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IL-10 Limits Parasite Burden and Protects against Fatal Myocarditis in a Mouse Model of Trypanosoma cruzi Infection

Ester Roffe,* Antonio Gigliotti Rothfuchs,†,‡ Helton C. Santiago,§ Ana Paula M. P. Marino,* Flavia L. Ribeiro-Gomes,§ Michael Eckhaus,‖ Lis R. V. Antonelli,‖,‡ and Philip M. Murphy*

Chagas’ disease is a zoonosis prevalent in Latin America that is caused by the protozoan Trypanosoma cruzi. The immunopathogenesis of cardiomyopathy, the main clinical problem in Chagas’ disease, has been extensively studied but is still poorly understood. In this study, we systematically compared clinical, microbiologic, pathologic, immunologic, and molecular parameters in two mouse models with opposite susceptibility to acute myocarditis caused by the myotropic Colombiana strain of T. cruzi: C3H/HeSnJ (100% mortality, uncontrolled parasitism) and C57BL/6J (<10% mortality, controlled parasitism). T. cruzi induced differential polarization of immunoregulatory cytokine mRNA expression in the hearts of C57BL/6J versus C3H/HeSnJ mice; however, most differences were small. The difference in IL-10 expression was exceptional (C57BL/6J 8.7-fold greater than C3H/HeSnJ). Consistent with this, hearts from infected C57BL/6J mice, but not C3H/HeSnJ mice, had a high frequency of total IL-10-producing CD8+ T cells and both CD4+ and CD8+ subsets of IFN-γ+ IL-10+ double-producing T cells. Furthermore, T. cruzi infection of IL-10−/− C57BL/6J mice phenocopied fatal infection in wild-type C3H/HeSnJ mice with complete loss of parasite control. Adoptive transfer experiments indicated that T cells were a source of protective IL-10. Thus, in this system, IL-10 production by T cells promotes T. cruzi control and protection from fatal acute myocarditis. The Journal of Immunology, 2012, 188: 000–000.

Consistent with the intracellular lifestyle of T. cruzi, studies in animal models and with human subjects strongly suggest that it induces a Th1-polarized immune-mediated process in affected organs (4). Mice genetically deficient in the signature Th1 cytokines IL-12 or IFN-γ, the IFN-γ–induced effector enzyme inducible NO synthase (5), the Th1-associated chemokine receptors CCR2 (6) or CCR5 (7), and the Th1-associated ligands for chemokine receptor CXCR3 (8) all develop increased parasitism postinfection (p.i.), and some have high acute mortality. Persistent parasitism and type 1 responses may promote progression to the chronic phase. In animal models of chronic infection, parasite DNA and Ags are still found in infected tissues in the presence of cell infiltration and fibrosis, indicating an inappropriate or ineffective immune response. In chronic human T. cruzi infection, the cytokine profile in the heart is also Th1 polarized (9), and PBMCs produce high levels of IFN-γ and low levels of IL-10 (10).

A problem in interpreting results from mouse models of T. cruzi infection is that many different models have been used with many different parasite strains and inocula and diverse mouse strains, with variable outcomes from the many laboratories working in the field. In the current study, we have systematically and directly compared the clinical, parasitologic, pathologic, immunologic, and molecular correlates of infection using the myotropic Colombiana strain of T. cruzi (11) in two experimental mouse models with opposite outcomes: C57BL/6J, which control parasitism and survive infection, and C3H/HeSnJ, which fail to control parasitism and die. This approach allowed us to identify a strong association of IL-10 and IL-10–producing cells with resistance to T. cruzi infection (12–15) and provides new insights with regard to the source of IL-10 and its effects on parasitism.
Materials and Methods

Animals and parasite

The following mice were obtained from The Jackson Laboratory (Bar Harbor, ME); Wild-type (WT) male C57BL/6J mice (stock number 666); WT male C3H/HeNj mice (stock number 661); IL-10–deficient male mice (stock number 2251; back-crossed for 10 generations onto the C57BL/6J background); RAG-1–deficient male mice (stock number 2216, back-crossed for 10 generations onto the C57BL/6J background); C3LW/H2bSn male mice (stock number 0438); and C3H/HeJ male mice (stock number 659). Inbred WT male C3H/HeNj mice and WT male C57BL/6Nj mice were obtained from Taconic Farms (Hudson, NY). Parasitemia and mortality were similar after T. cruzi infection of the C57BL/6 and C3H/He lines obtained from The Jackson Laboratory and from Taconic Farms (data not shown). Mice were infected at 8–10 wk of age and housed in cages under specific pathogen-free conditions. All female mice were used under the auspices of a protocol approved by the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee. The myotropic Colombian strain of Trypanosoma cruzi, obtained from Dr. Fuyuki Tokumasu and Dr. James Dvorak (Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health), was maintained by serial passage in Swiss Webster mice (Taconic Farms) every 21 d. Animals were infected i.p. with 1000 blood-stage trypomastigote forms of T. cruzi. Parasitemia levels were determined by light microscopy by counting the number of parasites in an unstained 5-μl drop of whole blood drawn from the tail vein and mounted between a microslide and cover slip at ×40 magnification, as described (16).

Quantification of parasite tissue loads by real-time PCR

Real-time PCR was performed as previously described (17), with minor modifications, using tissues from infected and control mice. Total RNA was isolated from hearts using an RNeasy kit (Qiagen, Valencia, CA), and real-time PCR was performed on an ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA) using SYBR Green PCR Master Mix (Applied Biosystems) after reverse transcription of 1 μg RNA using M-MLV reverse transcriptase (Promega, Madison, WI). The relative level of gene expression was determined by the comparative threshold cycle method as described by the manufacturer, in which data for each level of gene expression was determined by the comparative threshold cycle method as described by the manufacturer, in which data for each sample were normalized to hypoxanthine phosphoribosyltransferase and expressed as fold-change compared with uninfected controls. The primer sequences are available upon request.

Histopathologic analysis

Mouse organs were removed and washed in sterile PBS. Immediately after drying the surfaces by tamping with absorbent paper, organs were fixed in 4% buffered paraformaldehyde and processed for paraffin embedding. Six-micrometer-thick sections were cut with a microtome, then stained with H&E to investigate inflammation and parasitism or with Gomori’s trichrome to assess collagen content. Cardiac parasitism and inflammation were analyzed with a Zeiss integrating eyepiece with 100 hits (Oberkochen, Germany) at a final magnification of ×400. A total of 3000 hits were evaluated in each section of cardiac tissue. The infection and inflammation indices represent the number of hits covered by amastigote nests and inflammatory cells, respectively.

Flow cytometry

Flow cytometry was performed as previously described (19), with modifications. Briefly, animals were first euthanized under CO2 anesthesia. Spleens were removed, injected with Liberase Cl (0.45 mg/ml; Roche Applied Sciences, Indianapolis, IN), placed in serum-free RPMI 1640 (Invitrogen, Carlsbad, CA), quickly vortexed, and then incubated at 37°C for 30 min. Spleen single-cell suspensions were prepared by passing tissue through a 40-μm nylon cell strainer in PBS containing 2% FBS (Hyclone, Thermo Scientific, Waltham, MA) and EDTA. Four to five hearts were pooled and minced in Liberase Cl (0.45 mg/ml), quickly vortexed, and incubated at 37°C for 30 min. The cells were centrifuged in 35% Percoll (Amersham-Pharaceutics Biotech, Piscataway, NJ) for 15 min at 700 × g. Spleen and heart suspensions were hemolyzed in ACK buffer (Lanza BioWhittaker, Walkersville, MD). A total of 106 freshly isolated cells were analyzed immediately ex vivo for surface marker expression or apoptosis/necrosis markers (annexin V/propidiod iodide [PI]; Annexin V Apoptosis Detection Kit conjugated to FITC; eBioscience, San Diego, CA) or after being cultured 3 days in 96-well plates alone or with plate-bound anti-CD3 (3 μg/ml) for intracellular cytokine expression. During the last 3 h of culture, brefeldin A and monensin (1 μg/ml each; BD Pharmingen, San Jose, CA) were added for intracellular cytokine staining. Before adding Abs, cells were washed in PBS and incubated for 30 min with an amine-reactive fluorescent dye to exclude dead cells (Live/Dead Fixable Violet Dead Cell Stain Kit; Invitrogen). The following labeled rat or hamster anti-mouse mAbs were used: anti-TCR β-Alexa fluoro 700 (clone H57-597), anti-CD8–APC-eFluor 780 (clone 53-6.7), anti-Gr1–PECy5 (clone RB6-8C5), all from eBioscience; anti-CD11b–FITC (clone M1/70) and anti–I-A/I-E–biotin (clone 2G9), from BD Pharmingen; and anti-CD4–Pacific orange (clone RM4-5), from Caltag-Invitrogen (Carlsbad, CA). Cell suspensions were then fixed and permepabilized according to the manufacturer’s instructions (Fix/Perm kit; BD Pharmingen). To analyze activation markers, the cell suspensions were stained with the following labeled rat anti-mouse mAbs: anti-CD4–Pacific blue (clone IM7; BioLegend, San Diego, CA) and anti-CD62L–PECy7 (clone MEL-14; eBioscience). Intracellular staining was performed using the following labeled rat or hamster anti-mouse mAbs: anti-IL-10–PE (clone JES5-16E3) and anti-IL-17A–PerCP-Cy5.5 (clone TC11-18H10), from BD Pharmingen; anti–IFN-γ–PE (clone XMG1.2), anti–TNF-α–Pacific blue (clone MP6-XT22), and anti-Foxp3–FITC (clone FJK-16S), from eBioscience. To analyze cell proliferation, cell suspensions were stained with mouse anti-human Ki-67–PE (clone B56; BD Pharmingen). Quantum dot 605-conjugated streptavidin (Invitrogen) was added during intracellular staining to bind anti-1-A/I-E–biotin. Data were collected using an LSR II (BD Immunocytometry Systems) with Diva software (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Adaptive transfer

T cells were pooled from spleens and lymph nodes of five naive WT or five IL-10 KO mice by negative selection (Ficoll Cell Isolation Kit; Miltenyi Biotech). CD3+ T cells were further purified with PE-labeled anti-CD3e (145-2C11; BD Biosciences) by cell sorting using a FACSAria instrument (BD Biosciences). A total of 106 FACS-purified CD3+ T cells from WT or IL-10 KO mice were injected i.v. into RAG-1–deficient mice, which were then infected 24 h later with T. cruzi and analyzed for parasitemia and mortality.

Statistical analyses

Results are shown as means ± SEM. Differences between groups were compared using the Student t test (two sets of data) or one-way ANOVA (three or more sets of data), followed by the Student-Newman-Keuls post hoc test. Survival curves were analyzed using the log-rank test. Differences were considered significant at p < 0.05.

Results

C57BL/6J mice, but not C3H/HeSnj mice, control parasitism and survive after infection with T. cruzi

During experiments designed to study immunoregulation in the acute phase of T. cruzi (Colombiana strain)-induced myocarditis using candidate gene KO mice on the C57BL/6J background, we found that acute mortality in WT mice was very low, and myocarditis was mild to moderate. This outcome differs from other reports using this strain of T. cruzi with C57BL/6 mice (20, 21). In contrast, during experiments designed to study immunoregulation of the chronic phase of T. cruzi (Colombiana strain)-induced cardiomyopathy using C3H/HeSnj mice, 100% of animals died by 40 d p.i., and myocarditis was severe. This outcome differs
from other reports using this strain of *T. cruzi* with C3H/He mice (19, 22, 23). We do not know why these unexpected outcomes occurred, although possibilities include 1) differences in experimental protocol, including gender and inoculum differences; 2) mutations that may have accrued over time in either the parasite or mouse strain; and 3) environmental differences including changes in the microbiome associated with different animal facilities. Whatever the reason, the existence of these two models of disease using the same parasite strain, same inoculum, same sex, and same mouse facility provided an opportunity to evaluate immunoregulatory correlates of resistance versus susceptibility during the acute phase of infection directly and in a systematic manner.

*T. cruzi* infection of WT C3H/HeSnJ mice resulted in low levels of parasitemia until day 14 p.i. when it accelerated dramatically, peaking at $2 \times 10^7$ parasites/ml of whole blood (Fig. 1A). In contrast, parasitemia in WT C57BL/6J mice was much lower than that in C3H/HeSnJ mice after day 14 p.i., 20-fold less at peak (32 d p.i.), and slowly and progressively declined until by day 78 p.i., when measurements were discontinued, only an occasional parasite could be detected in some animals (Fig. 1A). Consistent with this, survival of infected C57BL/6J mice in the acute phase was 91.7% until at least 210 d p.i., when the experiments were stopped (Fig. 1B and data not shown).

We also compared parasite burden in the two mouse lines in heart, spleen, and liver p.i. (Fig. 1C). Consistent with previous reports that the Colombiana strain is myotropic, *T. cruzi* DNA levels were much greater in the hearts from both lines of mice than in either spleen or liver from the corresponding line. However, C3H/HeSnJ parasite load was greater than C57BL/6J parasite load in all three organs. This was particularly striking in the heart, where parasite burden was 160 times greater for C3H/HeSnJ than for C57BL/6J mice on day 30 p.i. (Fig. 1C). This fold-increase was 16 and 30 times greater than those observed in spleen and liver, respectively.

**C3H/HeSnJ mice, but not C57BL/6J mice, develop severe myocarditis and cardiac failure in the acute phase of *T. cruzi* infection**

Because *T. cruzi* is able to infect many organs and multiple cell types, we next performed comprehensive anatomic and histopathologic analysis to determine the extent of disease and the proximate cause of death. All C3H/HeSnJ mice examined were either found dead or were euthanized after fulfilling protocol criteria for euthanasia at 28–31 d p.i. C57BL/6J mice were euthanized on day 32 p.i. in good clinical condition. Seven of 10 C3H/HeSnJ mice examined had pleural effusions secondary to cardiac failure caused by severe myocarditis, which appeared to be the cause of death. The three mice that did not have pleural effusion still had severe myocarditis. Histopathologic analysis showed that the heart (Fig. 2, Table I) and skeletal muscle (data not shown) were the most differentially affected organs in C3H/HeSnJ versus C57BL/6J mice. In addition to severe myocarditis and moderate fibrosis (Fig. 2), the hearts of infected C3H/HeSnJ mice had moderate to severe multifocal necrosis, multifocal mineralization, and numerous amastigote pseudocysts (Fig. 2, Table I). Eight of ten animals analyzed had endocardial thrombi in the right atrium and/or right ventricle. In contrast, infected C57BL/6J mice had only mild to moderate myocarditis and mild fibrosis, without signs of cardiac necrosis or mineralization and very few amastigote pseudocysts (Fig. 2, Table I). Myocarditis was primarily lymphocytic in both C3H/HeSnJ and C57BL/6J mice, but polymorphonuclear leukocytes were also found in C3H/HeSnJ hearts in association with thrombi (data not shown).

In the spleen, both C3H/HeSnJ and C57BL/6J mice had moderate extramedullary hematopoiesis and moderate lymphoid hyperplasia. The livers of both lines of mice had mild extramedullary hematopoiesis. Infected C3H/HeSnJ mice had mild–moderate inflammatory cell infiltrates in all other organs where parasites were detected (brain, spinal cord, brown fat, lung, and esophagus); kidney, adrenal gland, thyroid, and pancreas lacked both cellular infiltrates and detectable parasites by light microscopy. At the cellular level, parasites were detected in C3H/HeSnJ mice in cardiac myocytes, skeletal muscle myofibers, and brown fat adipocytes in 100% of mice examined (n = 9 or 10); in lung myocytes from 6 of 10 mice examined; in brain neurons and glial cells from 3 of 6 mice examined; in spinal cord inflammatory cells and glial cells from 4 of 9 mice examined; and in esophageal myocytes from 3 of 10 mice examined.

**FIGURE 1.** C57BL/6J mice, but not C3H/HeSnJ mice, control parasitism and survive infection with *T. cruzi*. A, Parasitemia. Data are the mean ± SEM from a single experiment (n = 15 animals in each group) representative of three independent experiments. B, Survival. Data are from the same experiment shown in A (n = 15 animals per group), representative of three independent experiments. p < 0.0001 (log-rank test). C, Quantification of *T. cruzi* DNA in tissues of *T. cruzi*-infected mice. Heart, spleen, and liver were collected from C3H/HeSnJ and C57BL/6J mice 30 d p.i., and *T. cruzi* burden was quantitated by PCR. Results are expressed as mean ± SEM of 10 animals in each group. **p < 0.01, ***p < 0.001 (comparing infected C3H/HeSnJ mice versus infected C57BL/6J mice).
Myocardial infiltrates after *T. cruzi* infection have a higher frequency of Gr1<sup>hi</sup>CD11b<sup>-</sup>TCRβ<sup>-</sup> cells but a lower frequency of CD8<sup>+</sup> T cells in C3H/HeSnJ mice compared with C57BL/6J mice

Because C57BL/6J mice appeared to mount a clinically effective and appropriate response to the pathogen, whereas C3H/HeSnJ mice responded ineffectively, we next tested whether this difference in susceptibility could be linked to MHC haplotype, as previously suggested (24). C3H/HeSnJ mice are H-2k, whereas C57BL/6J mice are H-2b. When we infected C3H/HeSnJ mice, which are C3H/HeSnJ animals that carry MHC haplotype H-2b (the same as C57BL/6J), parasitemia remained uncontrolled, and mortality was still 100% (data not shown).

Therefore, we next investigated the local immune responses in the heart to identify specific differences at the cellular and molecular level that might contribute to opposite outcomes in these mice. First, we isolated leukocytes from hearts for flow cytometric analysis. In both uninfected C57BL/6J mice and uninfected C3H/HeSnJ mice, very few leukocytes were detectable in the heart by either histopathologic examination (data not shown) or by FACS analysis (data not shown). Postinfection, leukocytes accumulated in greater numbers in C3H/HeSnJ mice relative to C57BL/6J mice, and the distribution of subsets was different. In particular, the frequency of CD8<sup>+</sup> TCRβ<sup>-</sup> cells was significantly higher in C57BL/6J than in C3H/HeSnJ mice (Fig. 3). Conversely, the frequency of Gr1<sup>hi</sup>CD11b<sup>-</sup>TCRβ<sup>-</sup> cells, which contain neutrophils, was significantly lower in C57BL/6J mice than in C3H/HeSnJ mice (Fig. 3). There was no significant difference in the two lines in the frequency of CD4<sup>+</sup> T cells or monocyte/macrophages (Gr1<sup>int</sup>CD11b<sup>-</sup>TCRβ<sup>-</sup> cells) in the heart (Fig. 3). The frequency of Foxp3<sup>+</sup>CD4<sup>+</sup> regulatory T cells (Tregs) was also similar in the heart (0.67 ± 0.02% versus 1.23 ± 0.41% for C57BL/6J and C3H/HeSnJ mice, respectively). Regarding potential mechanisms, the frequency of Gr1<sup>hi</sup>CD11b<sup>-</sup>TCRβ<sup>-</sup> cells in the spleen before infection was lower in C57BL/6J mice than in C3H/HeSnJ mice (0.6 ± 0.1% versus 1.9 ± 0.4%, p = 0.006), perhaps accounting in part for the reduced Gr1<sup>hi</sup>CD11b<sup>-</sup>TCRβ<sup>-</sup> cell frequency found in infected C57BL/6J hearts relative to C3H/HeSnJ hearts (Supplemental Fig. 1A). No statistically significant difference was found in the spleen in the frequency of any other cell type analyzed in the two strains before infection: CD8<sup>+</sup> T cells (32.3 ± 2.4% versus 27.5 ± 1.9%), CD4<sup>+</sup> T cells (51.6 ± 7.6% versus 61.2 ± 4.1%), monocyte/macrophages (9.1 ± 2.0% versus 7.9 ± 0.7%), and Tregs (5.4 ± 0.7% versus 6.1 ± 0.4%) for C57BL/6J mice and C3H/HeSnJ mice, respectively (Supplemental Fig. 1A).

As in the heart, the spleens of infected C57BL/6J mice contained a higher frequency of CD8<sup>+</sup> TCRβ<sup>-</sup> cells than in spleens from infected C3H/HeSnJ mice (45.5 ± 7.8% versus 28.9 ± 2.3%; p = 0.0002) (Supplemental Fig. 1A). The frequency of the splenic CD4<sup>+</sup> TCRβ<sup>-</sup> population was slightly lower in infected C57BL/6J mice than in infected C3H/HeSnJ mice (30.1 ± 4.0% versus 36.6 ± 3.0%; p = 0.02). There was no significant difference between the two strains in the frequency of splenic monocyte/macrophages and polymorphonuclear leukocytes p.i. (Supplemental Fig. 1A).

To search for molecular candidates that might control outcome after *T. cruzi* infection of C57BL/6J mice and C3H/HeSnJ mice, we performed a survey of immunoregulatory cytokines and chemokines, effector enzymes, and transcription factors using RNA isolated from the heart 30 d p.i. Of 44 factors tested, infection with *T. cruzi* induced expression of IL-10 and other major immunoregulatory cytokines, effector enzymes, and transcription factors using RNA isolated from the heart 30 d p.i. Of 44 factors tested, infection with *T. cruzi* induced expression of IL-10 and other major immunoregulatory cytokines, effector enzymes, and transcription factors. Postinfection, we found strong induction in the hearts of both lines of mice for genes encoding the CXC chemokines Cxc2, Cxc9, and Cxc11; the chemokine receptors Cxcr3, Ccr1, Ccr3, Ccr4, and Ccr6; the cytokines IL-13 and IL-17A; and the effector enzymes NO synthase-2 (NOS-2) and arginase-1 (ARG-1). Postinfection, we found strong induction in the hearts of both lines of mice for genes encoding Ccl4 and Ccl5 (the ligands for Ccr5 and for Ccr1, Ccr3, and Ccr5, respectively) and IL-12 p40; however, the differences between strains for each gene were only ~2-fold. The only factor tested that was strongly overexpressed p.i. (8.7-fold) in C57BL/6J heart relative to C3H/HeSnJ heart was IL-10 (Table II).

Myocardial infiltrates after *T. cruzi* infection have a higher frequency of IL-10<sup>+</sup>CD4<sup>+</sup> and IL-10<sup>+</sup>CD8<sup>+</sup> effector T cell subsets in C57BL/6J mice compared with C3H/HeSnJ mice

We next defined *T. cruzi* induction of IL-10 and other major immunoregulatory cytokines at the protein and cell levels by FACS analysis of leukocytes that accumulated in the heart 28–30 d p.i. When leukocytes isolated from either heart or spleen of *T. cruzi*-infected mice were stimulated with either culture medium or *T. cruzi* lysate Ag, no intracellular cytokine production was detected.
able. However, after stimulation with anti-CD3, we were able to detect IFN-γ+, IL-10+, and TNF-α+ cells, but not IL-17+ cells, among both the CD4+ and CD8+ subsets of T cells isolated from infected hearts of both C57BL/6J mice and C3H/HeSnJ mice (Fig. 3). No IL-10+Gr1int/2CD11b+IA+TCRβ2 myeloid cells were identified. Also, we were not able to detect these four intracellular cytokines in splenocytes isolated from uninfected mice from either strain after stimulation with anti-CD3 (data not shown). Consistent with these findings, we investigated the histopathology of hearts isolated from infected C3H/HeSnJ and C57BL/6J mice 28–31 d.p.i. (Table I). We found that both strains of infected mice had multifocal, primarily lymphocytic, interstitial inflammation obscuring nests of T. cruzi in the heart. Endocardial, in right atrium and/or right ventricle, presence of neutrophils was also frequently observed. Moderate EMH, moderate lymphoid hyperplasia, mild EMH, and mild lipidosis were also observed in the spleen and liver, respectively. Ten C3H/HeSnJ mice and seven C57BL/6J mice were either found dead or were euthanized after fulfilling protocol criteria for euthanasia 28–31 d.p.i.; organs were paraffin-embedded and stained with H&E.

Table I. Histopathologic evaluation of organs from T. cruzi-infected C3H/HeSnJ or C57BL/6J mice 28–31 d.p.i.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Inflammation</th>
<th>Thrombosis</th>
<th>Myodegeneration or Necrosis</th>
<th>Parasitism</th>
<th>Mineralization</th>
<th>Spleen</th>
<th>Liver</th>
</tr>
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<tbody>
<tr>
<td>C3H/HeSnJ</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>Multi focal</td>
<td>Moderate EMH, moderate lymphoid hyperplasia</td>
<td>Mild EMH, mild lipidosis</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Mild to moderate EMH, mild to moderate lymphoid hyperplasia</td>
<td>Mild EMH</td>
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Ten C3H/HeSnJ mice and seven C57BL/6J mice were either found dead or were euthanized after fulfilling protocol criteria for euthanasia 28–31 d.p.i.; organs were paraffin-embedded and stained with H&E.

FIGURE 3. Myocardial infiltrates after T. cruzi infection have a higher frequency of Gr1int/CD11b+ TCRβ- cells but a lower frequency of CD8+TCRβ+ cells in C3H/HeSnJ mice compared with C57BL/6J mice. A, Gating of leukocytes isolated from heart 28–30 d.p.i. Death denotes staining with LIVE/DEAD (Invitrogen). B, Subset analysis in the heart: representative experiment of two experiments performed. Numbers correspond to the frequency of the adjacent gated population. The top panels are for C57BL/6J mice and the bottom panels for C3H/HeSnJ mice. C, Subset analysis: summary data. The frequency is shown for the indicated cell types in the heart of T. cruzi-infected C3H/HeSnJ (gray bars) or C57BL/6J (black bars) mice. Data represent mean ± SEM of two pools of four to five hearts in each group. *p < 0.05, **p < 0.01 (comparing C3H/HeSnJ and C57BL/6J mice).
with increased gene expression for Cxcr3 and IFN-γ in infected C3H/HeSnJ mice relative to infected C57BL/6J mice, we observed a higher frequency of IFN-γ+/CD4+ and IFN-γ+/CD8+ T cells in the hearts of infected C3H/HeSnJ mice relative to infected C57BL/6J mice, although the differences were less than 2-fold. There were also differences in the frequency of TNF-α–producing CD4+ and CD8+ T cells and IL-10–producing CD4+ T cells p.i. between C57BL/6J mice and C3H/HeSnJ mice, but again, the differences were at most ~2-fold. In contrast, the frequency of IL-10–producing CD8+ T cells was much greater, ~10-fold, in infected hearts from C3H/HeSnJ mice compared with that in hearts from infected C3H/HeSnJ mice (Fig. 4C).

Analysis of double-cytokine–producing cells in the heart upon T. cruzi infection revealed additional large differences between the two strains related to IL-10. None of the IL-10+/CD4+ or IL-10+/CD8+ T cells in either infected C57BL/6J hearts or infected C3H/HeSnJ hearts was also producing TNF-α (data not shown). Yet, in infected C57BL/6J hearts, most (~99%) of the IL-10+/CD4+ and IL-10+/CD8+ T cells were also producing IFN-γ (Fig. 4B, 4C). In striking contrast, in infected C3H/HeSnJ hearts there were almost no IL-10+/CD8+ T cells (~1%) (Fig. 4B, 4C) and very few IL-10+/CD4+ T cells (6%); most of the IL-10+/CD4+ T cells were also producing IFN-γ (Fig. 4B, 4C). All IFN-γ+/CD8+ T cells from infected C3H/HeSnJ hearts failed to express IL-10 and thus appeared to have a classic Th1 immunophenotype (Fig. 4B, 4C), whereas in infected C57BL/6J hearts, 35% of the IFN-γ+/CD8+ T cells were also positive for IL-10 (Fig. 4B, 4C); infected hearts from neither strain of mice contained IL-10+/IFN-γ−CD8+ T cells (Fig. 4B, 4C).

Regarding TNF-α, the frequency of IFN-γ+/TNF-α+/CD8+ T cells was similar in infected C57BL/6J and C3H/HeSnJ hearts (Fig. 4B, 4C), and the frequency of IFN-γ+/TNF-α+/CD4+ T cells was only ~2-fold greater in infected C3H/HeSnJ hearts than that in infected C57BL/6J hearts (Fig. 4B, 4C). Very few TNF-α+/IFN-γ cells were detectable in either the CD4+ or CD8+ T cell population in either strain of infected mice (Fig. 4B, 4C). In the spleens of infected C57BL/6J mice, in both the CD4+ and CD8+ TCRβ+ populations, single-positive cells for IFN-γ and TNF-α and double-positive cells for IFN-γ/TNF-α were increased compared with those in infected C3H/HeSnJ mouse spleens (Supplemental Fig. 1B, 1C). Thus, the level of IL-10 production in the heart by infiltrating CD8+ T cells stood out as a major correlate of sus-

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<th>Factor</th>
<th>C3H/HeSnJ</th>
<th>C57BL/6J</th>
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<tbody>
<tr>
<td>Chemokines</td>
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<tr>
<td>Cxcl1</td>
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<td>5.3 ± 0.5</td>
</tr>
<tr>
<td>Cxcl2</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>Ccl7</td>
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<tr>
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<td>1.9 ± 0.4</td>
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<td>29.0 ± 4.4</td>
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<tr>
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<td>152.9 ± 72.4</td>
<td>310.9 ± 23.5*</td>
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<tr>
<td>IL-13</td>
<td>43.3 ± 22.3*</td>
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<tr>
<td>IL-21</td>
<td>77.5 ± 7.9</td>
<td>14.1 ± 2.6</td>
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<tr>
<td>IL-22</td>
<td>124.4 ± 58.0</td>
<td>24.7 ± 7.1</td>
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<td>Arg-1</td>
<td>1890.0 ± 484.2**</td>
<td>198.2 ± 66.6</td>
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<tr>
<td>NOS-2</td>
<td>97.2 ± 29.0*</td>
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<td>3.9 ± 0.3</td>
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<tr>
<td>Gata-3</td>
<td>6.9 ± 2.1*</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>Foxp3</td>
<td>21.4 ± 9.6</td>
<td>4.1 ± 1.3</td>
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</table>

Data are the average of five samples for each factor normalized to hypoxanthine phosphoribosyltransferase and expressed as a fold-change compared with heart RNA from uninfected isogenic control mice. Factors appearing in boldface were differentially expressed in C3H/HeSnJ versus C57BL/6J.

\( * \text{p} < 0.05, ** \text{p} < 0.001, *** \text{p} < 0.001. \)
ceptibility in C3H/HeSnJ versus C57BL/6J mice infected with T. cruzi.

Because IL-10 is an important regulator of cytokine synthesis by APCs, it is possible that under conditions where it is present in low amounts, as in infected C3H/HeSnJ mice, T cells become overwhelmingly activated and die. To test this possibility, we analyzed activation, proliferation, and apoptosis markers in T cell populations. A slightly higher percentage of activated CD8+ T cells (CD62LloCD44hi) was detected in infected hearts of C57BL/6J mice compared with that in infected hearts of C3H/HeSnJ mice (Supplemental Fig. 2A). No difference was detected in the expression of the proliferation marker Ki-67 in CD4+ and CD8+ T cell populations isolated from infected hearts in both strains (Supplemental Fig. 2B). Finally, staining with annexin V and PI for measurement of apoptotic and necrotic cells, respectively, revealed a higher percentage of CD4+, but not CD8+, T cells undergoing cell death in hearts from infected C57BL/6J mice compared with that in hearts from infected C3H/HeSnJ mice (Supplemental Fig. 2C, 2D). Thus, the data do not support massive overactivation and death of T cells in C3H/HeSnJ mice as a likely mechanism for failure to control the parasite in this strain.

A particularly striking finding from this analysis is that in the infected heart, the frequency of IL-10+IFN-γ− cells was higher in C57BL/6J mice than that in C3H/HeSnJ mice in both the CD8+ and CD4+ T cell populations (Fig. 4B, 4C), whereas the frequency of IFN-γ+IL-10− cells was higher in C3H/HeSnJ mice than in C57BL/6J mice in both CD8+ and CD4+ T cell populations (Fig. 4B, 4C). Together, these observations suggested that IL-10 coming from both IFN-γ+IL-10+ double-producer and IL-10 single-producer T lymphocytes might prevent the intense inflammatory response and parasitemia observed in T. cruzi-infected C3H/HeSnJ mice.

T. cruzi infection in a highly resistant C57BL/6J model is uniformly fatal in the absence of IL-10

To test the functional importance of IL-10 in T. cruzi-infected mice, we infected IL-10−/− mice on the C57BL/6J background. As expected, parasitemia in WT control mice peaked at day 30 and was cleared by day 50 (Fig. 5A). In contrast, IL-10−/− mice on the C57BL/6J background failed to clear parasitemia, which increased linearly until death of all animals, just as we had observed with infected WT C3H/HeSnJ mice (Fig. 5A). Death occurred between 20 and 42 d p.i., whereas 95% of WT control mice survived through day 90, when the experiment was terminated (Fig. 5B). Notably, IL-10 KO mice began to die before the WT and KO parasitemia curves began to diverge. Similar to what we had observed with low IL-10–producing C3H/HeSnJ mice p.i., hearts from infected IL-10 KO mice had significantly higher levels of parasite DNA (Fig. 5C), more amastigote nests (Fig. 5D), and more intense myocarditis (Fig. 5D, 5E) compared with infected WT C57BL/6J mice.

IL-10 deficiency skews the distribution, proliferation, and survival of T cells in the hearts of T. cruzi-infected mice. We next compared T cell activation, proliferation, apoptosis, and necrosis in the hearts of IL-10−/− mice and WT C57BL/6J mice. As we had observed for C3H/HeSnJ mice (Fig. 3C), we observed p.i. a reduced frequency of CD8+ T cells in hearts of IL-10−/− mice compared with that in hearts of C3H/HeSnJ mice (Fig. 6A). However, unlike infected C3H/HeSnJ mice (Fig. 3C) and C57BL/6J mice, infected IL-10 KO mice had a predominance of CD4+ T cells in the myocardium.
In addition, the frequency of Foxp3+CD4+ Tregs was significantly reduced in hearts isolated from infected IL-10–deficient mice compared with that in hearts isolated from infected control mice (Fig. 6B). We also found a slightly decreased percentage of CD8+ T cells that were Ki-67+ in the hearts of infected IL-10 KO mice (Fig. 6C), suggesting a lower proliferative capacity compared with controls. Regarding activation, however, no difference between the two infected strains was identified in CD44 and CD62L expression by cardiac CD4+ or CD8+ T cells. Of note, double staining by both annexin V and PI was increased in both CD4+ and CD8+ T cells isolated from infected IL-10 KO hearts compared with controls (Fig. 6E), but single-stained annexin V+ cells were similar in frequency (Fig. 6F), suggesting that a higher percentage of the cells is undergoing late apoptosis or necrosis in infected IL-10 KO mice.

In the spleen, we observed an inverted CD4/CD8 ratio in infected IL-10 KO mice compared with controls, with the frequency of CD4+ T cells significantly increased compared with infected WT mice (Supplemental Fig. 3A). In addition, splenic CD4+ T cells from infected IL-10 KO mice had a slightly increased percentage of activated cells (CD44hiCD62Llo) compared with infected WT mice (Supplemental Fig. 3A). No difference was observed in expression of the proliferation marker Ki-67 (Supplemental Fig. 3A). However, in the CD8+ T cell population (Supplemental Fig. 3B), we found a decreased percentage of activated and proliferating cells (CD44hiCD62Llo and Ki-67+, respectively) (Supplemental Fig. 3B). With regard to cell death, there was an increased frequency of both CD4+ and CD8+ T cells undergoing apoptosis in infected WT mice compared with infected IL-10 KO mice (Supplemental Fig. 3A, 3B). However, an increased percentage of CD4+ annexin V+ PI+ cells was observed in IL-10–deficient mice (Supplemental Fig. 3A), suggesting that an increased rate of late apoptosis/necrosis was occurring in the spleens of these mice.

Adoptive transfer of T cells from WT mice, but not from IL-10 KO mice, prolongs survival of T. cruzi-infected RAG-1–deficient mice

We hypothesized that the source of IL-10 protecting mice from fatal T. cruzi infection in C57BL/6J mice was the T cell. To test this, we transferred sorted CD3+ T cells isolated from either WT or IL-10 KO mice into T. cruzi-infected RAG-1–deficient mice, which produce no mature T or B cells and have been previously shown to be extremely susceptible to T. cruzi infection (13). Confirming this, we found that 100% of RAG-1–deficient mice succumbed to infection by day 24 p.i., with parasitemia increasing linearly with time, reaching levels as high as 3.5 × 10^7 parasites/ml at death (Fig. 7). Consistent with our hypothesis, transfer of CD3+ T cells isolated from naive WT mice into infected RAG-1–deficient mice significantly prolonged survival, although not to WT levels, with the final mouse dying on day 37 p.i. (p < 0.0001, Fig. 7). Moreover, in adoptively transferred mice, parasitemia appeared to be well controlled until day 23 p.i., after which it increased linearly until death of the animals, reaching levels as high as 4.2 × 10^6 parasites/ml (Fig. 7). In contrast, CD3+ T cells isolated from naive IL-10 KO mice were not protective when injected into infected RAG-1 KO mice. On the contrary, they accelerated...
mortality slightly, with 100% of mice dying on either day 19 or 20 p.i. \((p = 0.0008, \text{Fig. 7B})\).

**Discussion**

In the current study, we have demonstrated that IL-10 is a strong protective factor against fatal acute myocarditis in a mouse model of *T. cruzi* infection. Our results are consistent with previous reports in which genetic inactivation or immunologic neutralization of IL-10 increased mortality and acute myocarditis in mouse models of *T. cruzi* infection (12–15) and extend those reports in four important ways: 1) by identifying the T cell as a critical source of protective IL-10 through adoptive transfer studies; 2) by delineating the precise immunophenotypes of IL-10+ T cell subsets accumulating in the hearts of infected mice; 3) by showing that IL-10 deficiency results not only in exaggerated immunopathology in the hearts of infected mice but also in loss of parasite control; and 4) by showing that *T. cruzi* infection of IL-10 KO C57BL/6J mice phenocopies infection in WT parasite-susceptible C3H/HeSnJ mice, in which IL-10 induction by the parasite is weak.

With regard to finding no. 3, earlier reports had also described exaggerated immunopathology in *T. cruzi*-infected IL-10 KO mice, but in contrast to our study, this occurred in the face of reduced parasitemia levels (12–15). The discrepancy may be due to the fact that parasitemia was simply not measured at the relevant time points in the previous studies. In fact, we also found reduced parasitemia in IL-10 KO mice at early stages p.i., when parasitemia is also quite low in WT mice (day 14 in Figs. 1 and 5). However, the major point is that there is a critical time point, 14–21 d p.i. in mice, beyond which there is a loss of control, and parasitemia increases inexorably with time.

In the hearts of infected parasite-resistant C57BL/6J mice, we found that both CD4+ and CD8+ T cells express IL-10, and, consistent with a functional role, that transfer of CD3+ T cells from WT but not from IL-10 KO mice delayed mortality in susceptible RAG-1 KO mice. Additional work will be needed to define precisely whether the relevant CD3+ T cell source of IL-10 in the model is CD4+, CD8+, or both. Moreover, because rescue of infected RAG-1 KO mice with WT CD3+ T cell transfer was incomplete, other IL-10–producing leukocytes outside the T cell lineage may also contribute to protection in the model. Notably, the effect on parasitemia was also intermediate in RAG-1–deficient mice transferred with WT CD3+ T cells, which suggests that either a threshold has been crossed or else that other factors collaborate with parasitemia level to determine the mortality rate in the model. The role of CD8+ T cells in *T. cruzi* infection is likely to be complex. Although they are thought to contribute to immunopathology in patients with chronic chagasic cardiomyopathy (9, 25, 26), in mice they are highly protective during acute infection. In both CD8- and CD4-deficient animals, parasitemia is exacerbated, showing that CD4+ and CD8+ T cells are crucial for parasite control (27). CD8-deficient mice have been reported to die earlier than CD4-deficient mice (27).

A complete description of how IL-10 limits parasitemia and immunopathology in the model is not yet available, and previous work in this area has provided some conflicting results. Thus, *in vitro* IL-10 has been reported to inhibit IFN-γ-dependent (28, 29) but to promote LPS-dependent (30) elimination of the para-
Almost all IL-10+ cells within both CD4+ and CD8+ T cell subpopulations also expressed IFN-γ. The importance of the IL-10/IFN-γ double-producing CD8+ T cell subset was also suggested by the almost complete and selective absence of this subset in hearts from parasite-susceptible C3H/HeSnJ mice. In contrast, IL-10/CD4+ T cells were only modestly decreased in hearts from infected C3H/HeSnJ mice. IFN-γ/IL-10+ conventional T-bet+ Foxp3+ Th1 cells were first described in 2007 in the context of a mouse model of Toxoplasma gondii infection (33) and subsequently in the context of influenza infection (34). In T. gondii infection, these cells were predominantly CD4+ T cells, whereas in the influenza model they were predominantly CD8+ T cells. The IFN-γ/IL-10+ T cell population was shown to be crucial for immunoregulation of the response developed during infection with both pathogens (33, 34), and the IL-10/IFN-γ population displayed potent effector function against T. gondii (33). It is well known that IFN-γ-deficient mice have dramatic parasitism and mortality when infected with T. cruzi (35). However, our results show that C3H/HeSnJ mice are able to produce high levels of IFN-γ in the heart, even more than in infected C57BL/6J mice, at least after 30 d of infection. Thus, this cytokine may be necessary for protection, but it is not sufficient. With regard to other functionally important cytokines, TNF-α has been shown to synergize with IFN-γ for optimal NO production to eliminate intracellular parasitism in macrophages (29). In contrast, TNF-α has been suggested to promote heart tissue damage during T. cruzi infection (36). Our data show that the absolute levels of IFN-γ and TNF-α poorly correlate with outcome, as C3H/HeSnJ mice have a higher frequency of IFN-γ/TNF-α double-producing T cells in the heart. So, higher amounts of TNF-α could be a factor that drives immunopathology in C3H/HeSnJ mice, but this does not explain why parasitism is not controlled.

Although our results clearly establish a protective role for IL-10 in T. cruzi-infected C57BL/6J mice, the precise contribution of low IL-10 production to susceptibility in infected C3H/HeSnJ mice and the molecular basis for its deficiency were not addressed directly by our study. Next steps include mapping the gene(s) responsible for the susceptibility phenotype difference between the two strains of mice. It has been known for years that the phenotype of the F1 progeny of C3H/HeJ × C57BL/6J, as well as other H-2k × H-2b matings, is complex. F1 mice have been reported to have low parasitemia, with mortality differing by gender: females survive infection >120 d, whereas males die later than susceptible parents but earlier than resistant parents (24). Thus, the genes responsible for low parasitemia appear to be expressed in a dominant manner (24). However, IL-10 was not measured in these previous studies. A plausible mechanism for the IL-10 defect in infected C3H/HeSnJ mice is defective innate immune recognition, as TLRs and NLRs are important for host resistance against T. cruzi infection (37) and induce IL-10 production. Stimulation of innate immune cells by T. cruzi-derived GPI anchors and DNA has been reported to activate TLR2, 4, 7, and 9 (38–41), leading to production of parasiticidal agents, optimal activation of APCs, and proper activation of adaptive immune responses. With regard specifically to TLR4, we observed no difference in parasitemia or mortality for the Jackson C3H/HeJ line no. 659, which has a spontaneous mutation inactivating the TLR4 gene, and the Jackson C3H/HeSnJ line no. 661, which has a WT TLR4 gene (data not shown). In a related report, Oliveira et al. (39) have shown that C3H/HeJ and TLR4 KO mice are slightly more susceptible than C3H/HeN mice to infection with...
the reticulotropic Y strain of T. cruzi. The difference relative to our experiment could relate to differences in the source of the mice and/or the precise parasite strain that was used, but in any case was not large. Additional work will be needed to define the role of other pattern recognition receptors in IL-10 deficiency in the model.

In conclusion, we show that C3H/HeSnJ mice provide a model of acute fulminant myocarditis after T. cruzi (Colombiana strain) infection, the result of an exuberant but ineffective immune response against the pathogen, whereas C57BL/6J mice provide a model of mild acute cardiac disease, the result of a more effective immune response. IL-10 strongly correlated with outcome, and we directly demonstrated its functional importance in the model in C57BL/6J mice. T cells constitute a source of protective IL-10, and both CD4+ and CD8+ subsets in the hearts of infected mice produce it; however, other T cell-independent sources appear to exist and remain to be defined. Almost all of the IL-10+ T cells in the heart also produce IFN-γ, however the precise functional role of these cells remains to be delineated. Based on all these considerations, we hypothesize that upon T. cruzi (Colombiana strain) infection, IL-10 deficiency abrogates expansion of a cardioprotective IFN-γ+IL-10+CD8+ T cell population in C57BL/6J mice. Why infected C3H/HeSnJ mice are selectively impaired at generating this population of T cells in the heart is an important question for future research. Together, our results support a role for IL-10 as a potentially beneficial immunomodulatory agent in T. cruzi infection.

Acknowledgments
We thank Dr. Fuyuki Tokumasu and Dr. James Dvorak (Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health) for valuable ideas and infectants of chemokine mRNA expression during infection with Trypanosoma cruzi. J. Infect. Dis. 159: 1723–1733.


