Both CD1d Antigen Presentation and Interleukin-12 Are Required To Activate Natural Killer T Cells during *Trypanosoma cruzi* Infection

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Mechanisms of natural killer T (NKT)-cell activation remain unclear. Here, we report that during *Trypanosoma cruzi* infection, interleukin-12 (IL-12) deficiency or anti-CD1d antibody treatment prevents normal activation. The required IL-12 arises independently of MyD88. The data support a model of normal NKT-cell activation that requires IL-12 and TCR stimulation.

Detection of liver NKT cells is reduced at day four of *T. cruzi* infection. Natural killer T (NKT) cells help control infections by *Trypanosoma cruzi* and other pathogens (5, 9, 10, 13, 28). How NKT cells become activated during these infections remains unclear. Possibilities include T-cell receptor (TCR) stimulation by CD1d presentation of pathogen-derived or self-derived glycolipid antigens or non-TCR stimulation through other NKT-cell receptors, such as the interleukin-12 (IL-12) receptor (2, 4, 11, 18, 29). To explore possible mechanisms of NKT-cell activation during *T. cruzi* infection, we developed an assay based on NKT-cell activation-induced surface receptor down modulation (14, 31) and our previous observation that during *T. cruzi* infection NKT cells undergo TCR and NK1.1 down modulation. Initially, we inoculated 7- to 10-week-old wild-type mice (C57BL/6 mice; Charles River, Wilmington, Mass.) with 2×10^5 CL strain *T. cruzi* trypomastigotes (23, 33) and monitored alterations in liver mononuclear cell TCRs and NK1.1. We found on day four of the infection a reproducible decrease in the number of detectable liver NKT cells and in the intensity of NK1.1 staining (Fig. 1), indicating liver NKT-cell activation.

During *T. cruzi* infection, IL-12, but not MyD88, is required for normal NKT-cell activation. IL-12 and IL-18 can activate or contribute to NKT-cell activation (6, 11, 20, 21). During infections, these cytokines may arise as a consequence of TLR signaling. In addition, NKT cells express TLR, so it is possible that direct recognition of pathogen-associated molecular patterns leads to NKT-cell activation (25). We analyzed the liver NKT-cell populations of IL-12p40^{-/-} (The Jackson Laboratory, Bar Harbor, Maine) and MyD88^{-/-} mice. MyD88 is an adaptor molecule that is critical for IL-18 signaling and required for many TLR signaling pathways (1, 17, 27). While *T. cruzi* infection reduced the detectable NKT cells in wild-type and MyD88^{-/-} mice, the number of NKT cells and the intensity of NK1.1 staining did not decline in IL-12^{-/-} mice (Fig. 1). This observation indicates that during *T. cruzi* infection, IL-12 is required for normal NKT-cell stimulation and receptor down modulation. Furthermore, the data argue that IL-12 can

be generated independently of MyD88-dependent TLR signaling. Accordingly, splenocytes placed in ex vivo culture from infected wild-type and infected MyD88^{-/-} mice produced similar amounts of IL-12 (Fig. 2). These data are in agreement with a recent report that demonstrates that during *T. cruzi* infection, IL-12 is stimulated independently of MyD88 (8). Taken together, our data argue that during *T. cruzi* infection, IL-12 signals, but neither IL-18 nor MyD88-dependent TLR signals, are required for normal NKT-cell activation.

During *T. cruzi* infection, CD1d is required for protective NKT-cell activation. We have previously demonstrated that CD1d gene-deficient (CD1d^{-/-}) mice develop greater parasitemia and tissue inflammation than wild-type mice, but since these mice develop without NKT cells, they cannot be used to investigate how NKT cells are activated (9, 10, 26). To investigate if during the infection NKT cells were activated by CD1d antigen presentation, we treated C57BL/6 mice with a blocking anti-CD1d antibody (clone 20H2; American Type Culture Collection, Manassas, Va.) or a control antibody (rat immunoglobulin G; Sigma, St. Louis, Mo.) and then inoculated the mice with *T. cruzi* (24). On day 4 of the infection, the liver NKT cells of the control antibody-treated mice were decreased in number and in NK1.1 staining intensity, whereas those of the anti-CD1d antibody-treated mice were decreased neither in number nor in NK1.1 staining intensity (Fig. 1). These data argue that CD1d antigen presentation was required for NKT-cell activation and that treatment with anti-CD1d would block protective NKT-cell functions during *T. cruzi* infection (9, 10). As expected, anti-CD1d antibody-treated mice, compared to control antibody-treated mice, suffered increased infection-induced weight loss and earlier death following a lethal inoculum of *T. cruzi* (Fig. 3A and B). Furthermore, following a sublethal inoculum, anti-CD1d monoclonal antibody treatment caused increased tissue inflammation (Fig. 3C and D). Together, these data strongly argue that during *T. cruzi* infection, TCR stimulation by CD1d antigen presentation is required for normal NKT-cell activation and protective functions.

To identify a *T. cruzi* CD1d-restricted antigen, CD1d-transfected L cells were pulsed with various trypomastigote preparations (live trypomastigotes, dead trypomastigotes, trypomastigote-conditioned media, and trypomastigote lysate) or 100 ng of α -galactosylceramide (α -GalCer)/ml (as a positive control).

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FIG. 1. IL-12 and CD1d are required for NKT-cell activation during *T. cruzi* infection. Liver mononuclear cells were prepared from mice that were either uninfected, infected 4 days previously (2×10^5 trypomastigotes), or inoculated 4 days previously with dead *T. cruzi* (2×10^5 trypomastigotes). Heating at 55°C for 5 min generated dead trypomastigotes. Mononuclear cell populations were prepared and incubated first with the anti-Fc γ R antibody 2.4G2 and then with fluorescent-conjugated anti-NK1.1 (clone PK136) and an anti-TCR β -chain (clone H57-597) (both from BD PharMingen). Representative flow cytometry plots from wild-type, IL-12^{-/-}, MyD88^{-/-}, anti-CD1d antibody (Ab)-treated and control antibody-treated mice are shown; the circled areas indicate the NKT cells, and the numbers within the plots indicate the percentages of NKT cells within the plots. Below each set of representative cytometry plots is listed the mean percentage of reduction in the numbers of NKT cells of that infected group compared to its uninfected group. These data were derived from wild-type mice (uninfected, $n = 17$; infected, $n = 15$), IL-12^{-/-} mice (uninfected, $n = 13$; infected, $n = 7$), MyD88^{-/-} mice (uninfected, $n = 4$; infected, $n = 8$), anti-CD1d antibody-treated mice (uninfected, $n = 6$; infected, $n = 4$), control antibody-treated mice (uninfected, $n = 6$; infected, $n = 4$), and wild-type mice (mock-inoculated, $n = 2$; dead trypomastigote inoculated, $n = 4$). *P* values were determined by using Student's *t* test. SEM, standard errors of the means.

FIG. 2. During *T. cruzi* infection, IL-12 production occurs independently of MyD88 signaling. Wild-type and MyD88^{-/-} mice were uninfected or infected with 2×10^5 trypomastigotes for 3 days. Splenocytes (5×10^6) were cultured for 48 h in each well of a 24-well plate (Corning, Inc., Corning, N.Y.) in 1 ml of RPMI 1640 supplemented with 5% heat-inactivated fetal calf serum and 50,000 U of penicillin-streptomycin. Supernatants were analyzed by using an IL-2 enzyme-linked immunosorbent assay (BD PharMingen). Results are shown as the means and standard deviations (uninfected, $n = 1$, infected, $n = 3$).

After washing, the antigen-pulsed CD1d-transfected L cells (2×10^5) were incubated overnight with a mouse invariant NKT-cell hybridoma (DN32.D3; 5×10^4 cells) (7), and the culture supernatants were assayed for IL-2. α -GalCer stimulated IL-2 production, unlike all of the trypomastigote preparations, suggesting that *T. cruzi* trypomastigotes do not express a CD1d-restricted NKT-cell-specific antigen (data not shown). In support of these in vitro data, the inoculation of mice with dead trypomastigotes did not activate NKT cells in vivo (Fig. 1). Together, these data suggest that during *T. cruzi* infection, the NKT-cell antigens may be self antigens. Live trypomastigotes might damage host cells and tissues to generate increased amounts of these self antigens.

This study demonstrates that during *T. cruzi* infection, normal NKT-cell activation requires (i) live *T. cruzi* inoculation, (ii) IL-12, and (iii) CD1d antigen presentation. The data support a model of NKT-cell activation during infections that requires CD1d presentation of an endogenous self antigen and IL-12 (6). The self antigens might provide weak stimulation that is normally incapable of activating NKT cells without IL-12 (6). These data are consistent with the previous findings that IL-12 enhances NKT-cell activation following exposure to low doses of α -GalCer and facilitates NKT-cell activation by self antigens during *Salmonella enterica* serovar Typhimurium

FIG. 3. CD1d is required for protection during *T. cruzi* infection. C57BL/6 mice were injected intraperitoneally with 200 μ g of anti-CD1d antibody or rat immunoglobulin G. Antibodies were injected 1 day before infection, on the day of infection, and every second day thereafter until day 14 of infection. (A and B) Antibody-treated female C57BL/6 mice (five mice per group) were inoculated with 5×10^5 *T. cruzi* trypomastigotes. (A) On the indicated days, each mouse was weighed, and the means and standard errors of the percentages of weight change compared to the preinfection weight for each mouse is displayed. (B) Survival of the mice is shown and was analyzed by using the log rank statistic of Kaplan-Meier survival analysis (SPSS, Inc., Chicago, Ill.). For weight change and survival of anti-CD1d antibody-treated mice compared to control antibody-treated mice, the *P* value was <0.05 . (C) C57BL/6 mice were inoculated with 2×10^5 *T. cruzi* trypomastigotes and BALB/c mice were inoculated with 1×10^5 *T. cruzi* trypomastigotes. Skeletal muscles were collected from antibody-treated mice on day 28 of infection and assessed for inflammation. Investigators blinded to the experimental conditions obtained five random $\times 100$ images (Eclipse E200 microscope and Coolpix 4500 camera; Nikon, Tokyo, Japan) of the left and right quadriceps (10 images per mouse). The images were overlaid with a grid of 49 evenly dispersed points, and the percentage of points intersected by nuclei was determined. The percentage of points intersected by nuclei of uninfected quadriceps images was subtracted. Three mice per group were analyzed to provide 30 scores per group for statistical analysis by Student's *t* test. Representative hematoxylin and eosin-stained sections are shown. (D) The muscle inflammatory scores are presented as the means and standard errors.

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FIG. 3—Continued.

infection (6, 19, 30). IL-12 might cause the antigen-presenting cells to increase expression of costimulatory molecules and thereby facilitate NKT-cell activation (12, 15, 16, 32). Alternatively, analogous to NK cells, IL-12 might act directly on NKT cells by countering inhibitory signals and thus allow activation of the NKT cells (11, 22). In contrast to our data and these studies, a recent report demonstrates that NKT-cell activation during *Leishmania donovani* infection is triggered by parasite-derived glycolipid antigens independently of IL-12 (3). It remains unclear which antigens stimulate NKT cells during *T. cruzi* infection. A better understanding of how NKT cells become activated may guide therapies to alleviate infection-induced morbidity and mortality.

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