum was prepared by centrifugal fractionation. Serum alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, creatinine, and creatine kinase were determined using Test WAKO kit (Wako Pure Chemical Industries, Osaka, Japan), according to the manufacturer's directions.

Flow cytometric analysis

Prepared cells were stained with FITC-labeled anti-CD4 mAb, PE-labeled anti-CD62L mAb, and PE-Cy5–labeled anti-CD44 mAb (all from BD Biosciences, San Jose, CA) for the detection of effector $CD4^+$ T cells ($CD4^+/CD44^{high}/CD62L^{low}$) and PerCP-Cy5.5–labeled anti-CD69 mAb (BD Biosciences) for the detection of activated $CD4^+$ T cells ($CD4^+/CD69^{high}$). The following Abs against surface markers (BD Biosciences) were used to stain cells in the tissue: PE-labeled anti-CD4, FITC-labeled anti-CD8, PE-labeled anti-NK1.1, FITC-labeled anti-CD3, PE-labeled anti–Mac-1, FITC-labeled anti-B220, PE-labeled anti-Ly6G (1A8), FITC-labeled anti-neutrophils (7/4), and FITC-labeled anti–Gr-1 mAb. For the analysis of intracellular cytokines, the isolated cells (1×10^6) were cultured for 4 h with 10 ng/ml PMA (Sigma-Aldrich, St. Louis, MO) plus 500 ng/ml ionomycin (Sigma-Aldrich) in the presence of 2 μM monensin (Sigma-Aldrich) plus GolgiPlug (BD Biosciences). Cells were stained with FITC- or PE-labeled anti-CD4 mAb, FITC-labeled anti-CD8, NK1.1, γδ TCR, or Gr-1 mAb and PerCP or allophycocyanin-labeled anti-CD3 mAb (BD Biosciences), fixed, and permeabilized with the Cytofix/Cytoperm Plus kit (BD Biosciences), according to the manufacturer's directions. Cells were then stained with FITC-labeled anti–IFN-γ mAb or PE-labeled anti–IL-17 mAb (BD Biosciences). The expression of surface markers and cytokines was analyzed using FACSCalibur (BD Biosciences).

Histological analysis

Tissues were removed and fixed with 10% formaldehyde neutral buffer solution (Nacalai, Kyoto, Japan) at the indicated days after infection. Then, specimens were embedded in paraffin and stained with H&E, and structural changes and cellular infiltrations were evaluated with a light microscope.

In vitro assessment of IL-17A and Th17 function on macrophage and neutrophil activation

Spleen $CD4^+$ cells were isolated from WT or *IL-17A*$^{-/-}$ mice at 14 d after *T. cruzi* infection by MACS using anti-CD4 mAb. In contrast, peritoneal macrophages were collected from uninfected WT mice treated with 3% thioglycolate (Difco, Detroit, MI) for 3 d and seeded in 48-well culture plates at 5×10^5 cells/well. These macrophages were infected with 2.5×10^4 *T. cruzi* trypomastigotes and cocultured with the isolated WT or *IL-17A*$^{-/-}$ $CD4^+$ cells for 18 h. After the culture, cytokines and nitrite (NO) levels in culture supernatant were measured with the DuoSet ELISA Development System (R&D Systems, Minneapolis, MN) and the Griess Reagent Kit for Nitrite Determination (Molecular Probes, Eugene, OR), respectively. In another experiment, the thioglycolate-induced peritoneal exudate macrophages (5×10^5 cells/well) were treated with 25 ng/ml recombinant murine IL-17A for 18 h, and NO secretion was measured. NO production was also evaluated after stimulation with 100 ng/ml LPS, 25 ng/ml IFN-γ, or 1×10^6 soluble *T. cruzi* Ag (STA), with or without 25 ng/ml IL-17A. STA was prepared by repeated freeze and thaw of 1×10^8 trypomastigotes in 100 μl culture medium. For the preparation of neutrophils, peritoneal exudate cells were collected at 4–16 h after 3% thioglycolate injection. The cell suspension was put on 60% Percoll (GE Healthcare, Uppsala, Sweden) and centrifuged at $1000 \times g$ for 30 min. The cell pellet, containing >80% neutrophils ($7\text{-}4^+Ly6G^+$ cells), was resuspended with culture medium and used in in vitro experiments as follows. The prepared neutrophils (5×10^5 cells) were treated with indicated stimuli, with or without recombinant mouse IL-17A (25 ng/ml) for 2 h. These cells were washed with PBS two times and then resuspended in PBS containing 10 μM 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate (Invitrogen, Carlsbad, CA), a detection reagent for reactive oxygen species (ROS). After incubation for 30 min at 37° C in the dark, levels of ROS production were evaluated by flow cytometry. Induction of mRNA expression of myeloperoxidase (MPO) and 5-lipoxygenase (5-LOX) in the stimulated neutrophils for 16 h was analyzed by RT-PCR, as described below.

Isolation and culture of cells

Splenocytes, mesenteric lymph node (MLN) cells, and liver mononuclear cells (MNCs) were isolated from *IL-17A*$^{-/-}$ and WT mice at the indicated days after *T. cruzi* infection. Single-cell suspensions were prepared with a culture medium (RPMI 1640, Sigma-Aldrich) supplemented with 10% FBS (ThermoTrace, Melbourne, Australia) and penicillin/streptomycin (Invitrogen). Prepared cells (5×10^5 cells/well) were cultured in 96-well culture plates (Nunc, Roskilde, Denmark) for 3 d, and the culture supernatants were analyzed for cytokine production with ELISA, according to the manufacturer's directions (DuoSet ELISA Development System, R&D Systems).

Quantitative real-time PCR analysis

Total RNAs were extracted from cells using TRIzol reagent (Invitrogen) and reverse-transcribed with a ReverTra Plus kit (TOYOBO, Osaka, Japan). Expression levels of cytokines and neutrophilic enzymes were determined relative to that of β-actin with HotStar Taq DNA polymerase (Qiagen, Valencia, CA), supplemented with SYBR Green (Molecular Probes), using an ABI PRISM 7000 sequence-detection system, according to manufacturer's instructions. The sequence of PCR primers were as follows: IFN-γ, 5′-TCA AGT GGC ATA GAT GTG GAA GAA-3′ and 5′-TGG CTC TGC AGG ATT TTC ATG-3′; IL-4, 5′-ACA GGA GAA GGG ACG CCA T-3′ and 5′-GAA GCC CTA CAG ACG AGC TCA-3′; TNF-α, 5′-CAT CTT CTC AAA ATT CGA GTG ACA A-3′ and 5′-TGG GAG TAG ACA AGG

TAC AAC CC-3′; IL-6, 5′-GAG GAT ACC ACT CCC AAC AGA CC-3′ and 5′-AAG TGC ATC ATC GTT GTT CAT ACA-3′; MPO, 5′-ATC ACG GCC TCC CAG GAT ACA ATG-3′ and 5′-ACC GCC CAT CCA GAT GTC AAT G-3′; and 5-LOX, 5′-TGC CAT CCA GCT CAA CCA AAC-3′ and 5′-GCG ATA CCA AAC ACC TCA GAC ACC-3′.

Data analysis

Experiments were repeated at least two times. Values are expressed as mean ± SEM. Differences among groups were analyzed using unpaired Student *t* tests. A value of $p < 0.05$ was considered statistically significant.

Results

Induction of IL-17A expression by T. cruzi *infection*

First, we assessed IL-17A expression during *T. cruzi* infection. In uninfected mice, percentages of IL-17A–expressing cells were very low (<0.2%) in spleen and MLN. After *T. cruzi* infection, percentages of IL-17A–producing ($CD3^+CD4^+$ and $CD3^+CD4^-$) cells in spleen and MLN were increased; they reached a peak at 21 d after the infection and then decreased to basal levels (Fig. 1*A*). IFN-γ–producing cells also increased after *T. cruzi* infection as similar time-course in the case of IL-17A-producing cells (Fig. 1*A*). In contrast, although IL-17A–expressing cells existed in uninfected liver MNCs (~0.5%), the percentages were relatively constant during *T. cruzi* infection (Fig. 1*A*). Furthermore, $CD8^+$ T, NKT, and γδT cells in spleen, MLN, and liver also produced IL-17A against *T. cruzi* infection at 21 d after the infection (Fig. 1*B*). Therefore, it was demonstrated that *T. cruzi* infection induced production of IL-17A by various cell lineages.

High mortality accompanied by sustained severe parasitemia and aggravated multiple organ failure in IL-17A$^{-/-}$ mice

To clarify the role of IL-17A, we infected *IL-17A*$^{-/-}$ and WT mice with *T. cruzi*. As shown in Fig. 2*A*, the survival rate was markedly decreased in the *IL-17A*$^{-/-}$ mice compared with WT mice 21 d after the infection. In WT mice, expansion of the parasites showed a small peak at ~14 d postinfection but returned to sublethal levels in almost all mice (Fig. 2*B*). In contrast, *IL-17A*$^{-/-}$ mice showed prolonged, more severe parasitemia compared with WT mice up to 28 d after the infection (Fig. 2*B*). At 33 d postinfection, although the parasitemia in *IL-17A*$^{-/-}$ mice seemed similar to that in WT mice (†; Fig. 2*B*), this resulted from the fact that data were obtained only from a few survivors in the *IL-17A*$^{-/-}$ mice group. The survivors maintained the parasitemia at relatively milder levels than did others in the group during the acute phase of the infection, the parasitemia did not worsen again, and the mice

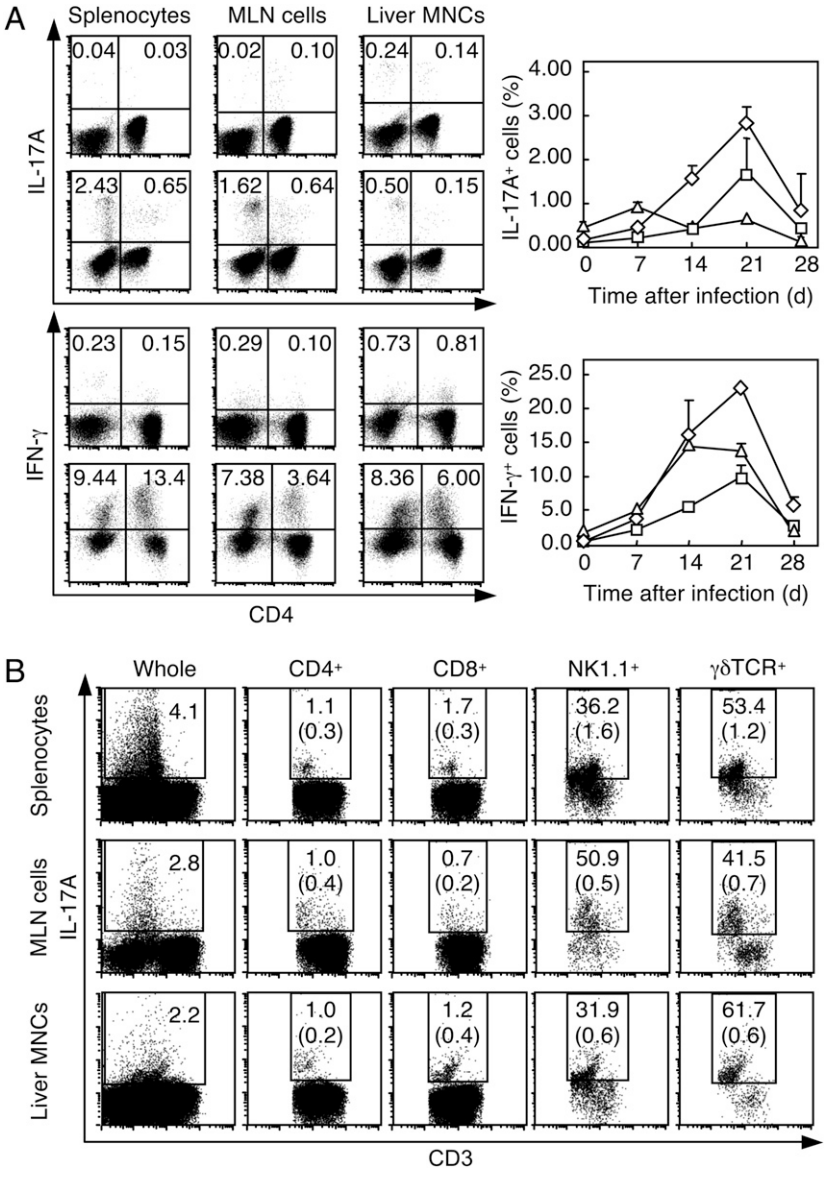

FIGURE 1. Induction of IL-17A from $CD4^+$ and other cell lineages during *T. cruzi* infection. *A*, *left panels*, Splenocytes, MLN cells, and liver MNCs prepared from WT mice on day 0 (*upper panels*) and day 21 (*lower panels*) of infection were stained for surface markers and intracellular IL-17A or IFN-γ and analyzed with FACS, as described in *Materials and Methods*. Numbers shown are the percentages of cells contained in gated $CD3^+$ cells. Experiments were repeated three times with similar results. *Right panels*, Splenocytes (◇), MLN cells (□), and liver MNCs (△) were prepared from WT mice at the indicated days postinfection, and the percentage of cells expressing IL-17A and IFN-γ was assessed. *B*, Splenocytes (*top row*), MLN cells (*middle row*), and liver MNCs (*bottom row*) collected from WT mice on day 21 of infection were stained for lineage surface markers and intracellular IL-17A. Numbers shown in each square are the percentages of the cells contained in each gated lineage; percentages of whole living cells are given in parentheses.

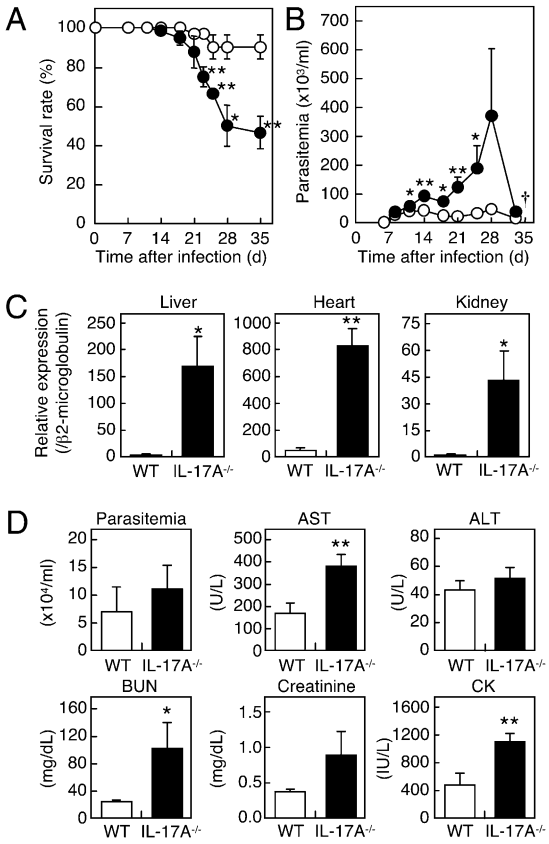

FIGURE 2. Increased mortality accompanied by sustained severe parasitemia and aggravated multiple organ failure in *IL-17A*$^{-/-}$ mice. *A*, WT (○) and *IL-17A*$^{-/-}$ (●) mice were infected i.p. with 2×10^3 blood trypomastigotes of *T. cruzi*. Mortality was monitored at the indicated days postinfection. Data are mean ± SEM from three independent experiments (n = 10–20 each). *B*, Parasitemia was monitored at the indicated days postinfection as in *A*. Data are mean ± SEM (n = 18–20). Experiments were repeated at least three times with similar results. *C*, Seventeen days postinfection, parasitism in liver, heart, and kidney was measured with a *T. cruzi* 195-bp-repeat DNA specific-PCR, as described in *Materials and Methods*. Data are mean ± SEM (n = 4). Experiments were repeated at least two times with similar results. *D*, Serum was prepared from WT and *IL-17A*$^{-/-}$ mice on day 21 of infection and analyzed for parasitemia and serologic marker concentrations, as described in *Materials and Methods*. Experiments were repeated at least four times with similar results. Data are mean ± SEM (n = 4). $*p < 0.05$; $**p < 0.01$ versus WT samples; †, Mean parasitemia in survivors at 33 d postinfection. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CK, creatine kinase.

survived >3 mo postinfection (data not shown). This may indicate that some factors, such as IL-17F, may compensate for IL-17A during the infection. Nonetheless, it was shown that *IL-17A*$^{-/-}$ mice were essentially more sensitive to *T. cruzi* infection than WT mice, and lethal expansion of the parasites occurred in more than half of the infected *IL-17A*$^{-/-}$ mice (Fig. 2*A*, 2*B*). These results indicated that IL-17A plays an important role in the successful resolution of *T. cruzi* infection.

In the acute phase of infection, *T. cruzi* replicates and infects various target organs, which leads to tissue injury. Therefore, we assessed the tissue parasitism and the degree of tissue damage by measuring serum concentrations, including aspartate aminotransferase and alanine aminotransferase for liver injury, blood urea nitrogen and creatinine for renal damage, and creatine kinase for heart failure. As shown in Fig. 2*C*, *IL-17A*$^{-/-}$ mice showed significantly greater parasitism in liver, heart, and kidney compared with WT mice. Furthermore, all markers measured were higher in *IL-17A*$^{-/-}$ mice compared with WT mice (Fig. 2*D*). These data suggested that *T. cruzi* infection leads to severe multiple organ failure resulting from physical damage by the parasites in *IL-17A*$^{-/-}$ mice, which was assumed to be a reason for the increased mortality.

Equivalent cellular infiltration in the liver of WT and IL-17A$^{-/-}$ *mice*

We then explored mechanisms of IL-17A resistance to *T. cruzi* infection. Liver is a secondary infection site of *T. cruzi*, and it is well known that IL-17A induces the recruitment of immune-related cells to infected sites. We initially assumed that the cellular accumulation in the infected liver was impaired in *IL-17A*$^{-/-}$ mice. However, cellular infiltration was unexpectedly equivalent between WT and *IL-17A*$^{-/-}$ mice (Fig. 3*A*). Also, microscopically discernible differences between WT and *IL-17A*$^{-/-}$ mice were not observed in the heart, which is another target organ of the infection (data not shown). As shown in Fig. 3*B*, the ratio of $CD4^+$ and $CD8^+$ T cells, NK and NKT cells, macrophages and B cells, and neutrophils in the infected liver of *IL-17A*$^{-/-}$ mice was similar to that of WT mice 21 d after the infection. Similar results were obtained ~14 d after the infection (data not shown). Total numbers of liver-infiltrated MNCs were also similar in WT and *IL-17A*$^{-/-}$ mice on days 14 and 21 (data not shown). To address whether IL-17A deficiency affected cell migration at very early phases of infection, cellular infiltration into the peritoneal

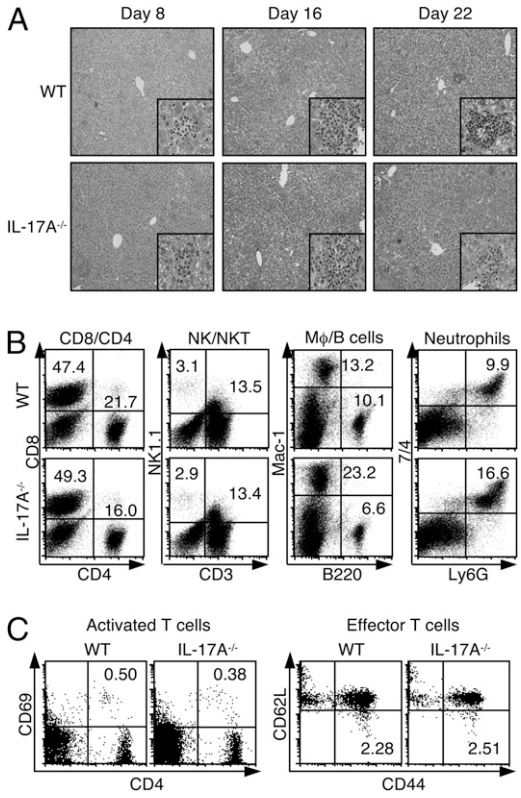

FIGURE 3. Equivalent cellular infiltration in the liver of WT and *IL-17A*$^{-/-}$ mice. *A*, Liver segments from WT and *IL-17A*$^{-/-}$ mice were stained with H&E for histological examination. Original magnification, ×200 (insets ×640). Experiments were repeated two times with similar results. *B* and *C*, Liver MNCs prepared from WT (*B, upper row*) and *IL-17A*$^{-/-}$ (*B, lower row*) mice on day 21 (*B*) or day 16 (*C*) of infection were stained for indicated surface markers and analyzed with FACS, as described in *Materials and Methods*. Numbers shown are the percentages of cells contained in each gated lineage. Experiments were repeated two times.

cavity was examined at 3 d after i.p. injection of *T. cruzi*. Despite our initial assumption, there was no significant difference between WT and *IL-17A*$^{-/-}$ mice with regard to the number of cells infiltrating the peritoneal cavity or the percentage of innate immune cells, such as NK/NKT (CD3$^-$NK1.1$^+$ or CD3$^+$NK1.1$^+$) cells, γδT (CD3$^+$γδTCR$^+$) cells, macrophages (CD11b$^+$Ly6G$^-$), and neutrophils (CD11b^{int}Ly6G$^+$) (Supplemental Fig. 1). Furthermore, expression levels of activation markers, such as CD69, CD44, and CD62L, on CD4$^+$ T cells in the infected liver were similar between WT and *IL-17A*$^{-/-}$ mice on day 16 (Fig. 3*C*).

In vitro assay of capacity of Th17 cells and IL-17A for macrophage and neutrophil activation

Next, we performed an in vitro assay to assess the helper function of IL-17A–producing CD4$^+$ T (Th17) cells to activate infected macrophages directly (Fig. 4*A*). Peritoneal exudate macrophages from thioglycolate-treated WT mice were infected with *T. cruzi* in vitro and then cocultured with spleen CD4$^+$ cells isolated from infected WT or *IL-17A*$^{-/-}$ mice. After 18 h of culture, the production of IL-6, TNF-α, and NO, which are mainly produced by activated macrophages, was enhanced by coculture with both CD4$^+$ cells compared with that in the absence of CD4$^+$ cells. However, there were no significant differences in the quantity of macrophage-derived factors between coculture with WT and *IL-17A*$^{-/-}$ CD4$^+$ cells (Fig. 4*A*). Therefore, a CD4$^+$ Th cell lineage other than Th17 was able to activate macrophages normally, even in the absence of IL-17A production. Furthermore, we examined the direct effect of IL-17A on macrophage activation; however, treatment with IL-17A did not enhance NO production by macrophages, even in the presence of costimulation with LPS and IFN-γ (Fig. 4*B*). In the thioglycolate-induced macrophages, STA could not induce NO production, whereas LPS and IFN-γ had a strong effect.

In addition, rIL-17A could not directly induce activation of neutrophils, as measured by upregulation of ROS production (Fig. 4*C*) and mRNA expression of MPO and 5-LOX (Fig. 4*D*). Interestingly, STA induced ROS production in the neutrophils more strongly than did 100 ng/ml LPS (Fig. 4*C*), and it was assumed that the *T. cruzi* infection immediately activated locally resident neutrophils or those recruited to the infection site. These results suggested that IL-17A produced from Th17 and other lineages in vivo is required for proper defense against *T. cruzi* by mechanisms other than the induction of cellular migration and direct activation of macrophages and neutrophils.

Cytokine production was impaired in IL-17A$^{-/-}$ *mice*

We then addressed the production of inflammatory cytokines, another important mechanism by which IL-17A plays a role during infection. As shown in Fig. 5*A*, cells from spleen, MLN, and liver of WT mice at 14 d postinfection produced substantial levels of IFN-γ and TNF-α in response to *T. cruzi* infection, which then decreased to 1/7 to 1/50 on day 21 (compare Fig. 5*A*, 5*B*), due to successful control of infection. At both time points, cytokine production by *IL-17A*$^{-/-}$ cells was impaired compared with that by WT cells derived from MLN and liver (Fig. 5*A*, 5*B*), demonstrating the influence of IL-17A on the production of inflammatory cytokines during *T. cruzi* infection. Of note, the high susceptibility of *IL-17A*$^{-/-}$ mice to infection was first apparent around day 14 and became more evident on day 21 and thereafter, as shown by greater mortality and more severe parasitemia (Fig. 2*A*, 2*B*). Similar results were obtained for mRNA expression in liver MNCs and MLN cells (i.e., expression of IFN-γ, TNF-α, and IL-6 were lower in *IL-17A*$^{-/-}$ mice than WT mice during *T. cruzi* infection) (Fig. 5*C*, data not shown). IL-4 expression was not affected by IL-17A deficiency. Interestingly, the impaired cytokine production was most evident in MLN cells and liver MNCs compared with splenocytes (Fig. 2*A*, 2*B*). These results indicate the differential requirement of IL-17A for cytokine production; IL-17A is required more in local sites, including draining lymph nodes and liver, rather than systemically, as shown for splenocytes. This might reflect observations

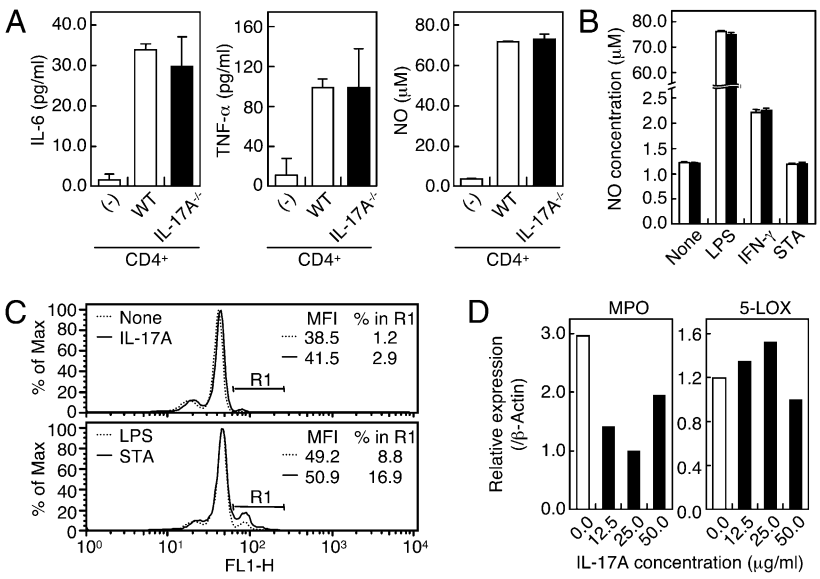

FIGURE 4. In vitro assays on function of Th17 and IL-17A in immune activation. *A*, Thioglycolate-induced macrophages (5×10^5 cells/well) infected with 2.5×10^4 *T. cruzi* trypomastigotes were cocultured for 18 h with spleen CD4$^+$ cells (1×10^5 cells/well) isolated from WT or *IL-17A*$^{-/-}$ mice at 14 d after *T. cruzi* infection. (-), Culture of infected peritoneal exudate macrophages without CD4$^+$ cells. After the culture, cytokines and NO levels in culture supernatant were measured. Data are mean ± SEM (n = 5–7). *B*, Thioglycolate-induced macrophages were treated with indicated stimuli for 18 h with or without 25 ng/ml IL-17A and then measured for NO secretion. Data are mean ± SEM (n = 6). *C*, Thioglycolate-induced neutrophils were incubated with none, 25 ng/ml IL-17A, 100 ng/ml LPS, or 1×10^6 STA. Two hours after the stimulation, ROS production was detected as described in *Materials and Methods*. Experiments were repeated two times with similar results. *D*, Thioglycolate-induced neutrophils were stimulated by the indicated concentrations of IL-17A for 18 h, and the mRNA expression of MPO and 5-LOX was measured. Experiments were repeated at least two times with similar results.

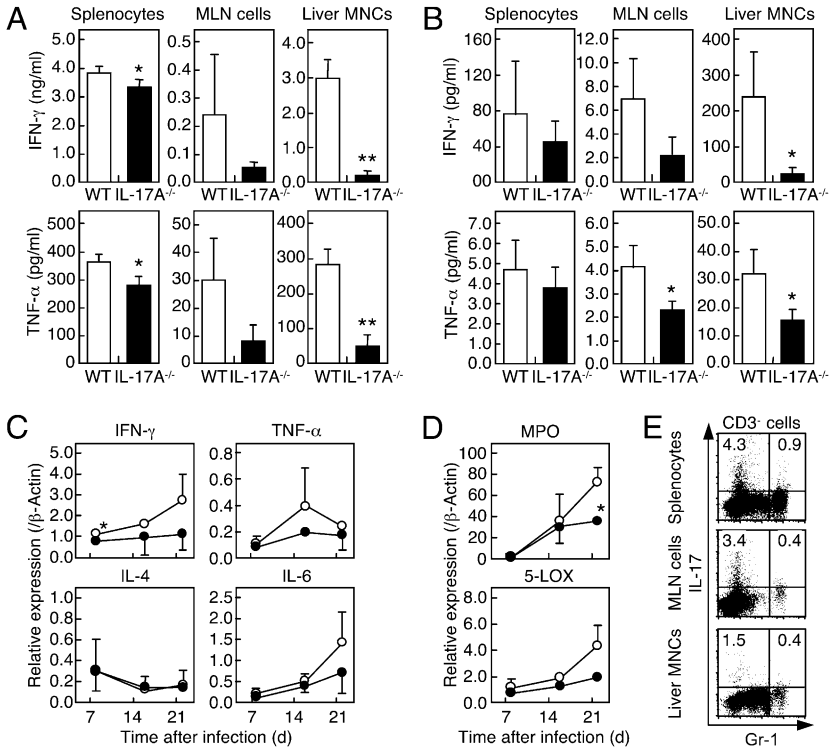

FIGURE 5. Attenuated cytokine production in *IL-17A*$^{-/-}$ mice during *T. cruzi* infection. Splenocytes, MLN cells, and liver MNCs prepared from WT and *IL-17A*$^{-/-}$ mice on day 14 (*A*) and day 21 (*B*) of infection were cultured for 64 h and analyzed for cytokine production, as described in *Materials and Methods*. Experiments were repeated at least three times with similar results. Data are mean ± SEM (n = 4). *C* and *D*, Liver MNCs were isolated from WT (○) and *IL-17A*$^{-/-}$ (●) mice at the indicated days postinfection and analyzed for mRNA expression by RT-PCR, as described in *Materials and Methods*. Experiments were repeated at least two times with similar results. Data are mean ± SEM (n = 2–4). *E*, Splenocytes (*top panel*), MLN cells (*middle panel*), and liver MNCs (*bottom panel*) collected from WT mice on day 21 of infection were stained for indicated surface markers and intracellular IL-17A. Numbers shown are the percentages of cells contained in each gated lineage. $*p < 0.05$; $**p < 0.01$ versus WT samples.

that producers of IL-17A, including NKT and γδT cells, exist and act in local sites, such as liver and intestinal tract. It is also known that marked pathologies of most autoimmune diseases caused by IL-17A are localized inflammation.

Furthermore, mRNA expression of neutrophilic enzymes, such as MPO and 5-LOX, was lower in *IL-17A*$^{-/-}$ mice compared with WT mice (Fig. 5*D*). Because MPO and 5-LOX participate in the biosynthesis of H_2O_2 and leukotrienes (34, 35), respectively, neutrophils in the infected *IL-17A*$^{-/-}$ mice are assumed to have weak trypanocidal activity compared with the WT neutrophils.

Neutrophils may also participate in the control of antiparasite immune responses as producers of IL-17A. As shown in Fig. 5*E*, CD3$^-$Gr-1$^+$ cells from spleen, MLN, and liver produced IL-17A in response to *T. cruzi* infection. Production of IL-17A by neutrophils was also observed in *Leishmania major* infection, as reported by Lopez Kostka et al. (36). However, the percentages of IL-17A$^+$ neutrophils were low, and production levels, as shown by intracellular cytokine staining, were also low compared with other types of cells (Fig. 1*B*). The contribution of neutrophil-derived IL-17A to defense against *T. cruzi* has not been examined in detail.

In conclusion, cytokine production and activation of lymphocytes, macrophages, and neutrophil were markedly impaired in the infected *IL-17A*$^{-/-}$ mice, whereas cellular migration was not affected by IL-17A deficiency. The attenuated immune activation in *IL-17A*$^{-/-}$ mice resulted in propagation of *T. cruzi* infection.

Discussion

Although IL-17A is known to participate in the induction of inflammation during infection of an intracellular protozoan parasite, *Toxoplasma gondii* (37), the pathogenic or protective roles of IL-17A in infection by other intracellular protozoan parasites are not well understood. In this study, we revealed that the production of IL-17A was induced against *T. cruzi* infection (Fig. 1*A*). As shown in Fig. 1*B*, although IL-17A was produced by a small population of CD4$^+$ T (Th17 cells) and CD8$^+$ T cells, its production was more potently induced in substantial numbers of NKT and γδT cells in response to *T. cruzi* infection (5–7). Some reports indicated that infection by *Leishmania amazonensis* and *L. braziliensis* induced the expression of IL-17A (38, 39), but there was no evidence for a relationship between IL-17A production and host protection in the protozoa infection. In this regard, we demonstrated, using *IL-17A*$^{-/-}$ mice, that IL-17A plays an important role in the successful resolution of *T. cruzi* infection (i.e., *T. cruzi*-infected *IL-17A*$^{-/-}$ mice showed prolonged, more severe parasitemia and exacerbated mortality compared with WT mice) (Fig. 2*A*, 2*B*).

Interestingly, the production of IFN-γ, which is a critical cytokine for achieving antitrypanosoma immunity (18, 19), was lower in *IL-17A*$^{-/-}$ mice during *T. cruzi* infection compared with WT mice (Fig. 5*A–C*). Therefore, the delay in parasite expulsion in *IL-17A*$^{-/-}$ mice might result from the weak IFN-γ responses against *T. cruzi* infection. Similar defects in IFN-γ production were observed in *IL-17A*$^{-/-}$ mice during *Mycobacterium* infection (30, 40). Furthermore, reduction of serum IL-17A by anti-IL-23p19 treatment also resulted in the attenuated production of IFN-γ, IL-6, and TNF-α in CNS during autoimmune encephalomyelitis (41). Unfortunately, the direct mechanisms for the decrease in IFN-γ production in *IL-17A*$^{-/-}$ mice have not been clarified. It was reported that TNF-α induces maturation of dendritic cells and the maturated dendritic cells drive IFN-γ production of CD4$^+$ T cells (42). Therefore, attenuated production of IFN-γ by IL-17A deficiency, as shown in Fig. 5, might result from the decrease in TNF-α production. Furthermore, activated production of TNF-α observed in steatohepatitis induced by fat/alcohol feeding with LPS injection was associated with increased expression of IFN-γ (43).

In addition to IFN-γ, IL-6 and TNF-α are well-known cytokines induced by IL-17A stimulation (8, 9). Production of these cytokines during *T. cruzi* infection was decreased in *IL-17A*$^{-/-}$ mice compared with WT mice (Fig. 5*A–C*). IL-6 and TNF-α were required for the successful resolution of *T. cruzi* infection (44–47). For example, IL-6 induces B cell terminal differentiation into plasma cells during *T. cruzi* infection, and *IL-6*$^{-/-}$ mice were

more susceptible to the infection (44, 45). TNF-α is known to act synergistically with IFN-γ on macrophages to augment killing of *T. cruzi* (22, 23), and a defect in TNF-α signaling by introduction of TNFR-Fc transgene or gene targeting of the receptor brought about increased susceptibility to *T. cruzi* infection (46, 47). Therefore, it is likely that poor production of IL-6 and TNF-α in *T. cruzi*-infected *IL-17A*$^{-/-}$ mice (Fig. 5*A–C*) was a factor contributing to the deviant expansion of the parasites and increase in mortality (Fig. 2*A*, 2*B*).

IL-17A induced during infection of bacteria and fungi and a protozoan parasite *Toxoplasma gondii* mobilizes neutrophils for elimination of the pathogens (28, 29, 31, 48). However, in the current study, deficiency in IL-17A did not affect the number of immune-related cells migrating into infected tissues (Fig. 3*A*, 3*B*). Therefore, IL-17A was not essential for the recruitment of neutrophils and other immune cells in *T. cruzi* infection. Presumably, cytokines, such as IL-17F, and other inflammatory cytokines produced during infection compensated for the lack of IL-17 for cell migration. Nevertheless, because neutrophilic enzyme activities were lower in *T. cruzi*-infected *IL-17A*$^{-/-}$ mice (Fig. 5*D*), IL-17A might be important for proper neutrophil activation required for killing of *T. cruzi* (34, 49). However, the activation of neutrophils by IL-17A seemed to be achieved indirectly, because IL-17A itself did not enhance the neutrophilic enzyme expression (Fig. 4*D*). Interestingly, neutrophils were activated for the enhanced ROS production by stimulation with *T. cruzi* Ags (Fig. 4*C*). Therefore, it was supposed that neutrophils participate in the protection against *T. cruzi* infection as effectors in host immune systems.

In conclusion, we demonstrated that IL-17A is induced in response to *T. cruzi* infection and results in efficient activation of the immune system critical for the killing of infected *T. cruzi*, mainly through sufficient production of INF-γ and other inflammatory cytokines. IL-17A is required for the elimination of bacteria, fungi, and *T. cruzi*; it controls cytokine production by T cells and macrophages, as well as neutrophil activation.

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Disclosures

The authors have no financial conflicts of interest.

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