INFECTION AND IMMUNITY, Jan. 2002, p. 36–48 Vol. 70, No. 1
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.1.36–48.2002
Copyright © 2002, American Society for Microbiology. All Rights Reserved.

During *Trypanosoma cruzi* Infection CD1d-Restricted NK T Cells Limit Parasitemia and Augment the Antibody Response to a Glycophosphoinositol-Modified Surface Protein

Malcolm S. Duthie,[1] Monika Wleklinski-Lee,[1] Sherilyn Smith,[1] Toshinori Nakayama,[2] Masaru Taniguchi,[2] and Stuart J. Kahn[1,3]*

Departments of Pediatrics[1] and Pathobiology,[3] University of Washington, Seattle, Washington 98195, and Department of Molecular Immunology, Chiba University, Chiba, Japan[2]

Received 18 May 2001/Returned for modification 25 July 2001/Accepted 1 October 2001

***Trypanosoma cruzi* is a protozoan parasite that chronically infects many mammalian species and in humans causes Chagas' disease, a chronic inflammatory disease. The parasite expresses glycophosphoinositol (GPI), which potently stimulates interleukin 12 (IL-12) production. During *T. cruzi* infection IL-12, and possibly GPI, might stimulate NK T cells to affect the protective and chronic inflammatory responses. Here we report that during *T. cruzi* infection CD1d-restricted NK T cells are stimulated as NK T-cell-deficient mice have greater parasitemia. Furthermore, during *T. cruzi* infection the percentages of NK T cells in the liver and spleen become decreased for prolonged periods of time, and in vitro stimulation of NK T cells derived from livers of chronically infected mice, compared to uninfected mice, results in increased gamma interferon and IL-4 secretion. Moreover, in NK T-cell-deficient mice the chronic-phase antibody response to a GPI-modified surface protein is decreased. These results indicate that, during the acute infection, NK T cells limit parasitemia and that, during the chronic phase, NK T cells augment the antibody response. Thus, during *T. cruzi* infection the quality of an individual's NK T-cell response can affect the level of parasitemia and parasite tissue burden, the intensity of the chronic inflammatory responses, and possibly the outcome of Chagas' disease.**

Trypanosoma cruzi chronically infects a variety of mammalian species (55). In Latin America 18 million people are infected (55). During the acute phase of the infection *T. cruzi* disseminates in the mammalian host and a detectable parasitemia occurs (55). Typically the acute phase resolves without significant pathology, but *T. cruzi* infection persists for the lifetime of the host and stimulates a chronic inflammatory process (55). In 30% of *T. cruzi*-infected humans, the chronic inflammatory process results in Chagas' disease, a chronic inflammatory disease that causes significant morbidity and mortality (55). The pathogenesis of the chronic inflammatory disease remains unclear.

Previous studies have demonstrated that β2-microglobulin$^{-/-}$ mice and mice deficient in CD4$^+$ T cells or NK1.1$^+$ cells have increased susceptibility to acute *T. cruzi* infection and have argued that major histocompatibility complex class I-restricted T cells, CD4 T cells, and NK cells provide protection (5, 12, 44, 45, 49). In all these mice, however, NK T cells were also depleted, and therefore these studies also support the possibility that NK T cells provide protection against *T. cruzi*. NK T cells are a subset of T cells distinct from conventional T cells and NK cells; they express some receptors of both cell types, e.g., the T-cell receptor (TCR) and the NK1.1 receptor (20). More than 50% of NK T cells in the liver and spleen are CD4$^+$ (20). Many NK T cells use an invariant TCR α chain (in mice Vα14-Jα281 and in humans Vα24-JαQ) paired with limited TCR β chains (mostly Vβ8.2 in mice and Vβ11 in humans) (20). In contrast with conventional T cells, NK T cells are stimulated by glycolipids presented by the major histocompatibility complex class I-like CD1d molecular complex composed of β2-microglobulin and the nonpolymorphic CD1d chain (20). Mice lacking *CD1d* or TCR Jα281 genes are deficient in NK T cells (14, 16, 39, 47). NK T cells can provide protection against infections by rapidly producing gamma interferon (IFN-γ) and interleukin 4 (IL-4), through cytolytic activity, or by stimulating NK cell responses (13, 19, 20). When an infection is not present, some NK T cells appear to secrete cytokines that inhibit self-damaging responses (20, 26). In several mouse models of autoimmune diseases and chronic inflammatory diseases, and in two human autoimmune diseases (diabetes and systemic scleroderma), the NK T-cell population is diminished and the ability of the cells to inhibit self-damaging responses appears to be impaired (20, 26).

NK T cells can be stimulated by IL-12 or TCR ligation to rapidly initiate effector functions and then, within hours, to undergo activation-induced cell death (AICD) (18, 37). The "natural" ligands of NK T-cell TCR remain unclear. The marine sponge-derived glycolipid α-galactosyl ceramide (α-GalCer) is the only known NK T-cell TCR ligand (35), and glycophosphoinositol (GPI) has been implicated as an NK T-cell TCR ligand (31, 46). During *Plasmodium berghei* infection of mice NK T cells have been reported to respond to GPI of *Plasmodium* proteins by secreting IL-4 that helps the immunoglobulin G (IgG) response to these proteins (46). *T. cruzi* expresses abundant GPI that is a potent stimulator of IL-12 production (2). Therefore, during *T. cruzi* infection, parasite-produced GPI may stimulate a protective NK T-cell response by ligating NK T-cell TCRs or by stimulating IL-12 production that then stimulates the NK T cells. Furthermore, if NK T cells

* Corresponding author. Mailing address: Department of Pediatrics, University of Washington, Box 356320, 1959 N.E. Pacific St., Seattle, WA 98195-6320. Phone: (206) 543-4424. Fax: (206) 221-5469. E-mail: stujk@u.washington.edu.

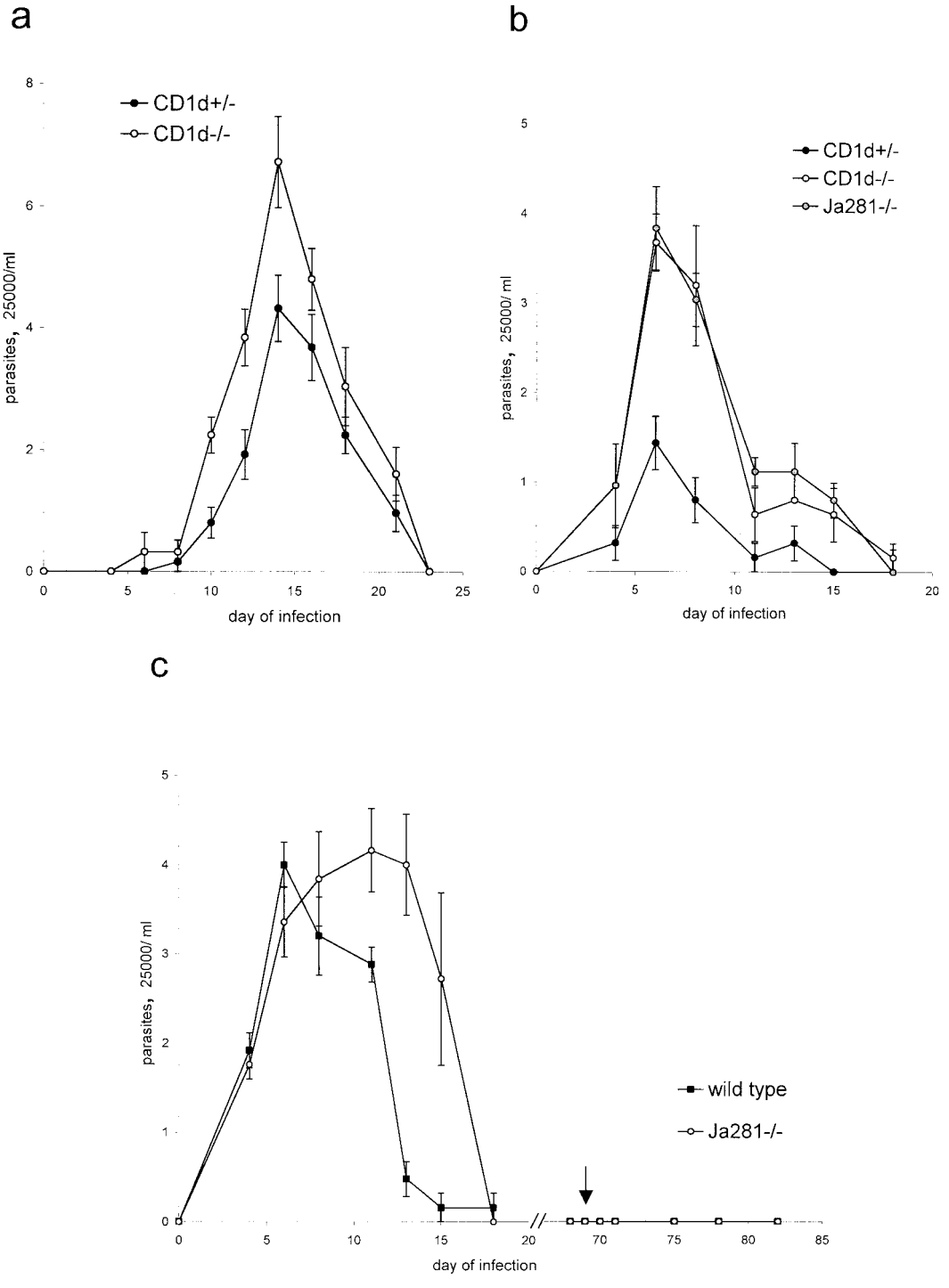


FIG. 1. During acute *T. cruzi* infection NK T-cell-deficient mice have increased parasitemia. In these experiments groups of five mice were infected with *T. cruzi* and parasitemia was monitored. (a) C57BL/6 $CD1d^{+/-}$ or $CD1d^{-/-}$ mice; (b) C57BL/6 $CD1d^{+/-}$, $CD1d^{-/-}$, or $J\alpha281^{-/-}$ mice; (c) BALB/c wild-type or $J\alpha281^{-/-}$ mice. C57BL/6 mice were infected with 10^5 trypomastigotes (a and b), and BALB/c mice were infected with 5×10^4 trypomastigotes (c). (c) On day 68 of the infection (arrow) the mice received a second inoculation of 10^6 trypomastigotes. The mean parasitemia and SEM (error bar) per group are shown. The cumulative parasitemia for each group was compared: (a) $P < 0.005$; (b) $CD1d^{-/-}$ or $J\alpha281^{-/-}$ versus $CD1d^{+/-}$, $P < 0.001$; (c) $P < 0.001$.

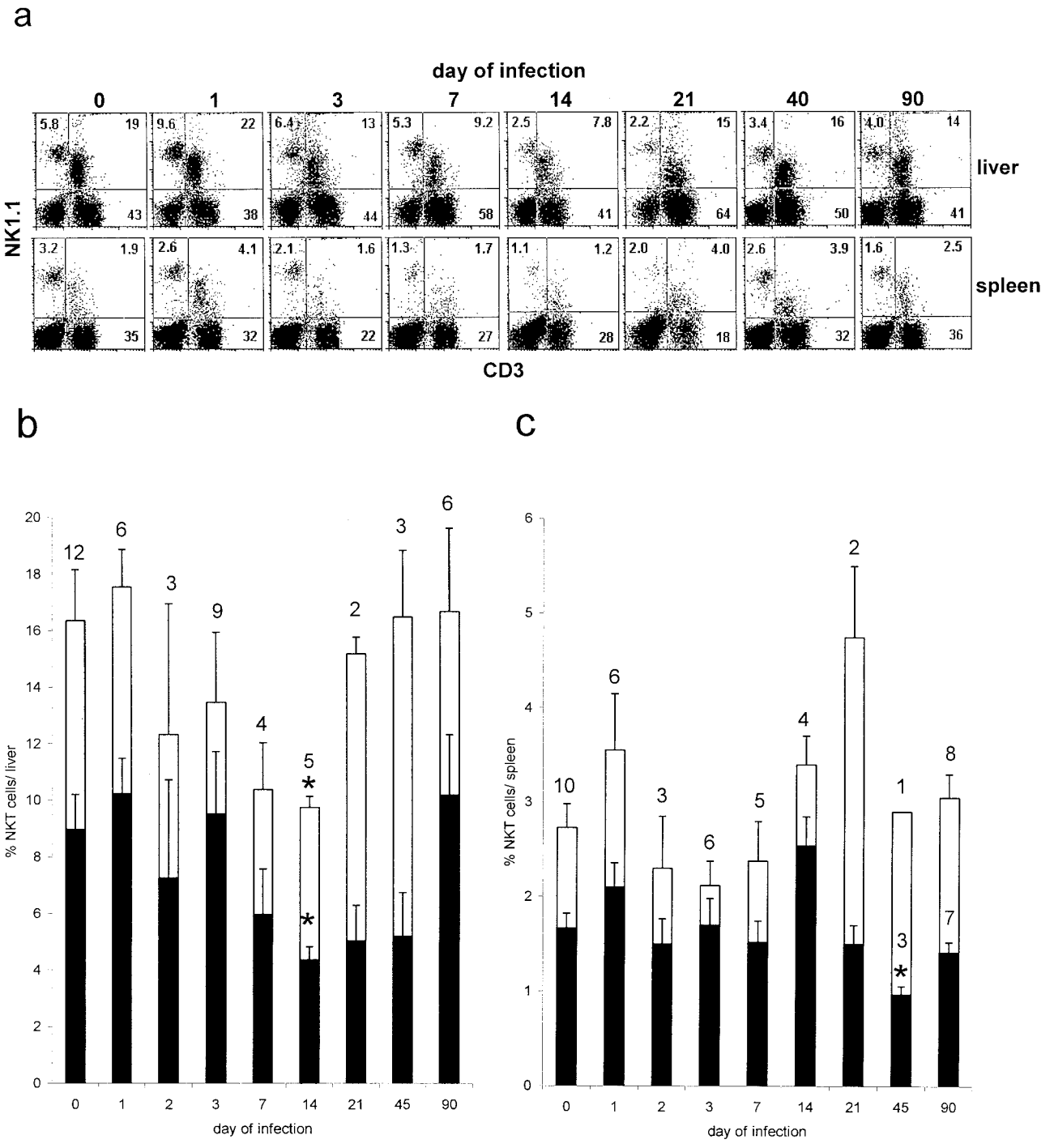


FIG. 2. During *T. cruzi* infection liver and spleen NK T-cell and NK cell population sizes are decreased. C57BL/6 mice were infected with 10^5 trypomastigotes, and at the indicated days of the infection liver and spleen mononuclear cells were stained with fluorescent MAbs to NK1.1 and CD3. (a) Each plot presents data of an individual representative mouse. (b and c) The mean percentages of NK T cells ($CD3^+$ $NK1.1^+$) (total bars) or $CD4^+$ $NK1.1^+$ cells (black bars) in livers (b) and spleens (c) of several mice are shown. The numbers above the bars indicate the number of mice analyzed on that day of the infection. (d) The mean percentages of NK cells ($CD3^-$ $NK1.1^+$) in livers (circles) or spleens (squares) are shown. On the different days of the infection the same numbers of livers or spleens were analyzed as indicated in panels b and c. Symbols: *, $P < 0.05$; **, $P < 0.01$ versus preinfection (day 0). Error bars (b, c, and d), SEM.

are stimulated during *T. cruzi* infection, this stimulation may cause AICD and the subsequent impairment in the ability of NK T cells to inhibit self-damaging chronic inflammatory responses. Here we report that Vα14-Jα281 NK T cells are stimulated to provide protection during acute *T. cruzi* infection and that during the chronic phase of the infection, Vα14-Jα281 NK T cells appear to develop an increased proinflammatory phenotype that contributes to the chronic inflammatory response.

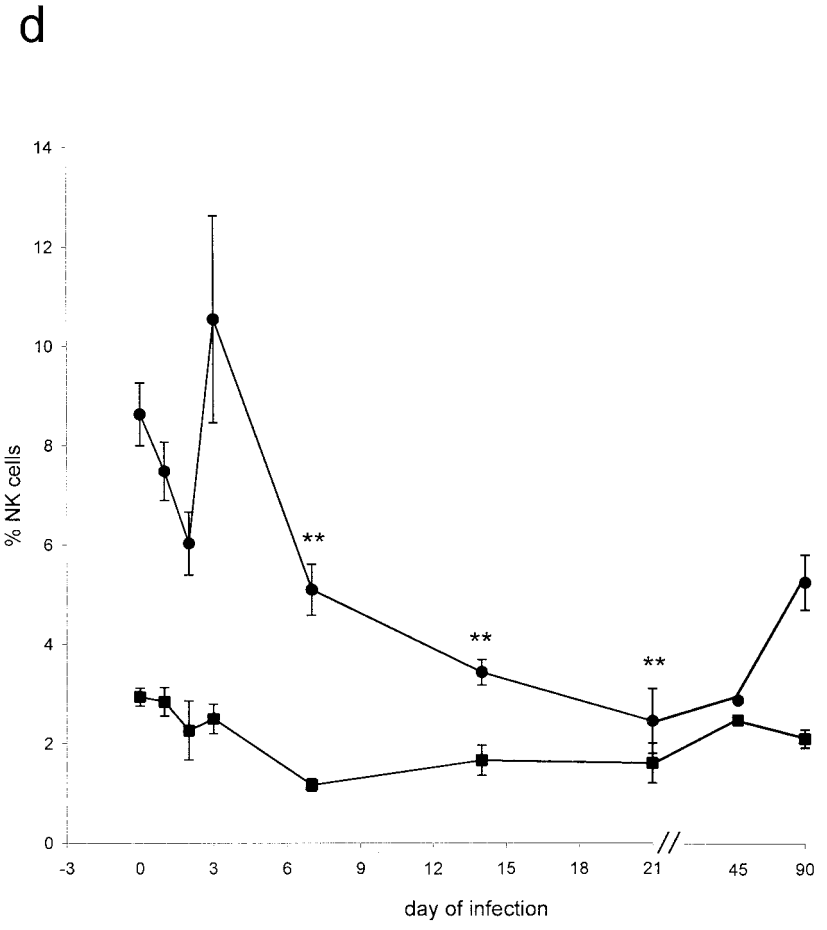


FIG. 2—*Continued.*

MATERIALS AND METHODS

***T. cruzi*.** A recently derived clone of the CL strain subclone 3 was used (43). Trypomastigotes were obtained from culture supernatants of infected 3T3 cells grown in Dulbecco's modified Eagle medium (BioWhittaker, Walkersville, Md.) supplemented with 10% heat-inactivated calf serum (BioWhittaker) and 50,000 U of penicillin-streptomycin (BioWhittaker).

Mice. Eight- to 10-week-old female wild-type C57BL/6 and wild-type BALB/c mice were obtained from Bantin & Kingman (Fremont, Calif.). $CD1d^{-/-}$ and $CD1d^{+/-}$ mice crossed a minimum of seven times to wild-type C57BL/6 mice were bred in the animal facilities at the University of Washington (47). C57BL/6 and BALB/c $J\alpha281^{-/-}$ mice were crossed a minimum of nine times to either wild-type C57BL/6 or BALB/c mice and bred in the animal facilities at the University of Washington (16).

***T. cruzi* infection of mice.** Mice were infected intraperitoneally (i.p.) with trypomastigotes. The size of the trypomastigote inoculations for each experiment is indicated in the Results section.

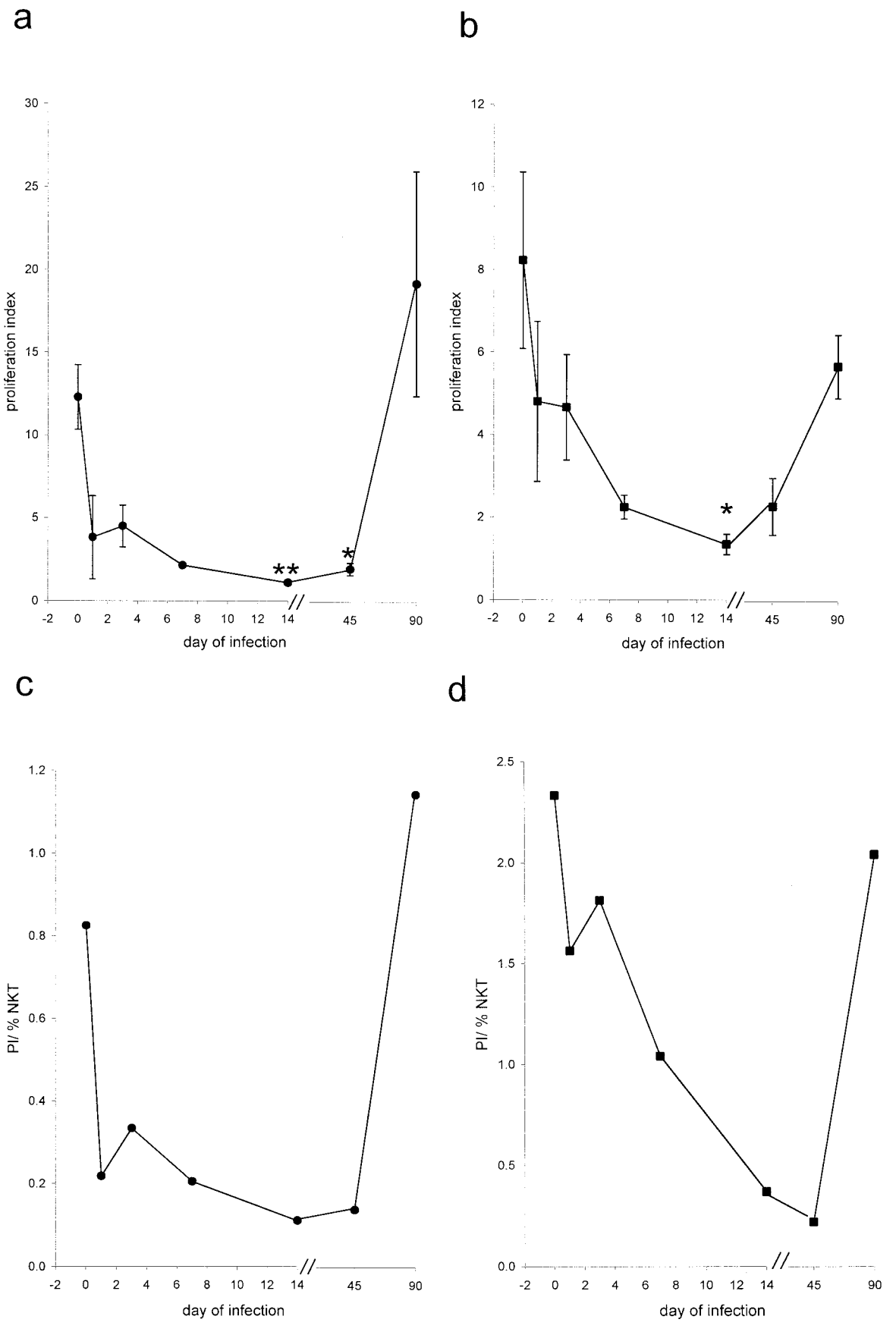
Parasitemia determination. Two microliters of blood, obtained by venesection of the tail, was diluted in 18 μl of 0.89% ammonium chloride in phosphate-buffered saline (PBS), and the trypomastigotes were counted on a hemacytometer (24).

Preparation of liver and spleen mononuclear cells. Liver cells were prepared by homogenizing the tissue using a 0.2-μm-pore-size screen, followed by centrifugation of the homogenate through a 33% Percoll gradient and recovering the cells in the pellet (22, 53). Splenocytes were prepared by disrupting spleens between the frosted ends of glass slides. In both preparations, red blood cells were removed by lyses in 1.66% NH_4Cl solution.

Flow cytometric analysis. Liver and spleen mononuclear cell preparations were incubated at room temperature with the anti-FcR monoclonal antibody (MAb) 2.4G2 for 20 min and then were washed and incubated at 4°C with an anti-NK1.1 phycoerythrin-conjugated MAb (clone PK136) (PharMingen), an anti-CD3 TriColor-conjugated MAb (clone H57-579) (PharMingen), and an anti-CD4 fluorescein isothiocyanate-conjugated MAb (clone GK1.5) in 50 μl of staining buffer (4% bovine serum albumin [BSA]–PBS–0.09% sodium azide, pH 7.4). The stained cells were analyzed by flow cytometry (in a FACScan device; Becton Dickinson, San Jose, Calif.). Minimums of 40,000 events were collected, and the data were analyzed with Joseph Trotter's WinMDI 2.7 (available at http://facs.scripps.edu/software.html).

Liver and spleen cell in vitro incubations with α-GalCer. α-GalCer (kindly provided by Y. Koezuka, Kirin Brewery Company, Gunma, Japan) was diluted in Tween 20 (Sigma). Liver or spleen mononuclear cells were prepared from uninfected and *T. cruzi*-infected C57BL/6 mice, seeded in 96-well plates (3×10^5 cells/well in triplicate) and cultured in media with α-GalCer (100 ng/ml) or diluent alone for 96 h. [^{3}H]thymidine (1 μCi/well) was added to each well for the final 24 h of culture. The proliferation index was calculated by dividing the mean counts per minute for samples incubated with α-GalCer by the mean counts per minute for samples incubated with diluent only. In all experiments the mean counts per minute for diluent-only samples was <200. IFN-γ and IL-4 concentrations in the supernatants were analyzed by enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions (PharMingen). Briefly, 96-well ELISA plates were coated overnight with capture antibody (IFN-γ, clone R4-6A2; IL-4, clone BVD4-1D11); the plates were blocked with 1% BSA–PBS for 2 h at room temperature; the samples and standards were added and incubated for 2 h at room temperature; the biotinylated antibodies (IFN-γ, clone XMG1.2; IL-4, clone BVD6-24G2) were added and incubated at room temperature for 1 h; and after extensive washing horseradish peroxidase-streptavidin (Genzyme) was added for 1 h, the samples were washed again, and 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS; Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added. The samples were analyzed at 405 nm (model EL800 device; BioTek Instruments Inc., Winooski, Vt.).

Analysis of antibody responses. Individual blood samples from *T. cruzi*-infected mice were collected by venesection of the tail and allowed to clot during overnight incubation at 4°C, and sera were prepared and stored at −20°C.


a
b
c
d
proliferation index
PI/ % NKT
day of infection
-2 0 2 4 6 8 10 12 14 45 90
**
*

Individual sera were analyzed by antibody capture ELISAs. ELISA plates were coated by adding PBS (50 μl/well) containing either 5×10^6 heat-inactivated trypomastigotes (5 min at 55°C) or SA85-1.1 recombinant protein (5 μg/ml). The preparation of SA85-1.1 recombinant protein has been described (32). After overnight incubation at 4°C the plates were washed with PBS-Tween, blocked with 4% BSA–PBS for 1 h at 37°C, and washed, and serum samples or anti-SA85-1.1 MAbs diluted in 1% BSA–PBS were added. The SA85-1.1 MAbs were used to generate the standard curves to calculate the serum antibody concentrations. The SA85-1.1-specific MAb used for the IgG standard curve was C1.2, and that for the IgG2a standard curve was NP2 (33). Plates were incubated at 37°C for 3 h and washed, and either biotinylated anti-IgG (PharMingen) or biotinylated anti-IgG2a (R19-15; PharMingen) antibodies were added (1 μg/ml in 1% BSA–PBS). The plates were incubated for 1 h at 37°C and washed three times, streptavidin-horseradish peroxidase (Genzyme) was added for 1 h at 37°C, the plates were washed four times, ABTS-H_2O_2 (Kirkegaard & Perry Laboratories) was added, and the plates were analyzed at 405 nm. The antibody concentrations of individual mice were extrapolated from the standard curves, and the mean and standard error of the mean (SEM) were calculated using Excel software (Microsoft Corporation, Redmond, Wash.).

Statistics. The *P* values were determined using Student's *t* test (Excel; Microsoft Corporation).

RESULTS

During acute *T. cruzi* infection NK T cells lower parasitemia. NK T-cell responses can be stimulated by IL-12 or by TCR glycolipid ligation (20). It has been reported that an NK T-cell TCR ligand is GPI (46). Since *T. cruzi* expresses abundant GPI-modified surface proteins and *T. cruzi* GPI is a potent stimulator of IL-12, *T. cruzi* might stimulate a protective NK T-cell response (2, 33). To examine this possibility C57BL/6 CD1d$^{-/-}$ mice (NK T-cell deficient) or CD1d$^{+/-}$ mice were infected with 10^5 trypomastigotes i.p. (a sublethal inoculum in wild-type C57BL/6 mice [data not shown]), and parasitemia and survival were monitored. Parasitemia was significantly higher in the CD1d$^{-/-}$ mice (Fig. 1a; $P < 0.005$ for comparison of the total parasitemia of each group of mice). Although the CD1d$^{-/-}$ mice appeared sicker during the parasitemic period (more ruffed fur and decreased activity), all the mice survived the acute phase and beyond 150 days of the infection. In both groups of mice parasitemia peaked on day 14 of the infection and resolved by day 23 (Fig. 1a). A similar experiment comparing C57BL/6 CD1d$^{-/-}$ and wild-type mice again resulted in an increase in the parasitemia of the CD1d$^{-/-}$ mice ($P < 0.001$) (data not shown). Again, all mice survived the infection. These data indicate that C57BL/6 CD1d$^{-/-}$ mice are susceptible to increased parasitemia and argue that the absence of NK T cells causes this susceptibility.

CD1d may provide functions unrelated to NK T-cell antigen presentation. For example, when intestinal epithelial cells are stimulated through CD1d, they secrete IL-10 (15). Since IL-10$^{-/-}$ mice are more susceptible to *T. cruzi* infection (27), a decrease in IL-10 secretion in CD1d$^{-/-}$ mice, rather than the absence of NK T cells, may contribute to the observed increase in parasitemia (Fig. 1a and data not shown). To confirm that the increased parasitemia in CD1d$^{-/-}$ mice was due to an absence of NK T cells rather than other CD1d functions, five NK T-cell-deficient Jα281$^{-/-}$ mice, five NK T-cell-deficient CD1d$^{-/-}$ mice, and five CD1d$^{+/-}$ mice were infected with 10^5 trypomastigotes i.p., and parasitemia and survival were monitored. In the Jα281$^{-/-}$ and CD1d$^{-/-}$ mice levels of parasitemia were similar and significantly higher than that in the CD1d$^{+/-}$ mice (Fig. 1b; $P < 0.001$ for comparison of the total parasitemia of either group of NK T-cell-deficient mice to the CD1d$^{+/-}$ mice). All mice survived the infection. These data indicate that Vα14-Jα281 NK T cells of C57BL/6 mice limit the parasitemia.

It is possible that the NK T cells of C57BL/6 limit the parasitemia by secreting protective IFN-γ and that during *T. cruzi* infection of BALB/c mice, a strain that is more susceptible to *T. cruzi* infection, the NK T cells would not limit parasitemia (56). Therefore, groups of BALB/c Jα281$^{-/-}$ (NK T-cell-deficient) and wild-type mice were infected with 5×10^4 trypomastigotes (a sublethal dose in wild-type BALB/c mice [data not shown]), and parasitemia and mortality were monitored. Parasitemia was prolonged in the NK T-cell-deficient Jα281$^{-/-}$ mice (Fig. 1c, $P < 0.001$), indicating that Vα14-Jα281 NK T cells provide protection in BALB/c and C57BL/6 mice and arguing that during *T. cruzi* infection of mice and other mammalian species Vα14-Jα281 NK T cells are stimulated and they lower the parasitemia.

If during *T. cruzi* infection Vα14-Jα281 NK T cells provide protection by rapidly secreting cytokines while the adaptive immune response is developing, then in mice that have already developed a memory response, the NK T cells may not be required for protection. To assess this possibility, NK T-cell-deficient Jα281$^{-/-}$ mice and wild-type BALB/c mice that had survived *T. cruzi* infection for 68 days were reinoculated with 10^6 trypomastigotes i.p. (a lethal dose to naïve wild-type BALB/c mice [data not shown]). Following this second inoculation parasitemia was not detected in either group (Fig. 1c) and all mice survived for >120 days. This result argues that NK T cells provide protection against *T. cruzi* in naïve mice but that in mice that have developed a parasite-specific lymphocyte memory response the NK T-cell role is diminished.

Analysis of NK T-cell populations during *T. cruzi* infection. Following NK T-cell stimulation by anti-CD3 MAb, IL-12, or α-GalCer, liver and spleen NK T cells rapidly produce IFN-γ and IL-4, stimulate NK cell responses, undergo AICD, and then repopulate the liver and spleen (13, 18, 19, 37). Furthermore, repeated stimulation with α-GalCer causes NK T cells to produce decreased IFN-γ and increased IL-4 (11). Stimulation of the Vα14-Jα281 NK T cells during acute *T. cruzi* infection could result in their AICD, and during the chronic phase, continuous stimulation of the NK T cells may prolong their depletion, or the Vα14-Jα281 NK T cells that develop in the

FIG. 3. The proliferative response following α-GalCer stimulation of liver and spleen cells isolated from *T. cruzi*-infected mice. Wild-type C57BL/6 mice were infected with 10^5 trypomastigotes and at different days of the infection liver (a) or spleen (b) cells were isolated and cultured for 72 h in the presence of α-GalCer (100 ng/ml) or diluent only. [^{3}H]thymidine was added for the last 24 h. (a and b) The mean proliferation index and SEM of three independent experiments are presented. In each independent experiment each sample was analyzed in triplicate, and the mean value was used to calculate the proliferation index. (c and d) The mean proliferation index is divided by the mean percentage of NK T cells. The percentage of NK T cells was determined as described in the legend to Fig. 2. Symbols: *, $P < 0.05$; **, $P < 0.01$ compared to uninfected mice (day 0).

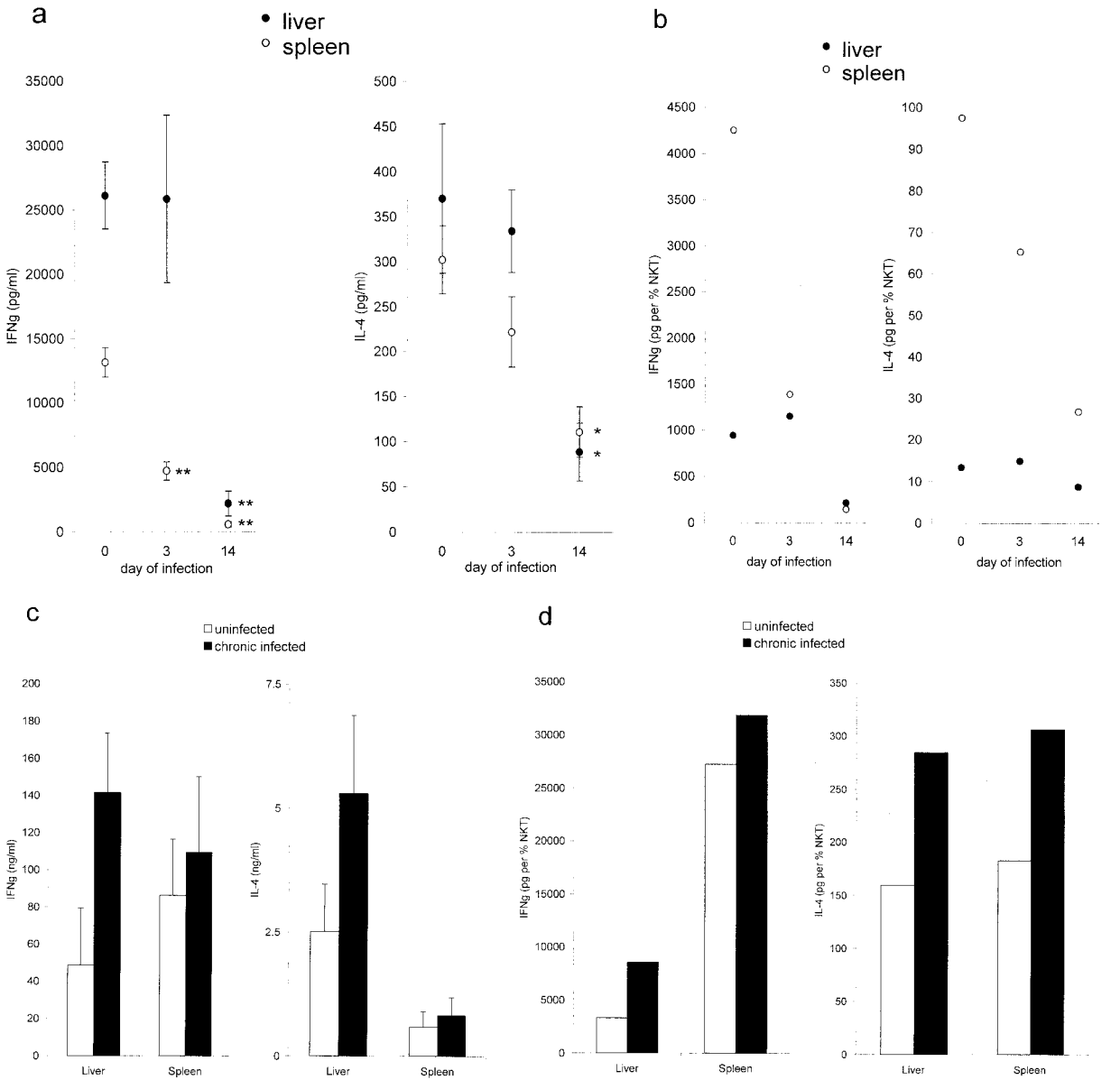


FIG. 4. IFN-γ and IL-4 production following α-GalCer stimulation of liver or spleen cells isolated from *T. cruzi*-infected mice. (a and b) Liver cells (closed circles) and spleen cells (open circles) were prepared from wild-type C57BL/6 mice that were uninfected or infected (10^5 trypomastigotes) for 3 or 14 days. Data of one of three experiments with similar results are presented. (c and d) Cells from uninfected mice (open bars) or mice infected (closed bars) (10^5 trypomastigotes) for 90 to 120 days were incubated at 3×10^5 per well for 96 h in media with α-GalCer (100 ng/ml) or diluent only, and the supernatants were analyzed by ELISA for IFN-γ or IL-4. The mean data of eight mice derived from three independent experiments are presented. The data represent cytokines detected following α-GalCer incubation minus cytokines detected following diluent-only incubation. Following diluent-only incubation no liver IFN-γ or spleen IL-4 was detected. The amounts of IL-4 detected in liver following diluent-only incubation were as follows: uninfected mice, 59 pg/ml; day 3 of infection, 187 pg/ml; day 14 of infection, 270 pg/ml. The amounts of IFN-γ detected in spleen following diluent-only incubation were as follows: uninfected, 26 pg/ml; day 3 of infection, 0 pg/ml; day 14 of infection, 330 pg/ml.

chronically infected mouse may have altered cytokine profiles. If the NK T cells are altered, then the ability of NK T cells to provide protection against other infections during the chronic phase of *T. cruzi* infection, or to inhibit inflammation, may be altered.

To analyze possible alterations in the NK T-cell populations during *T. cruzi* infection, wild-type C57BL/6 mice were infected with sublethal inocula of trypomastigotes, and at different times the liver and spleen NK T cells were analyzed. During the infection, alterations in the liver and spleen NK T-cell populations were observed (Fig. 2 and Table 1). The percentage of liver and spleen NK T-cell populations on day 1 of the infection appeared larger (Fig. 2a to c). After day 1, the percentage of liver NK T-cell population diminished, reached a nadir at 9.8% on day 14 of the infection, and returned to normal levels of approximately 14 to 18% by day 21 of infec-

tion (as parasitemia resolved) (Fig. 2a and b). In the spleen, NK T-cell populations also declined after the first day of the infection (Fig. 2a and c). The percentage of splenic NK T cells, in contrast to the liver NK T cells, appeared to reach a nadir of 2.1% on day 3 of the infection and then returned to normal levels by day 14 of the infection. Although normal percentages of NK T cells were restored to the liver and spleen after day 14 of the infection, the intensity of the NK1.1 staining of the NK T cells between days 21 and 45 of the infection was decreased (Fig. 2a, days 21 and 40, and data not shown), suggesting that the NK T cells that populate the liver and spleen after the acute phase of the infection have an altered phenotype that might affect their function. Further analysis of the cell populations revealed that as the percentage of NK T cells declines, the absolute number of $CD3^+$ $NK1.1^+$ cells actually increases and reaches a maximum on day 21 of infection, when the nonspecific polyclonal expansion of lymphocytes during *T. cruzi* infection is maximal (Table 1). Together, these data suggest the possibility that $NK1.1^{dim}$ cells on day 21 and day 45 of infection may represent expression of NK1.1 on polyclonally activated conventional T cells. A similar upregulation of NK1.1 on T cells has been reported during influenza virus infection (7).

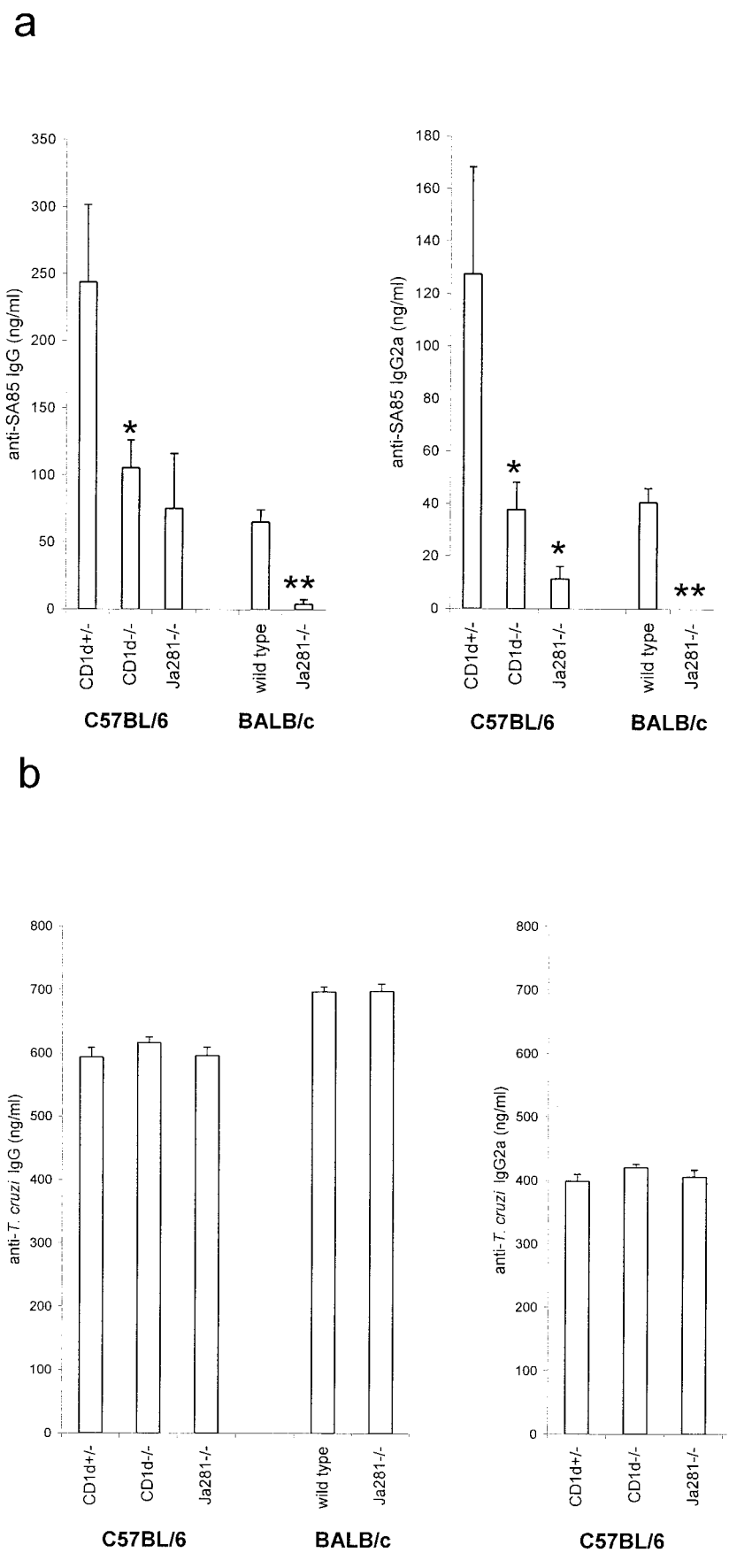
More than 50% of NK T cells are $CD4^+$, and $CD4^+$ NK T cells were shown to be protective in *Toxoplasma gondii* infection (17). Therefore, to determine during *T. cruzi* infection if $CD4^+$ NK T-cell populations were stimulated, the liver and spleen $CD4^+$ NK T-cell populations were analyzed (20). In the liver and spleen, through the first 2 weeks of the infection the percentage of $CD4^+$ NK T-cell populations increased and decreased in a similar fashion as the total NK T-cell population (Fig. 2b and c). In the liver, from day 14 through 45 of the infection, the percentage of $CD4^+$ NK T-cell population appeared to remain decreased while the percentage of total NK T cells returned to preinfection levels (Fig. 2b). Similarly, during this time period the splenic percentage of $CD4^+$ NK T cells was disproportionately decreased compared to the percentage of total NK T cells (Fig. 2c). In both the liver and spleen the percentage of $CD4^+$ NK T and $CD4^-$ NK T cells returned to normal levels by day 90 of the infection (Fig. 2a to c). These data indicate that during *T. cruzi* infection the NK T-cell populations ($CD4^-$ and $CD4^+$) undergo alterations for prolonged periods of time, and although the biological significance remains unclear, the data suggest that during the infection these alterations may affect their function.

Analysis of NK cell populations during *T. cruzi* infection. NK cells appear to provide protection during *T. cruzi* infection (3, 12, 23, 30, 52). NK cell responses can be stimulated by IL-12 or by stimulated NK T cells (9, 13, 19), and NK cells appear to participate in the homing of NK T cells to the liver (40). Therefore, during *T. cruzi* infection, the liver and spleen NK cell populations ($NK1.1^+$, $CD3^-$) were monitored (Fig. 2a and d). The percentages of both liver and spleen NK cell populations become decreased by day 7 of the infection and remain decreased on day 21 of the infection. In the liver the percentage of NK cells only returns to normal by day 90 (Fig. 2a and d). The data indicate that during acute *T. cruzi* infection NK cells are stimulated and suggest that during the infection the function of NK and NK T cells remains altered for prolonged periods of time.

The in vitro proliferative response to α-GalCer by liver and spleen cells of *T. cruzi*-infected C57BL/6 mice. To assess during *T. cruzi* infection alterations in the NK T-cell functions, mononuclear cells were isolated from the liver and spleen on different days of the infection and were stimulated in vitro with the CD1d-restricted NK T-cell-specific ligand, α-GalCer. Although the percentage of NK T cells appears to have increased on day 1 of the infection (Fig. 2a to c) and the absolute number of $NK1.1^+$ $CD3^+$ cells was increased on days 21 and 45 of infection (Table 1), the proliferative responses of liver (Fig. 3a) and spleen (Fig. 3b) mononuclear cells to α-GalCer were decreased on day 1 of the infection and remained decreased until the chronic phase (day 90). On days 3 through 14 of the infection in the liver and days 3 through 7 in the spleen, the decreased α-GalCer proliferative response paralleled the decrease in the percentage of NK T cells (Fig. 2a to c and 3a and b). The decreased proliferation to α-GalCer on days 1 and 45 (Fig. 3a and b) suggests either that the α-GalCer-specific NK T cells are selectively depleted while other $NK1.1^+$ T-cell subsets expand (Fig. 2a to c) or that the in vitro proliferative response of NK T cells to α-GalCer is inhibited. It is also interesting that between days 21 and 45 of the infection, when the α-GalCer proliferative response appears decreased, the NK T cells present in the liver and spleen are $CD4^-$ and have lower levels of NK1.1 staining (Fig. 2a to c), again suggesting that these cells may represent conventional T cells with upregulated NK1.1 expression.

To further examine during the infection the liver and spleen cell α-GalCer-specific proliferative responses, for each day of the infection, the α-GalCer proliferation index per percentage of NK T cells was calculated (proliferation index ÷ percent NK T cells) (Fig. 3c and d). These data demonstrate that during acute *T. cruzi* infection, the α-GalCer proliferative responses decrease more than the percentage of NK T cells decreases. This suggests that during the acute infection, α-GalCer-specific NK T cells are preferentially eliminated and that the NK T cells that remain during the acute infection are not α-GalCer specific (Fig. 3c and d). These data also suggest that the NK T cells within the liver and spleen by day 45 of the infection are not α-GalCer specific but that after day 45 of the infection, the NK T cells of the liver and spleen are α-GalCer specific (Fig. 2a and c and Fig. 3). These data support the previous data that during *T. cruzi* infection the NK T-cell populations are altered and that these alterations are likely to affect their functions.

The in vitro IFN-γ and IL-4 response to α-GalCer by liver and spleen cells of *T. cruzi*-infected C57BL/6 mice. To further analyze the response of liver and spleen NK T cells during acute *T. cruzi* infection, cells from uninfected C57BL/6 mice and C57BL/6 mice infected for 3 or 14 days were isolated and stimulated in vitro with α-GalCer, and the production of IFN-γ and IL-4 was determined by ELISA. On day 3 of the infection, liver α-GalCer-stimulated IFN-γ and IL-4 production is similar to that of cells from uninfected mice, whereas splenic IFN-γ and IL-4 production have begun to decline (Fig. 4a). By day 14 of the infection both liver and spleen α-GalCer-stimulated IFN-γ and IL-4 production are significantly decreased (Fig. 4a). Correction of the amount of IFN-γ and IL-4 produced by the percentage of NK T cells (cytokine produced ÷ percent NK T cells) argues that in the liver the α-GalCer-specific NK T cells that contribute to IFN-γ production are still present on


a
anti-SA85 IgG (ng/ml)
350
300
250
200
150
100
50
0
*
**
CD1d+/-
CD1d-/-
Ja281-/-
wild type
Ja281-/-
C57BL/6
BALB/c
anti-SA85 IgG2a (ng/ml)
180
160
140
120
100
80
60
40
20
0
*
*
**
CD1d+/-
CD1d-/-
Ja281-/-
wild type
Ja281-/-
C57BL/6
BALB/c
b
anti-T. cruzi IgG (ng/ml)
800
700
600
500
400
300
200
100
0
CD1d+/-
CD1d-/-
Ja281-/-
wild type
Ja281-/-
C57BL/6
BALB/c
anti-T. cruzi IgG2a (ng/ml)
800
700
600
500
400
300
200
100
0
CD1d+/-
CD1d-/-
Ja281-/-
wild type
Ja281-/-
C57BL/6
BALB/c

day 3 of the infection, but by day 14 of the infection these cells have been selectively depleted (Fig. 4b). In the spleen, the α-GalCer-specific NK T cells contributing to IFN-γ and IL-4 production appear to be selectively depleted on day 3 of the infection and further depleted by day 14 of the infection (Fig. 4b).

During the chronic phase of the infection, the NK T-cell populations have the same size and α-GalCer-stimulated proliferative responses as NK T-cell populations from uninfected mice (Fig. 2a and c and 3). Therefore, it was unexpected that liver cells from chronically infected mice, when stimulated in vitro with α-GalCer, compared to uninfected mice, produced approximately twofold more IL-4 ($P < 0.2$) and threefold more IFN-γ ($P < 0.06$) (Fig. 4c). The sizes of the uninfected and chronically infected NK T-cell populations were similar, and thus similar differences were observed when the cytokines produced per percent NK T cells were calculated (Fig. 4d). Spleen cells from chronically infected mice, compared to uninfected mice, produced smaller increases in cytokines (Fig. 4c and d). Finally, liver and spleen cells from uninfected and chronically infected J$\alpha281^{-/-}$ mice stimulated with α-GalCer did not produce detectable IFN-γ or IL-4, demonstrating the requirement of Vα14-Jα281 α-GalCer-specific NK T cells for this cytokine response (data not shown). Together these data argue that in *T. cruzi* chronically infected mice, liver and spleen NK T-cell stimulation leads to increased IFN-γ and IL-4 production and suggest that these increased cytokine responses may contribute to the chronic inflammatory response.

During chronic *T. cruzi* infection antibody responses of NK T-cell-deficient mice are altered. Some studies have argued that the GPI of GPI-modified proteins is an NK T-cell ligand that stimulates NK T-cell cytokine production that helps the antibody response to the GPI-modified proteins (46). Other studies have argued that GPI is not an NK T-cell TCR ligand and that antibody responses to GPI-modified proteins develop independently of NK T cells (41). In vitro α-GalCer stimulation of liver and spleen cells from chronically infected C57BL/6 mice produced increased IFN-γ (Fig. 4c and d), suggesting that in chronically infected mice NK T cells might stimulate more IFN-γ and thereby affect the antibody response. Previous studies have demonstrated in *T. cruzi* chronically infected mice robust IgG and IgG2a antibody responses to heat-inactivated trypomastigotes and to the SA85-1.1 protein, a GPI-modified protein (data not shown) (33). To investigate during *T. cruzi* infection if NK T cells affect the antibody response, the chronic-phase IgG and IgG2a responses to the SA85-1.1 protein and heat-inactivated trypomastigotes of CD1d$^{-/-}$ or J$\alpha281^{-/-}$ mice were compared to CD1d$^{+/-}$ mice or wild-type mice (Fig. 5). Consistently, in both C57BL/6 and BALB/c mice, the NK T-cell-deficient mice produced less anti-SA85-1.1 IgG and IgG2a (Fig. 5a). In contrast, no differences in the IgG or IgG2a responses to heat-inactivated trypomastigotes were detected (Fig. 5b). These data indicate that in *T. cruzi* chronically infected NK T-cell-deficient mice the antibody response to one GPI-modified protein is decreased (Fig. 5). These data argue that Vα14-Jα281 NK T cells can provide help for antibody responses. Furthermore, these data suggest that during *T. cruzi* chronic infection NK T cells contribute to a more robust cytokine response that affects the antibody response and possibly the inflammatory pathology.

DISCUSSION

NK T cells appear able to rapidly initiate protective immune responses against infections, to affect the adaptive immune response against pathogens, and to prevent unwanted self-damaging immune responses (20). In vivo stimulation of NK T cells with a TCR ligand (α-GalCer), anti-CD3, or IL-12 alters NK T-cell functions for prolonged periods of time (18, 37). This report presents results on the NK T-cell response during *T. cruzi* infection, a chronic infection that triggers a self-damaging inflammatory response. The data presented indicate that the NK T cells decrease the acute-phase parasitemia (Fig. 1), that during the acute infection, the NK T cells develop altered effector functions that persist into the chronic phase of the infection (Fig. 2 to 4), and that the NK T cells augment a *T. cruzi*-specific antibody response (Fig. 5).

It has been frequently hypothesized that NK T cells can rapidly respond to infectious pathogens and initiate protective immune responses (20). At this time, however, few reports have demonstrated that NK T cells are protective during infections. Several reports have demonstrated that immunization with α-GalCer stimulates NK T cells to provide protection, but these reports did not demonstrate a protective role for NK T cells during the natural infection (21, 34). Other investigations found that during *Mycobacterium bovis*, *Salmonella* sp., and *Listeria* sp. infections NK T cells are not protective (20). In addition, although it has been shown that NK T cells are stimulated by the injection of *Mycobacterium tuberculosis* cell walls, a protective role for NK T cells during *M. tuberculosis* infection has not been demonstrated (4). During infection of mice with an attenuated strain of *Toxoplasma*, NK T cells stimulate a protective response, and during *Plasmodium* and *Leishmania* infection of mice NK T cells decrease the parasite burden (17, 29, 42). Thus, the data in this report that NK T cells limit parasitemia caused by a strain of *T. cruzi* that naturally infects humans and mice provide further evidence that Vα14-Jα281 NK T cells provide protection during infections (Fig. 1). The data also suggest that against *T. cruzi* the NK T-cell protection occurs rapidly, possible within the first day of the infection, as α-GalCer-specific Vα14-Jα281 NK T cells appear to be absent or less responsive after the first days of the infection (Fig. 2 to 4).

It remains unclear how NK T cells limit parasitemia (Fig. 1).

FIG. 5. Comparison of antibody responses of chronically infected NK T-cell-deficient and normal mice to GPI-modified SA85-1.1 protein. Chronically infected NK T-cell-deficient and normal mice were infected with *T. cruzi*, and during the chronic phase, serum samples from individual mice were obtained and the anti-SA85-1.1 and anti-trypomastigote IgG and IgG2a antibody concentrations were determined. C57BL/6 mice were infected with 10^5 trypomastigotes, and BALB/c mice were infected with 5×10^4 trypomastigotes. The bars represent the mean antibody concentration of 10 C57BL/6 CD1d$^{-/-}$, 10 C57BL/6 CD1d$^{+/-}$, and 5 C57BL/6 J$\alpha281^{-/-}$ mice and 5 BALB/c J$\alpha281^{-/-}$ and 5 BALB/c wild-type mice. Symbols: *, $P < 0.05$ for NK T-cell-deficient versus CD1d$^{+/-}$ mice; **, $P < 0.001$ for NK T-cell-deficient mice versus wild-type mice.

TABLE 1. Number of cells per organ during *T. cruzi* infection

Day of infection	No. of mice	No. of cells (10^6)/organ (%)[a]							
		Liver cells				Spleen cells			
		Total	T	NKT	NK	Total	T	NKT	NK
0	12	5.8	2.06 (35.6)	0.95 (16.4)	0.50 (8.6)	92	28.8 (31.3)	2.5 (2.7)	2.7 (2.9)
1	6	5.3	1.89 (35.7)	0.93 (17.6)	0.40 (7.5)	75	24.5 (32.7)	2.7 (3.6)	2.1 (2.8)
2	3	9.5	3.61 (38.0)	1.17 (12.3)	0.57 (6.0)	83	26.4 (31.8)	1.9 (2.3)	1.9 (2.3)
3	9	8.8	2.96 (33.6)	1.19 (13.5)	0.93 (10.6)	90	24.9 (27.7)	1.9 (2.1)	2.3 (2.5)
7	4	22.7	13.78 (60.7)	2.36 (10.4)	1.16 (5.1)	140	35.8 (25.6)	3.4 (2.4)	1.7 (1.2)
14	5	26.9	15.55 (57.8)	2.64 (9.8)	0.91 (3.4)	242	74.8 (30.9)	8.2 (3.4)	4.1 (1.7)
21	2	30.4	20.49 (67.4)	4.62 (15.2)	0.76 (2.5)	190	37.2 (19.6)	9.1 (4.8)	3.0 (1.6)
45	3	11.7	ND[b]	1.93 (16.5)	0.34 (2.9)	92	ND	2.7 (2.9)	2.3 (2.5)
90	6	4.3	1.38 (32.2)	0.72 (16.7)	0.23 (5.3)	113	39.6 (35.0)	3.2 (2.8)	2.4 (2.1)

[a] Cells were prepared and analyzed as described in Materials and Methods. Abbreviations: T, $NK1.1^-$ $CD3^+$ cells; NKT, $NK1.1^+$ $CD3^+$ cells; NK, $NK1.1^+$ $CD3^-$ cells.
[b] ND, not determined.

Previous studies have demonstrated a critical role for IFN-γ in controlling *T. cruzi* infection (25, 38, 51). As reported here stimulation of Vα14-Jα281 NK T cells with α-GalCer results in IFN-γ secretion by liver and spleen mononuclear cells during the first days of *T. cruzi* infection (Fig. 4a and b). Thus, during *T. cruzi* infection, Vα14-Jα281 NK T-cell stimulation may result in early IFN-γ. Alternatively, NK T-cell cytolytic activity may limit parasitemia. In addition, previous studies have argued that during the first day of *T. cruzi* infection NK cells secrete protective IFN-γ (12, 52). These studies, however, used either an anti-NK1.1 antibody or anti-asialo GM1 antibody to remove IFN-γ-producing cells, and thus the cells removed could have been NK T cells as well as NK cells (12, 52). Finally, NK T cells can activate NK cells to produce IFN-γ and to become cytolytic (13, 19). Data presented here argue that NK cells become activated and are then depleted during acute *T. cruzi* infection (Fig. 2a and d), supporting the previous studies that NK cells provide protection (12, 52). It is possible that during *T. cruzi* infection, protection provided by NK cells is partially dependent on NK T-cell stimulation. Importantly, other protective mechanisms develop in NK $T^{-/-}$ mice, because these mice resolve parasitemia and survive through the acute infection, unlike β2-microglobulin$^{-/-}$ mice that also lack $CD8^+$ T cells (49).

It is unclear how during *T. cruzi* infection NK T cells are stimulated. It is known that NK T cells can be stimulated by IL-12, TCR ligation, or NK1.1 ligation (20). During *T. cruzi* infection IL-12 expression is induced and could stimulate the NK T-cell response (1, 2, 28). Alternatively, *T. cruzi* may express glycolipids that stimulate NK T cells through ligation of the TCR or the NK1.1 receptor, or during the infection self-antigens capable of stimulating NK T cells, which are not normally presented to NK T cells, may become available for presentation to NK T cells.

Following stimulation with α-GalCer, anti-CD3 MAb, or IL-12 NK T-cell populations are decreased (18, 37), and following multiple stimulations the population is decreased for a prolonged period, and it produces altered cytokines (11, 37). Therefore, it was of interest to observe how the NK T-cell populations responded during *T. cruzi* infection. The data argue that during the acute infection the percentage of liver and spleen NK T cells are decreased for an extended period of time (Fig. 2) but that the total number of $NK1.1^+$ $CD3^+$ cells are increased (Table 1). In addition, Vα14-Jα281 NK T cells with specificity for α-GalCer appear to be selectively decreased early during the acute infection (Fig. 3). Reagents that can distinguish between α-GalCer-specific NK T cells and other $NK1.1^+$ T cells will better define these alterations in NK T-cell populations.

The role of NK T cells in antibody responses is controversial. Some reports support a role for NK T cells in antibody responses (6, 46). In particular, GPI has been implicated as a ligand that stimulates NK T cells to help antibody responses (46). Other reports, however, have not supported a role for GPI in stimulating NK T cells or in NK T cells supporting antibody responses (14, 39, 41, 47). Data presented in this report demonstrate that NK T-cell-deficient C57BL/6 and BALB/c mice chronically infected with *T. cruzi* have decreased concentrations of anti-SA85-1.1 IgG and IgG2a antibodies (Fig. 5a). In contrast, the antibody concentrations to heat-inactivated trypomastigotes in the NK T-cell-deficient mice are unaltered (Fig. 5b). The SA85-1.1 protein becomes GPI modified during its synthesis (33). It remains unclear if during *T. cruzi* infection, GPI modification affects the SA85-1.1 protein NK T-cell response or antibody response. Additional studies comparing the antibody response during *T. cruzi* infection to *T. cruzi* non-GPI-modified proteins and GPI-modified proteins will be required. The decreased SA85-1.1 antibody response in NK T-cell-deficient mice indicates that NK T cells can affect antibody responses and that NK T cells can augment chronic inflammatory responses.

It is unusual for *T. cruzi* infections of humans or mammals to result in death during the acute phase (55). Most morbidity and mortality caused by *T. cruzi* are caused by chronic inflammation (55). The mechanisms causing *T. cruzi*-induced chronic inflammation remain unclear. Some data argue that the inflammation is stimulated by persistent parasites (10, 50). Other reports argue that the greater the acute-phase parasitemia is, then the greater the tissue burden of persistent parasites becomes, and that this increased parasite tissue burden causes greater chronic inflammation (8, 36). Thus, the demonstration here that Vα14-Jα281 NK T cells limit parasitemia (Fig. 1) argues that the response of these NK T cells can limit the chronic inflammatory pathology. These data also argue that

during *T. cruzi* infection the level of parasitemia and the development of chronic inflammatory sequelae may depend on the effectiveness of an individual's NK T response. Since prior stimulation of NK T cells with α-GalCer can dramatically decrease their subsequent responses for prolonged periods of time, then a person's antecedent exposure to NK T-cell stimulants may affect the outcome of *T. cruzi* infection (37).

NK T-cell dysfunction has been associated with mouse models of autoimmune diseases and two human idiopathic chronic inflammatory diseases (20, 26). In human systemic scleroderma and insulin-dependent diabetes the NK T-cell population is decreased (48, 54). Furthermore, in insulin-dependent diabetes the decreased NK T-cell population appears biased to a proinflammatory phenotype (54). It has been hypothesized that the cause of these and other idiopathic chronic inflammatory diseases is related to the immune response to infections. The data presented here suggest that NK T-cell responses in *T. cruzi* chronically infected mice are augmented. Thus, some *T. cruzi*-infected individuals might have augmented NK T-cell responses to a lifetime of infections and other environmental stimulants that can contribute to chronic inflammatory sequelae. It is also possible that *T. cruzi* infection compromises NK T-cell functions that inhibit unwanted immune responses and thus further contributes to the chronic inflammatory process.

ACKNOWLEDGMENTS

We thank Yasuhiko Koezuka, Kirin Brewery, Ltd., for providing α-GalCer.

This work was supported by a grant from the American Heart Association and the March of Dimes. Stuart Kahn is a Burroughs Wellcome New Investigator in Molecular Parasitology.

REFERENCES

1. **Aliberti, J. C., M. A. Cardoso, G. A. Martins, R. T. Gazzinelli, L. Q. Vieira, and J. S. Silva.** 1996. Interleukin-12 mediates resistance to *Trypanosoma cruzi* in mice and is produced by murine macrophages in response to live trypomastigotes. Infect. Immun. **64:**1961–1967.
2. **Almeida, I. C., M. M. Camargo, D. O. Procopio, L. S. Silva, A. Mehlert, L. R. Travassos, R. T. Gazzinelli, and M. A. Ferguson.** 2000. Highly purified glycosylphosphatidylinositols from Trypanosoma cruzi are potent proinflammatory agents. EMBO J. **19:**1476–1485.
3. **Antunez, M. I., and R. L. Cardoni.** 2000. IL-12 and IFN-gamma production, and NK cell activity, in acute and chronic experimental Trypanosoma cruzi infections. Immunol. Lett. **71:**103–109.
4. **Apostolou, I., Y. Takahama, C. Belmant, T. Kawano, M. Huerre, G. Marchal, J. Cui, M. Taniguchi, H. Nakauchi, J. J. Fournie, P. Kourilsky, and G. Gachelin.** 1999. Murine natural killer cells contribute to the granulomatous reaction caused by mycobacterial cell walls. Proc. Natl. Acad. Sci. USA **96:**5141–5146. (Erratum, **96:**7610.)
5. **Araujo, F. G.** 1989. Development of resistance to *Trypanosoma cruzi* in mice depends on a viable population of L3T4+ (CD4+) T lymphocytes. Infect. Immun. **57:**2246–2248.
6. **Araujo, L. M., A. Puel, C. Gouarin, A. Hameg, J. C. Mevel, Y. Koezuka, J. F. Bach, D. Mouton, and A. Herbelin.** 2000. NKT lymphocyte ontogeny and function are impaired in low antibody-producer Biozzi mice: gene mapping in the interval-specific congenic strains raised for immunomodulatory genes. Int. Immunol. **12:**1613–1622.
7. **Assarsson, E., T. Kambayashi, J. K. Sandberg, S. Hong, M. Taniguchi, L. Van Kaer, H. G. Ljunggren, and B. J. Chambers.** 2000. CD8+ T cells rapidly acquire NK1.1 and NK cell-associated molecules upon stimulation in vitro and in vivo. J. Immunol. **165:**3673–3679.
8. **Ben, Y.-C.-A., J.-M. Hontebeyrie, V. Tricottet, H. Eisen, M. Reynes, and G. Said.** 1988. Persistence of Trypanosoma cruzi antigens in the inflammatory lesions of chronically infected mice. Trans. R. Soc. Trop. Med. Hyg. **82:**77–83.
9. **Biron, C. A., K. B. Nguyen, G. C. Pien, L. P. Cousens, and T. P. Salazar-Mather.** 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu. Rev. Immunol. **17:**189–220.
10. **Brandariz, S., A. Schijman, C. Vigliano, P. Arteman, R. Viotti, C. Beldjord, and M. J. Levin.** 1995. Detection of parasite DNA in Chagas' heart disease. Lancet **346:**1370–1371.
11. **Burdin, N., L. Brossay, and M. Kronenberg.** 1999. Immunization with alpha-galactosylceramide polarizes CD1-reactive NK T cells towards Th2 cytokine synthesis. Eur. J. Immunol. **29:**2014–2025.
12. **Cardillo, F., J. C. Voltarelli, S. G. Reed, and J. S. Silva.** 1996. Regulation of *Trypanosoma cruzi* infection in mice by gamma interferon and interleukin 10: role of NK cells. Infect. Immun. **64:**128–134.
13. **Carnaud, C., D. Lee, O. Donnars, S. H. Park, A. Beavis, Y. Koezuka, and A. Bendelac.** 1999. Cutting edge: cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. J. Immunol. **163:**4647–4650.
14. **Chen, Y.-H., N. M. Chiu, M. Mandal, N. Wang, and C. R. Wang.** 1997. Impaired NK1+ T cell development and early IL-4 production in CD1-deficient mice. Immunity **6:**459–467.
15. **Colgan, S. P., R. M. Hershberg, G. T. Furuta, and R. S. Blumberg.** 1999. Ligation of intestinal epithelial CD1d induces bioactive IL-10: critical role of the cytoplasmic tail in autocrine signaling. Proc. Natl. Acad. Sci. USA **96:**13938–13943.
16. **Cui, J., T. Shin, T. Kawano, H. Sato, E. Kondo, I. Toura, Y. Kaneko, H. Koseki, M. Kanno, and M. Taniguchi.** 1997. Requirement for Vα 14 NKT cells in IL-12-mediated rejection of tumors. Science **278:**1623–1626.
17. **Denkers, E. Y., T. Scharton-Kersten, S. Barbieri, P. Caspar, and A. Sher.** 1996. A role for CD4+ NK1.1+ T lymphocytes as major histocompatibility complex class II independent helper cells in the generation of CD8+ effector function against intracellular infection. J. Exp. Med. **184:**131–139.
18. **Eberl, G., and H. R. MacDonald.** 1998. Rapid death and regeneration of NKT cells in anti-CD3ε- or IL-12-treated mice: a major role for bone marrow in NKT cell homeostasis. Immunity **9:**345–353.
19. **Eberl, G., and H. R. MacDonald.** 2000. Selective induction of NK cell proliferation and cytotoxicity by activated NKT cells. Eur. J. Immunol. **30:**985–992.
20. **Godfrey, D. I., K. J. Hammond, L. D. Poulton, M. J. Smyth, and A. G. Baxter.** 2000. NKT cells: facts, functions and fallacies. Immunol. Today **21:**573–583.
21. **Gonzalez-Aseguinolaza, G., C. de Oliveira, M. Tomaska, S. Hong, O. Bruna-Romero, N. T., M. Taniguchi, A. Bendelac, L. Van Kaer, Y. Koezuka, and M. Tsuji.** 2000. α-Galactosylceramide-activated Vα 14 natural killer T cells mediate protection against murine malaria. Proc. Natl. Acad. Sci. USA **97:**8461–8466.
22. **Goossens, P. L., H. Jouin, G. Marchal, and G. Milon.** 1990. Isolation and flow cytometric analysis of the free lymphomyeloid cells present in murine liver. J. Immunol. Methods **132:**137–144.
23. **Hatcher, F. M., R. E. Kuhn, M. C. Cerrone, and R. C. Burton.** 1981. Increased natural killer cell activity in experimental American trypanosomiasis. J. Immunol. **127:**1126–1130.
24. **Hoff, R.** 1974. A method for counting and concentrating living Trypanosoma cruzi in blood lysed with ammonium chloride. J. Parasitol. **60:**527–528.
25. **Holscher, C., G. Kohler, U. Muller, H. Mossmann, G. A. Schaub, and F. Brombacher.** 1998. Defective nitric oxide effector functions lead to extreme susceptibility of *Trypanosoma cruzi*-infected mice deficient in gamma interferon receptor or inducible nitric oxide synthase. Infect. Immun. **66:**1208–1215.
26. **Hong, S., and L. Van Kaer.** 1999. Immune privilege: keeping an eye on natural killer T cells. J. Exp. Med. **190:**1197–1200.
27. **Hunter, C. A., L. A. Ellis-Neyes, T. Slifer, S. Kanaly, G. Grunig, M. Fort, D. Rennick, and F. G. Araujo.** 1997. IL-10 is required to prevent immune hyperactivity during infection with Trypanosoma cruzi. J. Immunol. **158:**3311–3316.
28. **Hunter, C. A., T. Slifer, and F. Araujo.** 1996. Interleukin-12-mediated resistance to *Trypanosoma cruzi* is dependent on tumor necrosis factor alpha and gamma interferon. Infect. Immun. **64:**2381–2386.
29. **Ishikawa, H., H. Hisaeda, M. Taniguchi, T. Nakayama, T. Sakai, Y. Maekawa, Y. Nakano, M. Zhang, T. Zhang, M. Nishitani, M. Takashima, and K. Himeno.** 2000. CD4(+) Vα14 NKT cells play a crucial role in an early stage of protective immunity against infection with Leishmania major. Int. Immunol. **12:**1267–1274.
30. **James, S. L., T. L. Kipnis, A. Sher, and R. Hoff.** 1982. Enhanced resistance to acute infection with *Trypanosoma cruzi* in mice treated with an interferon inducer. Infect. Immun. **35:**588–593.
31. **Joyce, S., A. S. Woods, J. W. Yewdell, J. R. Bennink, A. D. De Silva, A. Boesteanu, S. P. Balk, R. J. Cotter, and R. R. Brutkiewicz.** 1998. Natural ligand of mouse CD1d1: cellular glycosylphosphatidylinositol. Science **279:**1541–1544.
32. **Kahn, S., T. G. Colbert, J. C. Wallace, N. A. Hoagland, and H. Eisen.** 1991. The major 85-kDa surface antigen of the mammalian-stage forms of *Trypanosoma cruzi* is a family of sialidases. Proc. Natl. Acad. Sci. USA **88:**4481–4485.
33. **Kahn, S. J., D. Nguyen, J. Norsen, M. Wleklinski, T. Granston, and M. Kahn.** 1999. *Trypanosoma cruzi*: monoclonal antibodies to the surface glycoprotein superfamily differentiate subsets of the 85-kDa surface glycoproteins and confirm simultaneous expression of variant 85-kDa surface glycoproteins. Exp. Parasitol. **92:**48–56.
34. **Kawakami, K., Y. Kinjo, S. Yara, Y. Koguchi, K. Uezu, T. Nakayama, M.**

Taniguchi, and A. Saito. 2001. Activation of Vα14$^+$ natural killer T cells by α-galactosylceramide results in development of Th1 response and local host resistance in mice infected with *Cryptococcus neoformans*. Infect. Immun. **69:**213–220.
35. **Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, H. Koseki, and M. Taniguchi.** 1997. CD1d-restricted and TCR-mediated activation of Vα14 NKT cells by glycosylceramides. Science **278:**1626–1629.
36. **Marinho, C. R., M. R. D'Imperio Lima, M. G. Grisotto, and J. M. Alvarez.** 1999. Influence of acute-phase parasite load on pathology, parasitism, and activation of the immune system at the late chronic phase of Chagas' disease. Infect. Immun. **67:**308–318.
37. **Matsuda, J. L., O. V. Naidenko, L. Gapin, M. Taniguchi, C. R. Wang, Y. Koezuka, and M. Kronenberg.** 2000. Tracking the response of natural killer cells to a glycolipid antigen using CD1d tetramers. J. Exp. Med. **192:**741–753.
38. **McCabe, R. E., S. G. Meagher, and B. T. Mullins.** 1991. Endogenous interferon-gamma, macrophage activation, and murine host defense against acute infection with Trypanosoma cruzi. J. Infect. Dis. **163:**912–915.
39. **Mendiratta, S. K., W. D. Martin, S. Hong, A. Boesteanu, S. Joyce, and L. Van Kaer.** 1997. CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4. Immunity **6:**469–477.
40. **Miyamoto, M., M. Emoto, V. Brinkmann, N. van Rooijen, R. Schmits, E. Kita, and S. H. Kaufmann.** 2000. Cutting edge: contribution of NK cells to the homing of thymic CD4+NKT cells to the liver. J. Immunol. **165:**1729–1732.
41. **Molano, A., S. H. Park, Y. H. Chiu, S. Nosseir, A. Bendelac, and M. Tsuji.** 2000. Cutting edge: the IgG response to the circumsporozoite protein is MHC class II-dependent and CD1d-independent: exploring the role of GPIs in NK T cell activation and antimalarial responses. J. Immunol. **164:**5005–5009.
42. **Pied, S., J. Roland, A. Louise, D. Voegtle, V. Soulard, D. Mazier, and P. A. Cazenave.** 2000. Liver CD4− CD8− NK1.1+ TCR alpha beta intermediate cells increase during experimental malaria infection and are able to exhibit inhibitory activity against the parasite liver stage in vitro. J. Immunol. **164:**1463–1469.
43. **Plata, F., F. Garcia-Pons, and H. Eisen.** 1984. Antigenic polymorphism of *Trypanosoma cruzi*: clonal analysis of trypomastigote surface antigens. Eur. J. Immunol. **14:**392–399.
44. **Rottenberg, M. E., M. Bakhiet, T. Olsson, K. Kristensson, T. Mak, H. Wigzell, and A. Orn.** 1993. Differential susceptibilities of mice genomically deleted of CD4 and CD8 to infections with *Trypanosoma cruzi* or *Trypanosoma brucei*. Infect. Immun. **61:**5129–5133.
45. **Rottenberg, M. E., D. A. Rodriguez, and A. Orn.** 1992. Control of Trypanosoma cruzi infection in mice deprived of T-cell help. Scand J. Immunol. **36:**261–268.
46. **Schofield, L., M. J. McConville, D. Hansen, A. S. Campbell, B. Fraser-Reid, M. J. Grusby, and S. D. Tachado.** 1999. CD1d-restricted immunoglobulin G formation to GPI-anchored antigens mediated by NKT cells. Science **283:**225–229.
47. **Smiley, S. T., M. H. Kaplan, and M. J. Grusby.** 1997. Immunoglobulin E production in the absence of interleukin-4-secreting CD1-dependent cells. Science **275:**977–979.
48. **Sumida, T., A. Sakamoto, H. Murata, Y. Makino, H. Takahashi, S. Yoshida, K. Nishioka, I. Iwamoto, and M. Taniguchi.** 1995. Selective reduction of T cells bearing invariant V alpha 24J alpha Q antigen receptor in patients with systemic sclerosis. J. Exp. Med. **182:**1163–1168.
49. **Tarleton, R. L., B. H. Koller, A. Latour, and M. Postan.** 1992. Susceptibility of beta 2-microglobulin-deficient mice to *Trypanosoma cruzi* infection. Nature **356:**338–340.
50. **Tarleton, R. L., L. Zhang, and M. O. Downs.** 1997. "Autoimmune rejection" of neonatal heart transplants in experimental Chagas disease is a parasite-specific response to infected host tissue. Proc. Natl. Acad. Sci. USA **94:**3932–3937.
51. **Torrico, F., H. Heremans, M. T. Rivera, M.-E. Van, A. Billiau, and Y. Carlier.** 1991. Endogenous IFN-gamma is required for resistance to acute Trypanosoma cruzi infection in mice. J. Immunol. **146:**3626–3632.
52. **Une, C., J. Andersson, M. L. Eloranta, D. Sunnemark, R. A. Harris, and A. Orn.** 2000. Enhancement of natural killer (NK) cell cytotoxicity and induction of NK cell-derived interferon-gamma (IFN-gamma) display different kinetics during experimental infection with Trypanosoma cruzi. Clin. Exp. Immunol. **121:**499–505.
53. **Watanabe, H., K. Ohtsuka, M. Kimura, Y. Ikarashi, K. Ohmori, A. Kusumi, T. Ohteki, S. Seki, and T. Abo.** 1992. Details of an isolation method for hepatic lymphocytes in mice. J. Immunol. Methods **146:**145–154.
54. **Wilson, S. B., S. C. Kent, K. T. Patton, T. Orban, R. A. Jackson, M. Exley, S. Porcelli, D. A. Schatz, M. A. Atkinson, S. P. Balk, J. L. Strominger, and D. A. Hafler.** 1998. Extreme Th1 bias of invariant Vα24JαQ T cells in type 1 diabetes. Nature **391:**177–181.
55. **World Health Organization** 1991. Control of Chagas disease 811. World Health Organization, Geneva, Switzerland.
56. **Wrightsman, R., S. Krassner, and J. Watson.** 1982. Genetic control of responses to *Trypanosoma cruzi* in mice: multiple genes influencing parasitemia and survival. Infect. Immun. **36:**637–644.

Editor: J. M. Mansfield