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Chagas' Disease Is Attenuated in Mice Lacking γδ T Cells

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The role of γδ T cells in the immunopathology of Chagas' disease is evaluated by monitoring the course of *Trypanosoma cruzi* infection in mice lacking γδ T cells after disruption of the T-cell receptor Cδ locus. Levels of parasitemia, states of lymphocyte activation, and levels of lymphokine production as well as tissue pathology are compared in δ knockout mice and their littermates in acute and chronic phases of infection. Although the levels of circulating parasites do not significantly differ in the two groups, mortality scores and numbers of inflammatory lesions of skeletal and cardiac muscles are lower in γδ T cell-deficient mice than in littermate controls. Furthermore, polyclonal lymphocyte activation, as measured by proliferative activities and numbers of B- and T-cell blasts in the spleen, are reduced in deficient mice in the acute and chronic phases of infection. Levels of gamma interferon mRNA obtained from total spleen cells, known to be a critical lymphokine in resistance to *T. cruzi* infection, are significantly higher in uninfected γδ T cell-deficient mice than in control animals and slightly above levels for littermates in the course of acute infection. Interestingly, however, in chronic phases, the levels of this lymphokine are not statistically different between the two groups of mice. These results indicate that γδ T cells do not play a crucial role in parasite clearance during the acute phase of the disease but contribute to the mechanisms leading to tissue damage and pathology.

Chagas' disease, caused by the protozoan parasite *Trypanosoma cruzi*, results from a disturbance of the host immune system. The immune response in the murine model is characterized by an intense polyclonal activation of all lymphocyte classes, both in the acute and chronic phases of infection (4, 23, 25). Previous studies revealed that most of this massive response is not specific to the parasite antigens (22).

Within the polyclonally activated B and T cells, $CD5^+$ B and double negative γδ T cells are preferentially expanded at the early stages of infection (21, 27). We have suggested that these cells may be directly or indirectly involved in the development of autoimmune lesions in the chronic phase of the disease, as both have been associated with autoimmune reactivities and pathological processes (2, 8–11, 18, 20, 41).

In order to assess the roles of γδ T cells in predisposing for susceptibility to infection and establishing tissue pathology, we studied *Trypanosoma cruzi* infection in Cδ knockout mice, which lack γδ T cells but maintain a normal development of all other lymphocyte classes (15). During acute infection, no differences are observed between γδ T cell-deficient and control littermates with regard to levels of circulating parasites. In contrast, mortality rates are lower in mutant mice than in controls. Although γδ T cells are preferentially expanded during the acute phase of *T. cruzi* infection in nondeficient animals, our results suggest that these cells do not seem to play a critical role in parasite clearance. However, the relatively reduced rates of mortality associated with attenuated tissue inflammatory lesions observed in the chronic phase might indicate that γδ T cells contribute instead to the establishment of tissue damage and pathology.

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MATERIALS AND METHODS

Animals and infection. Young adult (7- to 8-week old) Cδ-chain homozygous ($\delta^{-/-}$) mutant mice (clone B36, from 129/Ola × C57BL/6 crosses; *H-2*$^{b/b}$) and heterozygous ($\delta^{+/-}$) littermates (15) were obtained from Peter Mombaerts and Susumo Tonegawa (Department of Biology, Howard Hughes Medical Institute, Massachusetts Institute of Technology, Boston) and maintained in our animal facilities. Progenies of the heterozygous crosses were screened by PCR performed on peripheral blood genomic DNA, using a set of primers specific for the T-cell receptor (TCR) Cδ chain and for the neomycin resistance gene. Briefly, genomic DNA was prepared from peripheral blood with the Instagene purification matrix (Bio-Rad Laboratories, Inc., Ivry sur Seine, Paris, France). Five microliters of DNA was used for the PCR, using the following primer sequences at 50 pmol/100-μl reaction mixture: TCR Cδ sense, 5′-CAA ATG TTG CTT GTC TGG TG-3′; TCR Cδ antisense, 5′-GTC AGT CGA GTG CAC AGT TT-3′; neomycin sense, 5′-CTT GGG TGG AGA GGC TAT TC-3′; and neomycin antisense, 5′-AGG TGA GAT GAC AGG AGA TC-3′. PCR products were visualized in 1.5% agarose gels in the presence of ethidium bromide. Most of the δ TCR gene is deleted in the targeted mutation, and therefore the homozygous mutant mice show only the band corresponding to the neomycin resistance gene.

Mice were injected intraperitoneally with 10^4 bloodstream forms of the CL strain of *T. cruzi* (1a). Parasitemia was recorded every 3 to 5 days, as previously described (5).

[^{3}H]thymidine incorporation. Spleen cells were obtained on the 11th day of infection and cultured (2×10^5 cells per well) for either 3 h or 72 h alone or in the presence of concanavalin A (ConA; 5 μg/ml), lipopolysaccharide (LPS; 5 μg/ml), or anti-CD3 monoclonal antibody (MAb; 25% of anti-CD3-containing supernatants; clone 145-2C11). Proliferative activity was evaluated after either a 3-h or an 18-h pulse with 1 μCi of [^{3}H]thymidine (Amersham International, Little Chalfont, Buckinghamshire, United Kingdom). Cells were harvested, and incorporated radioactivity was measured in a β-plate counter (LKB Wallace, Saint-Quentin-en-Yvelines, France).

Antibodies and reagents. Anti-CD3-ε MAb (clone 145-2C11) (19) was used for in vitro spleen cell stimulation. Fluorescein isothiocyanate-conjugated anti-CD3-ε (see above), anti-CD8, and anti-CD5 antibodies; or phycoerythrin-conjugated anti-CD4 and anti-CD8 antibodies (Caltag Laboratories, South San Francisco, Calif.); or biotin-conjugated anti-μ (Becton Dickinson, Rungis, France) antibodies were employed in fluorescence-activated cell sorter (FACS) analyses. Anti-CD3, anti-Cδ (clone 3A10) (16), and anti-Cβ (clone H57-597) (17) MAbs were produced, purified, and labelled with fluorescein isothiocyanate or biotin in our laboratory. The supernatant from clone 2.4G2, containing anti-Fc_γII/III receptor (CD32/CD16) antibodies (46), was used when processing spleen cells for FACS analysis.

Flow cytometry (FACS). Spleen cells (10^6 cells) were directly stained with MAb diluted in balanced salt solution–1% fetal calf serum–0.01% azide. In these experiments cells were preincubated with anti-CD32/CD16 antibodies in order to block immunoglobulin nonspecific binding through Fc receptors. Biotinylated antibodies were further incubated with fluorescein isothiocyanate or phyco-

erythrin-streptavidin conjugates. Two-color analysis was carried out with a FACScan cytofluorometer (Becton Dickinson and Co., Mountain View, Calif.). Dead cells were excluded from the analysis by gating out propidium iodide-stained cells. Lymphocytes were gated on forward-light scatter, and 5×10^3 to 8×10^3 events were acquired. Results were analyzed with Lysis II software.

Semiquantitative PCR for mRNA detection. For details of primers and probe design, as well as determinations of the optimal cycle numbers for the PCR amplifications and the principles for standardization of the experimental samples, see references 24 and 30. Briefly, total spleen cell RNA from individual mice and poly(A^+) RNA from the HDK1-Th1 clone (the standard sample with a known mRNA concentration) were extracted and reverse transcribed. The resulting cDNAs were then amplified by PCR with primers for the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) in a thermal cycler (GeneAmp 9600 PCR system; Perkin-Elmer Cetus, Saint-Quentin-en-Yvelines, France) in the presence of thermalase DNA polymerase (one cycle of 2 min at 92°C, followed by 30 cycles of 10 s at 91°C, 25 s at 59°C, and 25 s at 72°C). Dot blots of the PCR products were hybridized with a specific [γ-^{32}P]ATP-labelled HPRT probe, internal to the amplified HPRT gene product. Membrane signals were quantitated after autoradiography (Kodak X-Omat film) with a Masterscan (Bionis, Richebourg, France) or analyzed with a PhosphorImager scanner (Molecular Dynamics, Ivry, France).

mRNA levels in the samples were then normalized to a given value of HPRT mRNA expressed from the standard titration of HDK1 PCR products (such that all samples contained similar levels of HPRT mRNA). Samples and the HDK1 cDNA (used to produce the standard curve) were then amplified with primers specific to gamma interferon (IFN-γ). Samples containing the same amount of HPRT mRNA and serial dilution of standard sample were examined by dot blot analysis and hybridized with an internal [γ-^{32}P]ATP-labelled IFN-γ probe. Units of IFN-γ gene expression in experimental samples were then calculated after quantifying the results of these final dot blots from the linear part of the (Th1)-HDK1 standard curve and expressed as equivalent to picograms of input RNA.

Measurement of TNF activity. Levels of tumor necrosis factor (TNF) were determined by the cytotoxic assay with TNF-sensitive L929 murine fibroblasts treated with actimomycin D (Sigma). L929 fibroblasts (3×10^4 cells per well) were cultured in 96-well flat-bottom plates in minimal essential medium supplemented with antibiotics and 5% fetal calf serum overnight at 37°C in a 5% CO_2 humidified atmosphere. The medium (50 μl) was replaced in parallel with the addition of a serial dilution of serum samples and recombinant TNF alpha (TNA-α) as the standard (Pharmigen, San Diego, Calif.). For serum-positive control of TNF activity, priming of C57BL/6 mice was done with 10^7 viable units of *Mycobacterium avium* BCG followed 21 days later by LPS challenge 1 h before bleeding. Actinomycin D (0.4 μg) was added to each well. After 20 h of incubation, plates were washed with 0.9% saline and cells were stained and fixed with a solution of 0.05% crystal violet in 20% ethanol. Optical densities at 595 nm were read with a Titertek Multiscan (Flow Laboratories). Lysis (50%) of L929 cells was obtained with a concentration of 65 pg of recombinant TNF-α. Values of TNF activity (in picograms per milliliter) for each sample were determined from the curve obtained with the recombinant TNF-α standard. Units of TNF activity were obtained in parallel by multiplying the reciprocal of the highest dilution resulting in 50% lysis by the dilution factor of the sample. Serum from C57BL/6 mice challenged with BCG-LPS, as described above, corresponded to 40,000 U/ml of biological activity per 5 μg of TNF-α per ml.

Histopathological analyses. Infected mice were killed after 52 days of infection, and tissue specimens were collected and fixed in paraformaldehyde (4% in PBS) for further processing. Extra $\delta^{-/-}$ and $\delta^{+/-}$ mice which were not killed by the infection or which were not used for the experiments on day 52 were maintained under the conditions of our animal care facilities, and tissue specimens were collected 2 years after infection and processed as described above. Paraffin-embedded tissue sections were stained by hematoxylin-eosin and subjected to microscopic analyses. Individual slides were analyzed in a blind fashion, and scores were ascribed according to the extent of inflammation (+++, frequent and very intense; ++, frequent and intense; +, occasional and attenuated; ±, rare and weak; and 0, absent).

Statistical analyses. The differences between the groups of mice used in this study were analyzed for statistical significance by the Student *t* test and expressed as arithmetic means ± the standard deviation (SD). The data concerning parasitemia, however, are presented as arithmetic means ± the standard error of the mean. With respect to mortality, the data were analyzed by the Mantel-Cox test. For the histopathological findings, data were analyzed with StatView-SE software for the Macintosh. The significance level between groups was calculated by the chi-square test. Significant differences were at the 0.05 confidence level, with 1 df.

RESULTS

The outcome of *T. cruzi* infection is different in δ-deficient and control mice. Mutant homozygous ($\delta^{-/-}$) mice and heterozygous ($\delta^{+/-}$) littermates were infected with *T. cruzi* intraperitoneally, and levels of parasitemia were scored individually every 3 to 5 days. Figure 1A shows the parasitemia profiles

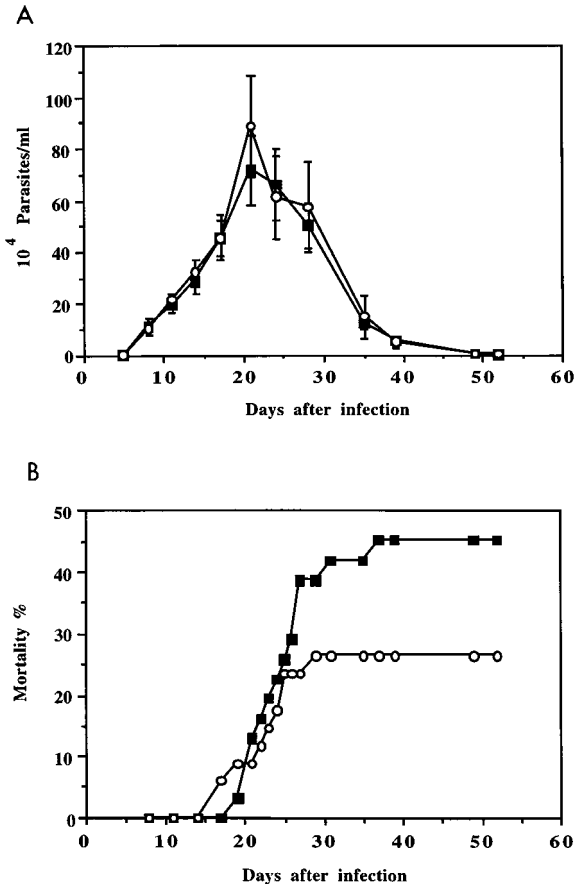

FIG. 1. (A) Numbers of circulating parasites during the course of infection with *T. cruzi* of $\delta^{+/-}$ (■) and $\delta^{-/-}$ (○) mice. Mice were infected with 10^4 bloodstream forms of the CL strain of *T. cruzi*. Levels of parasitemia were scored every 3 to 5 days, as described in Materials and Methods. The data are arithmetic means (± the standard error) of 30 to 40 mice per group analyzed individually. (B) Cumulative mortality rates of $\delta^{+/-}$ (■) and $\delta^{-/-}$ (○) mice after *T. cruzi* infection. Thirty-one $\delta^{+/-}$ and 34 $\delta^{-/-}$ mice were injected intraperitoneally with 10^4 parasites. Mortality rates were scored until 52 days postinfection. Mice were kept under constantly maintained conditions during the period analyzed, with no manipulation other than the monitoring of parasitemia. At least 14 mice per group were killed at day 52 for histopathological examination (see Table 1). $P = 0.1497$ (by the Mantel-Cox test).

obtained from 30 to 40 mice per group analyzed individually. As typically observed in normal mouse strains infected with the CL strain of *T. cruzi*, the number of parasites increased in the first 2 weeks of infection. Mice which were not killed by the infection then entered a chronic phase, with a progressive decrease in parasitemia levels after reaching peak levels of around 7.5×10^5 circulating parasites per ml by day 20. The blood-circulating parasites became undetectable after almost 2 months of infection. As shown in Fig. 1A, no significant differences were detected in the levels of parasitemia between both groups of mice during the entire period analyzed.

As can be seen in Fig. 1B, after 25 days of infection the numbers of surviving mice were similar for both groups (74.19 and 76.47%). Interestingly, however, from day 28 onwards, concomitant with the decrease in parasitemia levels, a dichotomy can be observed in the numbers of deaths in the two groups. Thus, at this time point 23.5% of γδ T cell-deficient animals had died whereas mortality was 38.7% among their heterozygous littermates. After this time point, until day 52 of infection, although the number of deaths remained constant in

TABLE 1. Histopathological findings for cardiac and skeletal muscles of mice after 52 days of infection

Muscle type	Score[a]	% of mice (*P* value) of individual type with[b]: Infiltrative focus		Focal myocytolytic necrosis		Interstitial edema		Neuritis		Vascular congestion		Parasite cluster	
		$\delta^{+/-}$	$\delta^{-/-}$	$\delta^{+/-}$	$\delta^{-/-}$	$\delta^{+/-}$	$\delta^{-/-}$	$\delta^{+/-}$	$\delta^{-/-}$	$\delta^{+/-}$	$\delta^{-/-}$	$\delta^{+/-}$	$\delta^{-/-}$
Skeletal	0/±/+	5.3	64.3	5.3	21.4	42.1	78.6	100.0	100.0	100.0	100.0	100.0	100.0
	++/+++	94.7	35.7 (0.0003)	94.7	78.6 (0.1597)	57.9	21.4 (0.0362)	0.0	0.0 (NC[c])	0.0	0.0 (NC)	0.0	0.0 (NC)
Cardiac	0/±/+	33.3	64.3	72.2	100.0	72.2	100.0	88.9	100.0			100.0	100.0
	++/+++	66.7	35.7 (0.0818)	27.8	0.0 (0.0318)	27.8	0.0 (0.0318)	11.1	0.0 (NC)			0.0	0.0 (NC)

[a] Clinical evaluation of pathology. The scores were ascribed upon the analysis of all tissue section fields. +++, frequent and very intense; ++, frequent and intense; +, occasional and attenuated; ±, rare and weak; and 0, absent.

[b] Results represent the percentages of mice per group with the indicated histopathological findings. The differences between groups with respect to pathology parameters were analyzed by the chi-square test. Individual slides obtained from 17 $\delta^{+/-}$ and 14 $\delta^{-/-}$ mice were examined in a blind fashion.

[c] NC, not calculable.

the γδ T cell-deficient animals (26.5%), mortality increased in the control group (45.2%). Although the percentage of survivors is consistently higher in the $\delta^{-/-}$ mice than in control mice, the probability observed is not significant ($0.10 < P < 0.15$).

It is interesting that the general appearance and behavior of the mice were not similar between the two groups in that the cachexia characteristic of the normal strains (and of $\delta^{+/-}$ mice) was not observed among the $\delta^{-/-}$ animals. Attempting to objectively determine the possible reasons for the decreased level of wasting observed in $\delta^{-/-}$ mice, we measured TNF activity by evaluating serum cytotoxic effects towards TNF-sensitive L929 fibroblasts (see Materials and Methods). Interestingly, however, no detectable TNF activity was observed in the serum of the $\delta^{+/-}$ or $\delta^{-/-}$ group of mice after 11 or 52 days of infection, compared with the activity observed in the serum of uninfected C57BL/6 mice challenged with BCG and LPS 1 h before bleeding (for which a 50% lysis of L929 cells was obtained with a 1/20,000 serum dilution [data not shown]) and no correlation between mouse type and the differences in cachexia and wasting could be established.

γδ T cell-deficient mice present attenuated inflammatory lesions in muscle tissues. Heart and skeletal muscle histology was carried out to examine whether the absence of γδ T cells could affect the extent of tissue damage. Tissue sections obtained from 17 $\delta^{+/-}$ and 14 $\delta^{-/-}$ mice were examined in a blind fashion to ascribe scores for different histopathological parameters of Chagas' disease, namely, infiltrative focus, focal myocytolytic necrosis, interstitial edema, interstitial fibrosis, parasite clusters, neuritis, and vascular congestion. Animals were killed at day 52, and on the basis of our previous experience, we considered these results to reflect a typical chronic tissue condition established at the end of visible parasitemia (1).

Slides containing tissue specimens were subjected to microscopic analysis, and scores were ascribed (see Materials and Methods and Table 1). The skeletal and cardiac muscle lesions were characteristic of *T. cruzi* chronic-phase infection with an accentuated mononuclear infiltrate and myocytolytic necrosis. However, significant differences were observed between the two groups of mice with regard to the extent of the inflammatory process (Table 1). Thus, $\delta^{-/-}$ mice presented a statistically significant lower level of lymphocytic infiltration ($P = 0.0003$) and interstitial edema ($P = 0.0362$) in skeletal tissues. Furthermore, levels of edema ($P = 0.0318$) and necrosis of myocardial fibers ($P = 0.0318$) were markedly reduced in the γδ T-cell deficient mice. Although the scores for focal myocytolytic necrosis and infiltrative foci in skeletal and cardiac muscles, respectively, were not particularly different, the levels of these histopathological parameters tended to be lower in $\delta^{-/-}$ mice than in $\delta^{+/-}$ mice. Rare parasite clusters were found in the tissues of both groups of mice. The scores attributed to neuritis, vascular congestion, and interstitial fibrosis were not significantly different between $\delta^{-/-}$ and $\delta^{+/-}$ mice.

Figure 2 is representative of the histopathological findings observed in both groups. In skeletal muscle, the infection of $\delta^{-/-}$ mice induced a diffuse mononuclear infiltrate with a discrete perivascular inflammatory process with mild fiber destruction (Fig. 2A). $\delta^{+/-}$ mice developed an inflammatory reaction that was more widespread and intense, with more important myocytolitic focal lesions (Fig. 2B). In heart muscle, focal inflammatory sites were observed in $\delta^{-/-}$ mice with occasional perivascular foci (Fig. 2C). Again, compared with the $\delta^{-/-}$ animals, the frequencies of inflammatory foci and myocytolytic focal necrosis were higher in the hearts from $\delta^{+/-}$ mice (Fig. 2D). Extra $\delta^{+/-}$ and $\delta^{-/-}$ mice that were not scheduled for histopathological analysis at 52 days of infection were kept alive in our animal care facilities. Interestingly, the results presented above are strengthened by the fact that only $\delta^{-/-}$ animals survive the chronic infection by 2 years. The analysis of cardiac muscle revealed that 100% of the mice presented rare and weak infiltrative focus, compared with the 64.3% scored at day 52 of infection. No other changes were observed concerning the other pathological parameters analyzed early in infection. However, while an attenuation in interstitial edema was scored in skeletal muscles (mice with occasional and attenuated edema = 100%), which was paralleled by no increases in levels of myocytolytic necrosis, $\delta^{-/-}$ mice showed a frequent and very intense infiltrative focus in skeletal muscles (data not shown).

Lymphocyte-proliferative activity in the acute phase of infection is reduced in γδ T cell-deficient mice. Acute *T. cruzi* infection induces polyclonal activation of all lymphocyte classes in peripheral lymphoid organs, probably the origin of later tissue inflammation. In order to examine whether the state of lymphocyte activation is modified in γδ T cell-deficient mice during acute infection, spleen cells were obtained from $\delta^{+/-}$ and $\delta^{-/-}$ mutant mice at day 11 of infection and compared with spleen cells from uninfected animals of both types. Uninfected mice of both types had comparable numbers of total spleen cells (around 8×10^7 cells) and of blastic lymphocytes (around 13%), as determined by light-scatter FACS analysis.

Upon infection, extensive cell activation was induced in the spleen (see below). Lymphocytes that are given [^{3}H]thymidine immediately after recovery provide a measure of the levels of proliferative activity in the spleen (ex vivo proliferation). As

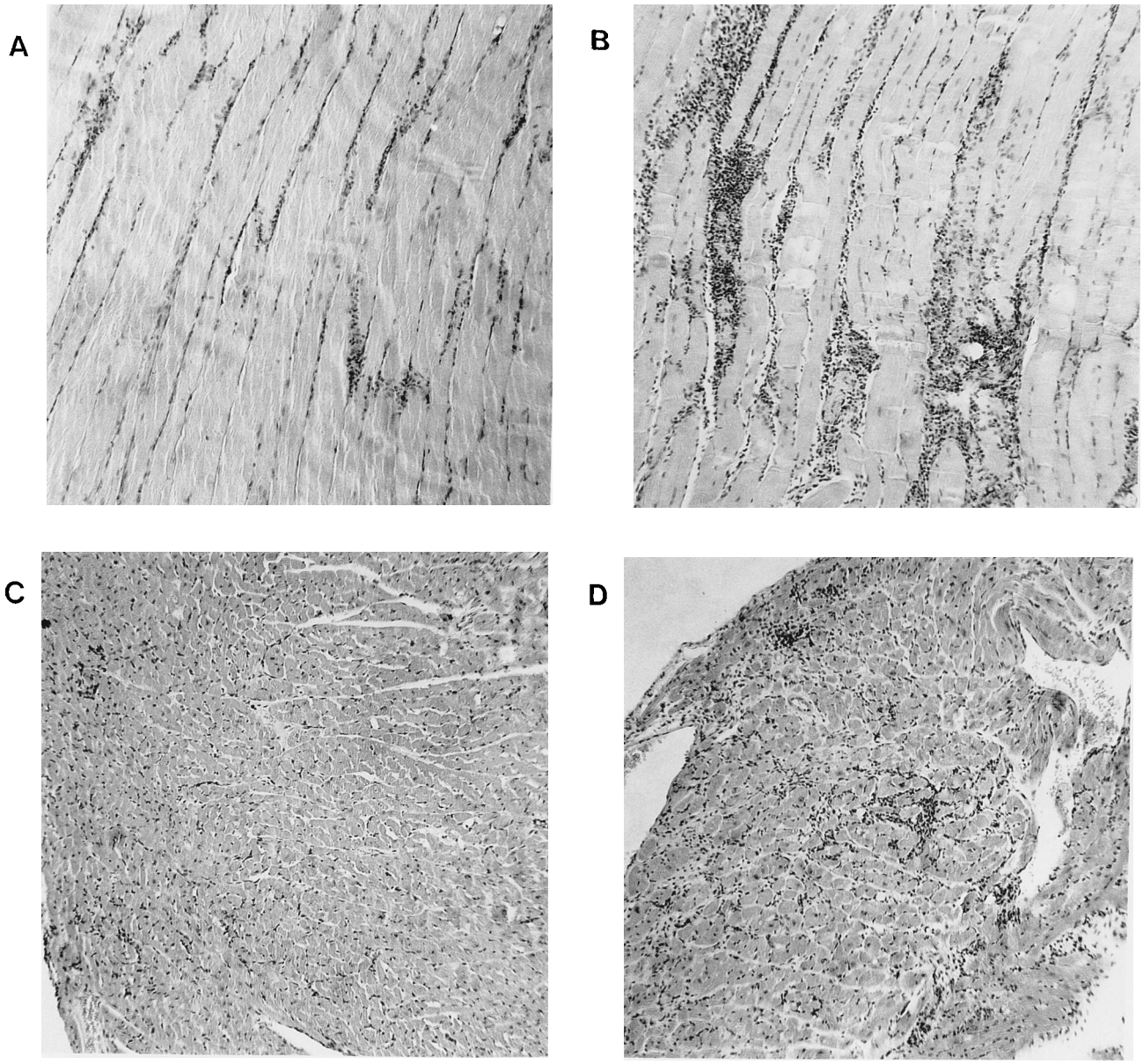

FIG. 2. Histopathological analysis of skeletal (A and B) and cardiac (C and D) muscles at day 52 postinfection from $\delta^{-/-}$ (A and C) and $\delta^{+/-}$ (B and D) mice. The tissues were removed, fixed in 4% paraformaldehyde, and embedded in paraffin. The specimens were processed for light microscopy after hematoxylin and eosin staining and evaluated under 100× magnification.

can be seen in Fig. 3A, spleen cells from both types of infected mice incorporated severalfold more thymidine than the spleen cells from uninfected mice of the corresponding type. The levels of thymidine incorporation by the lymphocytes from the infected $\delta^{-/-}$ or $\delta^{+/-}$ mice were similar to the levels obtained by lymphocytes from the well-characterized C57BL/6 mice (see the legend to Fig. 3). Interestingly, the level of spontaneous proliferation of cells from $\delta^{-/-}$ mice was significantly lower than that of cells from infected $\delta^{+/-}$ animals.

Previous reports have described that *T. cruzi* infection induces an intense immunosuppression of total spleen cell responses to T- or B-cell mitogens (27). Although the responses of both $\delta^{+/-}$ and $\delta^{-/-}$ mice to mitogenic stimulations are indeed suppressed when compared with responses of normal uninfected controls (see the legend to Fig. 3), we were unable to detect any significant differences in [^{3}H]thymidine uptake at 72 h in spleen cell cultures from both groups of infected mice stimulated by ConA, LPS, or anti-CD3 antibodies (Fig. 3B).

Reduced splenomegaly in γδ T cell-deficient mice at late stages of infection. We next evaluated the state of spleen lymphocyte activation in late stages of infection. Spleen cells from infected $\delta^{+/-}$ and $\delta^{-/-}$ mice at day 52 were compared with those obtained from normal and acutely infected animals. Cells were stained with appropriate lymphocyte surface markers and analyzed by FACS analysis.

During the first 11 days of infection, the total number of spleen cells doubled and the number of lymphocyte blasts increased by around fivefold in both $\delta^{+/-}$ and $\delta^{-/-}$ mice (Fig. 4A). However, on day 52 postinfection, although the numbers

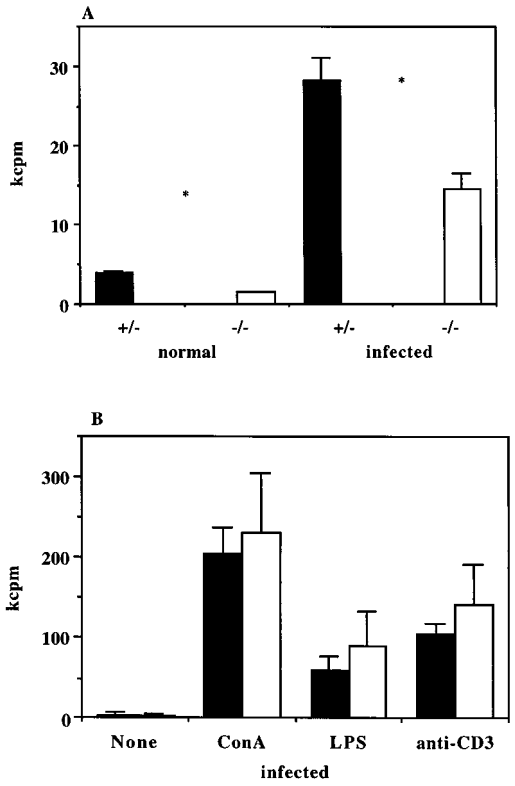

FIG. 3. (A) Spontaneous lymphocyte proliferation of spleen cells from $\delta^{+/-}$ (■) and $\delta^{-/-}$ (□) mice after 11 days of infection. Spleen cells (2×10^5) were cultured for 3 h in medium containing 1 μCi of [^{3}H]thymidine and then harvested to determine the levels of spontaneous incorporation. For comparison, spontaneous incorporation values obtained from normal or infected C57BL/6 mice were 4.4 kcpm and 45.0 kcpm, respectively. Data are arithmetic means (± SD) from 3 to 4 mice per group analyzed individually and are representative of different experiments. *, $P < 0.05$. (B) [^{3}H]thymidine uptake by spleen cells from $\delta^{+/-}$ (■) and $\delta^{-/-}$ (□) mice after mitogenic stimulation. Spleen cells (2×10^5) were cultured for 72 h with 5 μg of ConA per ml, 5 μg of LPS per ml, or 25% of 145-2C11 anti-CD3-containing supernatants. Cultures were pulsed with 1 μCi of [^{3}H]thymidine per culture for the last 18 h of culture, and counts were obtained with a β-plate counter. The amounts of [^{3}H]thymidine uptake by spleen cells from normal uninfected C57BL/6 mice stimulated by ConA, LPS, and anti-CD3 after 72 cultures were 302.8 ± 24.4 kcpm, 278.5 ± 68.2 kcpm, and 614.3 ± 65.1 kcpm, respectively.

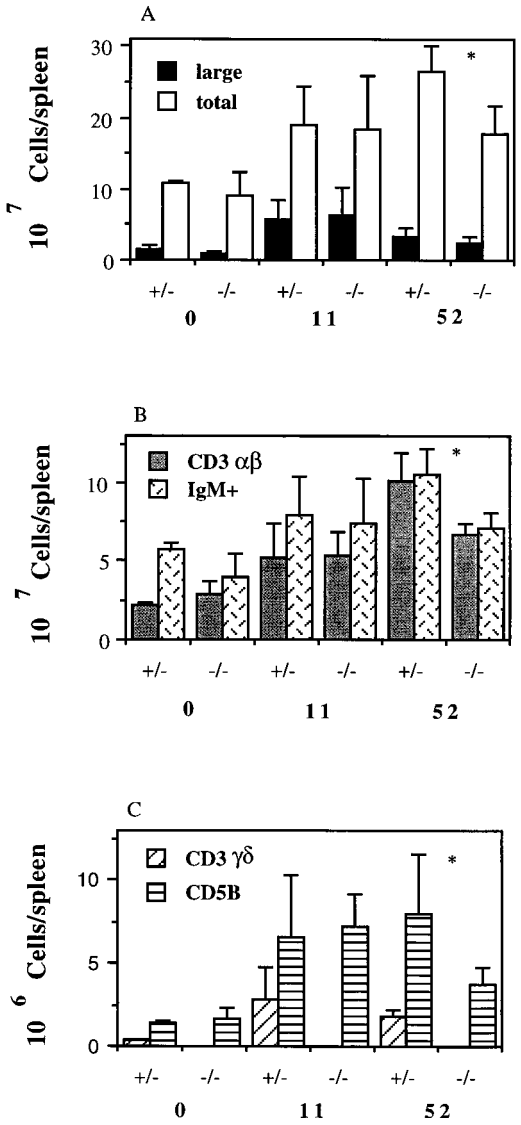

FIG. 4. Analysis of splenic lymphocyte populations obtained from normal $\delta^{+/-}$ and $\delta^{-/-}$ mice, $\delta^{+/-}$ and $\delta^{-/-}$ mice infected for 11 days (acute), and $\delta^{+/-}$ and $\delta^{-/-}$ mice infected for 52 days (chronic). Data are arithmetic means (± SD) of four mice per group analyzed individually and are representative of different experiments. (A) Total numbers of spleen cells and large cells within total lymphocytes. Numbers of large cells were obtained from light-forward-scatter distribution of the lymphocyte-gated population. Cells for which light forward scatter exceeded the normal gaussian distribution of small lymphocytes were considered large. (B) $CD3^+$ $\alpha\beta^+$ and IgM^+ cells. (C) $CD3^+$ $\gamma\delta^+$ and $CD5^+$ B cells. *, $P < 0.05$.

of total spleen cells are significantly lower in the knockout mice, compared with those for the heterozygous controls, both groups showed comparable numbers of activated lymphocytes.

As can be seen in Fig. 4B, the $\delta^{+/-}$ mice show typical increases in $CD3^+$ $\alpha\beta^+$ and immunoglobulin M^+ cell populations after 52 days of infection. Within $CD3^+$ cells, preferential increases in levels of $CD8^+$ lymphocytes were observed for both groups ($\delta^{+/-}$ and $\delta^{-/-}$ mice) over levels of $CD4^+$ cells (CD4/CD8 cell ratios of 1.2 and 1.0, respectively; data not shown). The expansion of $CD5^+$B cells recorded for $\delta^{+/-}$ mice was also significantly greater than that observed with $\delta^{-/-}$ animals (Fig. 4C). As already described for acute phases of infection (21, 27), increases in levels of $CD3^+$ $\gamma\delta^+$ T cells were observed in $\delta^{+/-}$ animals in the chronic phase.

γδ knockout mice constitutively produce high amounts of IFN-γ mRNA. In an attempt to reveal specific mechanisms associated with the relative resistance to infection of γδ T cell-deficient mice, we studied the expression of IFN-γ, previously shown to be essential to such resistance (24, 32). Analysis of IFN-γ gene expression was performed ex vivo in spleen cells from normal or infected mice of both groups. Total splenic RNA from individual mice was reverse transcribed, and the cDNA obtained was used for PCR amplifications. The samples were adjusted, as described in Materials and Methods, and equivalent amounts of input RNA were blotted and hybridized with specific internal probes.

The quantitative analysis of the resulting autoradiograms or membranes, done in parallel with a standard serial dilution of sample, showed that $\delta^{-/-}$ mice constitutively produce increased levels of mRNA for IFN-γ when compared with $\delta^{+/-}$ animals. Thus, as can be observed in Table 2, uninfected $\delta^{-/-}$ mice express significantly higher levels of IFN-γ mRNA than $\delta^{+/-}$ animals. Interestingly, although the parasite does not induce statistically different levels of expression of IFN-γ

TABLE 2. IFN-γ mRNA from total spleen cells in the course of *T. cruzi* infection in $\delta^{+/-}$ and $\delta^{-/-}$ mice

Group[b]	Amt (pg) of IFN-γ mRNA equivalent to 10^3 pg of HPRT mRNA[a] (*P* value) for:		
	Noninfected mice	Mice infected 11 days	Mice infected 52 days
$\delta^{+/-}$	34.07 ± 25.8	106.39 ± 53.72	133.92 ± 56.45
$\delta^{-/-}$	69.25 ± 37.85 (0.034)	150.09 ± 79.91 (0.072)	135.90 ± 65.02 (0.441)

[a] Before IFN-γ-specific amplification cDNA samples were made equivalent to 10^3 pg of housekeeping HPRT mRNA, as described in Materials and Methods. Equivalent amounts of cDNA were then amplified with IFN-γ-specific primers, and the same amounts of PCR products were blotted with a Minifold apparatus (Scheleicher & Schuell, Ceralabo, Aubervilliers, France). Membranes were hybridized with an IFN-γ-specific internal probe labelled with [γ-^{32}P]ATP. Autoradiographies were quantified with a Masterscan or by direct counting of the membranes with a PhosphorImager scanner. Results are arithmetic means of two independent experiments ± SD.

[b] Each group contained at least 6 to 10 animals analyzed individually.

throughout the period of infection in the two groups, on the 11th day, spleen cells from $\delta^{-/-}$ mice seem to express slightly increased levels of RNA encoding this lymphokine with the levels produced by their $\delta^{+/-}$ littermates.

DISCUSSION

We have previously described a preferential expansion of γδ T cells in the early phases of experimental *T. cruzi* infection and hence suggested that these cells could be either directly or indirectly associated with the mechanisms leading to pathology in the murine model of Chagas' disease (21, 27). In this study we show that the responses of mice lacking γδ T cells to *T. cruzi* infection are different from those of controls bearing a normal γδ T-cell repertoire. Although no differences were found in the number of circulating parasites during the acute phase of infection, γδ T cell-deficient mice showed relatively lower rates of mortality and reduced tissue inflammation in the chronic phase. Furthermore, blastogenic lymphocyte responses to infection were reduced in deficient mice later on in the infection. These results suggest that in Chagas' disease, γδ T cells are not implicated in the clearance of circulating parasites in early phases of infection but rather in the mechanisms leading to tissue damage and death. Our results, showing comparable levels of parasitemia in γδ T cell-competent and -deficient mice, support the notion that parasite clearance is due to αβ T cells and not γδ T cells.

In several infectious diseases, γδ T cells have been found in increased numbers and actually infiltrating the infected sites (8). In some infections, γδ T cells have been directly implicated in the mechanisms associated with resistance. For example, animals lacking γδ T cells (by in vivo cell depletion or by germ-line mutation of the TCR δ-chain gene) developed larger lesions containing increased parasite numbers after *Leishmania major* infection (37) and an increased load of *Plasmodium yoelii* (45) or *Listeria monocytogenes* (12, 28). In this last case, even enlarged granulomatous lesions or necrosis in the liver has been observed (7). In these infections, however, resistance has also been associated with mechanisms involving a major participation by $CD4^+$ Th1-type lymphocytes (29, 40). On the contrary, in *T. cruzi* infection, resistance and parasite clearance are dependent on the activities of both $CD8^+$ and $CD4^+$ αβ T cells (26, 27, 43). Moreover, mice susceptible to *T. cruzi* infection produce both Th1 and Th2 types of cytokines (24, 33), which is very different from the clear-cut Th1-Th2 dichotomy observed in the previously cited infections.

IFN-γ production has been shown to be related to resistance in experimental *T. cruzi* infection (24, 31, 32). After infection, the progression of disease is closely controlled by cytokines and the very early production of these cytokines may be essential for the determination of disease outcome. Interestingly, $\delta^{-/-}$ mice are better constitutive producers of IFN-γ than normal controls. It is noteworthy that after infection, similar levels of IFN-γ mRNA are detected in resistant and susceptible mice, even when considering different models (24, 33), a condition we observed 11 days after infection. These observations also suggest that γδ T cells may negatively regulate IFN-γ production by other cell types.

Concerning the class (Th1 or Th2) determination of the immune responses, other reports have suggested a contribution of γδ T cells in the discrimination of appropriate primary responses predisposing resistance (6) or in the maintenance of tissue-specific tolerance (3). Our results indicate that in *T. cruzi* infection these alternatives are less likely. We have shown that attenuated tissue aggression and relatively reduced levels of mortality occur in the γδ T cell-deficient groups of mice. In addition, given the probable autoimmune reactivities leading to chronic Chagas' disease, our results implicate γδ T cells in the development of tissue damage. This could be due either to direct cytotoxic activity (39) of γδ T cells towards parasite-infected cells, by direct interaction with (autoreactive) inflammatory $CD4^+$ cells, or by indirect participation in the mechanisms leading to tissue injury. These hypotheses are supported by the elevated numbers of γδ T cells that have been described for a variety of human autoimmune pathologies and murine models of autoimmunity (8), which suggests that these cells play a role in the establishment of progressive diseases (8, 13). Thus, it has been previously established the neuronal and muscular pathologies that dominate the chronic phases of *T. cruzi* infection are dependent on the activity of autoreactive $CD4^+$ T cells (14, 36) and also involve major infiltrates of $CD8^+$ T cells, including those bearing γδ TCR (34, 35, 42, 44).

Low levels of lymphocyte commitment and reduced inflammation in chronic phases in $\delta^{-/-}$ mice associated with constitutively high levels of IFN-γ sustain the differences in the outcome of infection and add biological meaning and consistency to the differences in mortality rates. Since susceptibility has been correlated with a very early and precise control of cytokine regulation following *T. cruzi* infection, including the production of high systemic levels of acute-phase cytokines (38), the relatively decreased level of mortality observed in deficient mice would be compatible with γδ T cell participation in the control of cytokine production.

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