NK cell activation and protection occur independently of natural killer T cells during *Trypanosoma cruzi* infection

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Abstract

Natural killer T (NKT) cells regulate aspects of pro-inflammatory and anti-inflammatory responses and contribute to the control of infections and chronic inflammatory diseases. During *Trypanosoma cruzi* infection both NKT cells and NK cells are critical to the protective response. How NKT cells interact and possibly regulate NK cells during infections remains uncertain. *In vivo* studies have demonstrated that specific activation of NKT cells with \( \alpha \)-galactosylceramide (\( \alpha \)-GalCer) leads to NK cell activation. These results suggest that during some infections activated NKT cells might regulate NK cell activation and functions. Therefore, using gene-deficient mice that lack NKT cells and antibody-treated mice that lack NK cells, we investigated the interactions of NKT cells and NK cells during experimental *T. cruzi* infection. We report here that during acute *T. cruzi* infection spleen and liver NK cell activation and cytolytic activity occur independently of NKT cells. Moreover, NK cell protection occurs independently of NKT cells. In contrast to these results that fail to demonstrate an interdependence, at day 4 of infection the number of liver NK cells is controlled by NKT cells. Thus, during *T. cruzi* infection, regulation of the number of liver NK cells requires NKT cells, but the activation of NK cells and protection by NK cells does not. The data presented here argue that during infections NK cell activation and protection occur independently of NKT cells.

Introduction

Natural killer T (NKT) cells are a subset of T cells distinct from conventional T cells and NK cells as they express some receptors of both cell types, e.g. the TCR and NK1.1. Unlike conventional T cells, NKT cells are stimulated by glycolipid antigens presented by the MHC-like CD1d molecular complex that is composed of \( \beta \)-2-microglobulin and the non-polymorphic CD1d protein (1). NKT cell selection is dependent on the CD1d molecular complex (1, 2). Many NKT cells express an invariant \( \alpha \)14-\( \beta \)18 TCR (3). Thus, mice lacking the \( \alpha \)18 gene (\( \alpha \)18 \(-/-\) mice) are deficient in the invariant NKT (iNKT) cells (4). NKT cell effector functions include cytolytic activity and the secretion of cytokines (e.g. IFN\( \gamma \), IL-4, IL-13) (5). NKT cells maintain an activated phenotype and respond rapidly following stimulation. The ability to respond rapidly is thought to enable NKT cells to regulate protective anti-pathogen responses and anti-inflammatory responses (6–8).

Similar to NKT cells, NK cells also contribute to the control of infections and chronic inflammatory diseases (6, 9–13). How NK cells and NKT cells interact during infections and chronic inflammatory diseases remains unclear. Several studies have examined the relationship of NKT cell and NK cell activation following stimulation with the NKT cell-specific glycolipid antigen \( \alpha \)-galactosylceramide (\( \alpha \)-GalCer). These studies indicate that \( \alpha \)-GalCer stimulation of iNKT cells results in IFN\( \gamma \) secretion that activates and potentiates NK cell functions (14–17). Thus, during infections stimulation of iNKT cells may affect NK cell functions.

Infection with the protozoan parasite *Trypanosoma cruzi* causes Chagas disease, a chronic inflammatory disease that affects several million people in endemic areas (18). *T. cruzi* replication occurs within the host’s cells and is limited by the host’s cellular immune response (19). Depletion of NK cells and NKT cells in mice with an anti-NK1.1 antibody results in elevated parasitemia and shortened survival (20, 21). Similar results have been obtained with the anti-asialo GM1 (ASGM1) antibody that selectively depletes NK cells (20).
Trypanosoma cruzi: NK cells protect without NKT cells

Studies have shown that mice deficient in iNKT cells suffer increased acute-phase parasitemia, morbidity and death (22, 23). Thus, both NK cells and NKT cells help control acute Trypanosoma cruzi infection, and it is possible that NKT cells might regulate aspects of the NK cell response. Previous in vitro studies demonstrated that splenocytes from nude mice cultured with T. cruzi produce IFNγ, suggesting that T. cruzi can stimulate NK cells independently of NKT cells (21). Based on these in vitro studies, we hypothesized that NK cells would be activated independently of NKT cells in vivo during T. cruzi infection. To examine this possibility and to better define the interactions of NKT cells and NK cells, we analyzed T. cruzi-infected mice that lacked NK cells, NKT cells or both cell types.

Methods

Mice

Wild-type C57BL/6 mice were purchased from Charles River (Wilmington, MA, USA). C57BL/6 CD1d−/− mice (2) (back-crossed a minimum of seven times) and C57BL/6 J Ax18−/− mice (4) (back-crossed a minimum of nine times) were bred in the animal facilities of the Corixa Corporation (Seattle, WA, USA). C57BL/6 MHC II−/− mice were a gift from Sally Clarke (University of Washington, Seattle, WA, USA). Mice between 7- and 10-week old were used. The Corixa Corporation Institutional Animal Care and Use Committee approved all of the animal procedures.

T. cruzi

The CL strain of T. cruzi was used (24, 25). Trypomastigotes were obtained from culture supernatants of infected 3T3 cells grown in DMEM supplemented with 10% heat-inactivated calf serum and 50 000 U penicillin/streptomycin (all from Bio-Whittaker, Walkersville, MD, USA). Mice were infected by intraperitoneal (i.p.) injection with T. cruzi trypomastigotes at the stated inoculum.

Cytotoxicity assays

Spleen and liver mononuclear cell populations were prepared as previously described (22). Mononuclear effector (E) cells were incubated with 51Cr-labeled YAC-1 target (T) cells for 4 h at 37°C at different E : T ratios. After 4 h of incubation, 51Cr released from target cells was counted using a gamma counter (Packard, Meriden, CT, USA). Specific lysis was calculated as follows: [(measured 51Cr release – spontaneous 51Cr release)/ (maximum 51Cr release – spontaneous 51Cr release)] × 100. Maximum release was determined based on detergent-lysed target cells. Spontaneous release was determined by incubating target cells in the absence of effector cells. As a positive control for cytotoxicity, mice were injected i.p. with 100 μg polyinosinic polycytidylic acid (poly I:C) (Sigma) 18 h before cell preparation.

NK cell depletion and mononuclear cell preparation

Mice were depleted of NK cells by i.p. injection of 500 μg α-ASGM1 antibody (Wako Chemicals, Richmond, VA, USA) 1 day before T. cruzi infection and on day 7 of the infection when indicated. Control mice were injected on the same days with 500 μg control rabbit Ig (Jackson ImmunoResearch, West Grove, PA, USA).

Quantitative analysis of spleen and liver cell populations

Total mononuclear cell populations were determined using a hemacytometer. The number of NK cells was determined by incubating cells at 4°C for 20 min with the anti-FcR mAb 2.4G2, washing and incubating for 30 min at 4°C with an anti-NK1.1 FITC- or PE-conjugated mAb (clone PK136) and an anti-TCRβ-chain Cy-Chrome-conjugated mAb (clone H57-597) (all PharMingen, San Diego, CA, USA) in 50 μl staining buffer (1% BSA/PBS/0.09% sodium azide, pH 7.4) followed by flow cytometry (FACScan®, BD Biosciences, San Jose, CA, USA). The data were analyzed with WinMDI 2.7 (Joseph Trotter, available at http://facs.scripps.edu/software.html) to determine the percentage of NK cells (NK1.1+TCRβ−). The percentage of the NK cell subset × the total number of cells = the absolute number of NK cells.

Determination of serum glutamic pyruvic transaminase

Blood was collected from individual mice by venesection of the tail, and sera were prepared and assayed for glutamic pyruvic transaminase (GPT) activity using a colorimetric test according to the manufacturers’ instructions (Sigma). The samples were analyzed at 490 nm (ELx808, Bio-Tek Instruments Inc., Winooski, VT, USA).

Statistics

The P-values for cytotoxicity and cell number were determined using the Student’s t-test (Microsoft Excel, Microsoft Corporation, Redmond, WA, USA). The P-values for Kaplan–Meier survival analysis were determined using the Wilcoxon statistic (Epi Info 2002, available at http://www.cdc.gov/epiinfo/).

Results

During early T. cruzi infection NK cell cytotoxicity is detected

NKT cells have been shown in non-infectious experimental systems to activate NK cells. During T. cruzi infection both NKT and NK cells are activated, suggesting that during this infection NKT cells might regulate NK cell activation. Previous studies detected cytotoxic NK cells within spleen or peritoneal exudate cells on day 2 of T. cruzi infection (26, 27). We postulated that in the liver, where a large number of NKT and NK cells reside, NK cell cytotoxicity would be detected at a similar time of the infection. At 24 h of infection, using YAC-1 cells as target cells, cytotoxicity was not detected (Fig. 1a). At 42 h, however, liver and spleen cells displayed cytotoxicity (Fig. 1a). Cytotoxic activity was only detected within liver and spleen cells collected from 36 to 42 h of infection (Fig. 1a and b). Cells collected later (at 48 or 69 h) did not exhibit cytotoxicity (Fig. 1c). Thus, from 36 to 42 h of the infection we detected cytotoxicity by liver and spleen mononuclear cells that is consistent with NK cell activation and cytotoxicity.

To determine if the cytotoxic activity was derived from NK cells, mice were injected with the anti-ASGM1 antibody, a treatment that specifically removes NK cells (28–30) but
During T. cruzi infection iNKT cells regulate liver NK cell numbers

In recent studies we observed that on day 14 and 21 of T. cruzi infection iNKT cells regulate, by an unknown mechanism, the number of liver and spleen cells (23). At day 14 and 21, in iNKT cell-deficient mice, compared with wild-type mice, the number of NK cells in the spleen and liver is increased by 2-fold. We wanted to evaluate if this effect of iNKT cells on NK cell numbers occurred earlier during the infection, and therefore day 4-infected wild-type and Jα18–/– mice were analyzed. In the spleen, wild-type mice and Jα18–/– mice displayed a mild increase in the total number of spleen cells (Fig. 3). The

NK cytotoxic activity is independent of NKT cells

To determine if NK cell cytotoxic activity was dependent on NKT cells, we analyzed cytotoxic activity of spleen and liver mononuclear cells from NKT cell-deficient mice. Jα18–/– mice (deficient in iNKT cells) and CD1d–/– mice (deficient in both iNKT and variant NK cells) were infected with 40 h, and the liver and spleen cells demonstrated cytotoxic activity comparable to that of wild-type mice (Fig. 2a and b). Jα18–/– mice treated with anti-ASGM1 antibody did not display cytotoxic activity (Fig. 2a). These data indicate that during T. cruzi infection NK cell activation and cytotoxic activity occur independently of CD1d-restricted NKT cells.

Fig. 2. Liver and spleen NK cells, but not iNKT cells, mediate Trypanosoma cruzi-induced cytotoxicity. C57BL/6 mice were infected with 1 × 10^5 trypanomastigotes and spleen and liver (effector) cells were assayed for cytotoxic activity against YAC-1 (target) cells. In (a) Jα18–/– mice and (b) CD1d–/– mice were compared with wild-type mice. In (a) wild-type and Jα18–/– mice were injected with either control antibody or anti-ASGM1 antibody to deplete NK cells. In (a), P < 0.001 for control antibody and anti-ASGM1 antibody-treated mice at the highest E:T ratio, and in (b) P < 0.01 for infected versus uninfected mice at the highest E:T ratio. Results are mean values of triplicate determinations of the percent-specific 51Cr release. Each line represents the cytotoxic activity of an individual mouse and the results are representative of three experiments that analyzed one or more mice.
Neither iNKT nor NK cells contribute toward T. cruzi-induced liver damage

During T. cruzi infection a mild liver damage can be detected by monitoring serum GPT. iNKT cells are critical to both Con A-induced hepatitis and α-GaICer-induced hepatitis, suggesting that during T. cruzi infection, the activated liver NK cells might be critical to the liver damage (31–33). Arguing against this possibility was an observation we made that by day 7 of T. cruzi infection, iNKT cell-deficient mice, compared with wild-type mice, displayed increased T cell infiltration of the liver, suggesting that iNKT cell-deficient mice might suffer more severe liver damage (23). To determine if during T. cruzi infection the presence or absence of iNKT cells contributed to liver damage, wild-type and Jα18−/− mice were infected, and serum GPT activity was monitored (Fig. 4a). While serum GPT levels became elevated during the infection, no significant differences were detected between the mouse strains (Fig. 4a). These data argue that iNKT cells are not critical to the T. cruzi-induced liver damage. In addition, because NK cells are a major component of the liver, and iNKT cells regulate the size of the liver NK cell population during the infection, we questioned if NK cells might contribute to the liver damage. Therefore, control antibody-treated and anti-ASGM1 antibody-treated mice were inoculated with T. cruzi and serum GPT levels were measured. NK cell depletion did not prevent the liver damage, arguing that NK cells are not critical to the T. cruzi-induced hepatitis either (Fig. 4b). These data argue that during T. cruzi infection iNKT cells and NK cells do not cause or control liver damage.

During T. cruzi infection NK cells provide protection in the absence of iNKT cells

The detection of NK cell cytotoxic activity in the absence of iNKT cells argues that during T. cruzi infection NK cell protection may occur independently of iNKT cells. To examine this possibility, we first demonstrated that NK cell depletion of wild-type mice with anti-ASGM1 antibody shortened the survival (Fig. 5a; P < 0.05). We then treated Jα18−/− mice with anti-ASGM1 antibody. The NK cell-depleted Jα18−/− mice died significantly earlier than the control-treated Jα18−/− mice (Fig. 5b; P < 0.005). The survival data indicate that during acute T. cruzi infection NK cells provide protection in the absence of iNKT cells. These data suggested that iNKT cell-deficient mice, depleted of NK cells, would have decreased survival compared with wild-type mice depleted of NK cells.

To allow direct comparison of wild-type and Jα18−/− mice depleted of NK cells, these mice were treated with either control antibody or anti-ASGM1 antibody and then inoculated with T. cruzi. In agreement with our recent data (23), an inoculum that is sub-lethal in wild-type mice (1 × 10^5 trypomastigotes) killed a significant number of the Jα18−/− mice (Fig. 5c; P < 0.05). Again, NK cell depletion of wild-type mice caused earlier death compared with control antibody-treated wild-type mice (Fig. 5c; P < 0.05). But surprisingly, NK cell depletion of Jα18−/− mice caused earlier death compared with Jα18−/− control antibody-treated mice (Fig. 5c; P < 0.05). Together the data indicate that during acute T. cruzi infection NK cells provide protection in the absence of iNKT cells. Interestingly, the Jα18−/− NK cell-depleted mice and

number of spleen NK cells (NK1.1^hi TCRβ−), however, did not increase by day 4 of the infection, and the number of spleen NK cells was similar in uninfected and 4 day-infected wild-type and Jα18−/− mice (Fig. 3).

In wild-type mice the liver total mononuclear cells or NK cells were not increased by day 4 of the infection (Fig. 3). In contrast, in Jα18−/− mice both liver total mononuclear cells and NK cells were increased 2-fold by day 4 of the infection (Fig. 3; P = 0.03 comparing NK cell numbers of infected wild-type and Jα18−/− mice). These data indicate that during T. cruzi infection iNKT cells play a critical role in maintaining liver mononuclear cell and NK cell homeostasis. A specific function for iNKT cells in controlling liver cell numbers during T. cruzi infection is supported by the observation that MHC class II-deficient mice do not display increases in liver mononuclear cells or NK cells (Fig. 3). Thus, in the liver during T. cruzi infection early NK cell activation and cytotoxicity occur independent of iNKT cells, but NK cell number homeostasis is dependent on iNKT cells.

Fig. 3. During Trypanosoma cruzi infection liver NK cells are increased in the absence of iNKT cells. C57BL/6 wild-type, Jα18−/− and MHC II−/− mice were either uninfected or infected with 2 × 10^5 trypomastigotes. Four-day-infected mice and uninfected mice were analyzed at the same time. Spleen and liver mononuclear cells were prepared, counted, incubated with anti-NK1.1 and anti-TCRβ fluorescent antibodies and analyzed by flow cytometry. Results are shown as the mean number of cells and SE. NK cells were identified as NK1.1^hi TCRβ−, and liver NK cell percentages were as follows: wild-type uninfected = 7.60, infected = 8.54; Jα18−/− uninfected = 10.35, infected = 12.64; MHC II−/− uninfected = 8.45, infected = 8.65. Twenty uninfected wild-type mice were compared with 17 infected wild-type mice, 8 uninfected Jα18−/− mice were compared with 14 infected Jα18−/− mice and 2 uninfected MHC II−/− mice were compared with 4 infected MHC II−/− mice. *P < 0.05 comparing liver NK cell numbers of infected wild-type and Jα18−/− mice.
the wild-type NK-depleted mice had similar survival arguing that NK cell depletion has a dominant affect over NKT cell deficiency during acute *T. cruzi* infection.

**Discussion**

NKT cells are immunoregulatory cells that contribute to protection against pathogens and prevention of pathological chronic inflammatory responses (6–8). How NKT cells provide these functions remains unclear. Their large numbers in the liver and spleen and their ability to rapidly respond to stimuli suggest that NKT cells are able to initiate and affect many aspects of ensuing immune responses. α-GalCer is an NKT cell-specific antigen (34). The specific activation of NKT cells by α-GalCer enabled investigators to demonstrate that α-GalCer-activated NKT cells secrete IFN-γ that subsequently activates NK cells (14–17). This ability of NKT cells to activate NK cells raises the possibility that during some infections NKT cell might activate or affect the NK cell response. During acute

**Fig. 4.** During *Trypanosoma cruzi* infection neither NK cells nor iNKT cells mediates liver damage. Groups of five female C57BL/6 mice were infected with $1 \times 10^7$ trypomastigotes and sera from individual mice were assayed for GPT activity. In (a), *Ja18−/−* mice and wild-type mice were analyzed. In (b), wild-type mice were injected with either anti-ASGM1 antibody (NK-depleted group) or control antibody. Results are shown as the mean and SE.

**Fig. 5.** During *Trypanosoma cruzi* infection NK cells provide protection independently of iNKT cells. Female C57BL/6 mice were injected with anti-ASGM1 antibody or control antibody 1 day before and 1 week after *T. cruzi* infection. In (a) wild-type mice were inoculated with $5 \times 10^5$ trypomastigotes and in (b) *Ja18−/−* mice were inoculated with $2 \times 10^5$ trypomastigotes. In (c) wild-type mice and *Ja18−/−* mice were inoculated with $1 \times 10^5$ trypomastigotes. Results are shown as percent survival.
The interaction of NKT cells and NK cells during T. cruzi infection both NKT cells and NK cells become activated and provide protection, but it is unknown if during the infection NKT cells activate or affect the NK cell response (20–22, 26). Here we report, as previously shown, that during T. cruzi infection both NK and NKT cells provide protection (20, 22, 23, 26). Moreover, we report that NK cell cytolytic activity and protection occur independently of NKT cells. Although early NK cell-mediated cytolytic activity and protection appear to occur independently of NKT cells, the size of the liver mononuclear cell population, including the NK cell population, is dependent on NK cells. Furthermore, monitoring of serum GPT levels indicates that NKT and NK cells do not contribute to the T. cruzi-induced liver damage, and despite increased liver mononuclear cells and liver NK cells in the iNKT cell-deficient mice, the liver damage was not affected.

The importance of NK cells in providing protection during T. cruzi infection has driven our interest in defining how NKT cells affect the NK cells. α-GalCer treatment has been shown to provide protection against different pathogens and tumors (35, 36). The ability of the α-GalCer-activated NKT cells to stimulate NK cells is often critical to these protective responses (37). However, not all α-GalCer-stimulated anti-pathogen responses require NK cells (38, 39). In addition, investigation of immune responses against different solid tumors demonstrates that NK cells and iNKT cells can provide independent protection (40). Thus, in different immune responses it appears that NK cells can function dependently or independently of NKT cells. To our knowledge no study has examined the interactions of NKT cells and NK cells during a natural infection (one that was not pre-treated with α-GalCer). Our data indicate that during T. cruzi infection NK cells are activated and protective in the absence of NKT cells and therefore support a model for infections in which protective NKT and NK responses are initiated independently.

On day 4 of T. cruzi infection of iNKT cell-deficient mice, compared with wild-type mice, an increase in both total liver mononuclear cells and liver NK cells is observed. This cellular increase was not evident in the spleen populations of iNKT cell-deficient mice. How iNKT cells influence the size of liver cell populations remains unclear. Parasite burden and microbial signaling are not likely to contribute to the increase in liver NK cells in the iNKT cell-deficient mice because in the MHC class II-deficient mice the number of liver NK cells was not increased even though there was greater parasitemia than that observed in the iNKT-deficient mice (data not shown). The iNKT cells might regulate the population sizes by direct cellular contact or by secreting soluble factors. Alternatively, NK cells may compete with NK cells for growth factors. These data suggest that during infections individuals with diminished iNKT cell populations might develop altered liver effector cell populations that could affect the inflammatory responses.

α-GalCer stimulation of iNKT cells results in liver damage that can be monitored with serum GPT (33, 41). Liver damage occurs during T. cruzi infection and the activated iNKT cells or NK cells could contribute to this liver damage (42). During the acute infection of wild-type mice, iNKT cell-deficient mice and NK cell-depleted mice the levels of detectable serum GPT were similar, arguing that iNKT cells and NK cells do not contribute to the liver damage. Even the large increase in liver NK cells observed in the iNKT cell-deficient mice did not alter the degree of apparent liver damage. Together, these data argue that during T. cruzi infection, iNKT cells and NK cells do not contribute to the liver damage. Rather, the liver damage might be caused by parasites as they invade and subsequently destroy hepatocytes (43, 44). Furthermore, the serum GPT levels during the infection revealed an initial peak at about day 7 of the infection, followed by a larger second peak at day 14 of the infection. These serum GPT peaks might reflect times of semi-synchronized trypomastigote escape from and destruction of the hepatocytes.

In summary, during T. cruzi infection NK cell activation and protection is independent of NKT cells. Although the NK cell activation appears independent, regulation of the size of the liver NK cell population during the first days of the infection appears dependent on NKT cells. We have recently observed that NKT cells dampen the pro-inflammatory response during the late acute phase of the infection. It is possible that this dampening effect of NKT cells involves inhibition of NK cells. Determining how NKT cells and NK cells are activated and inhibited is likely to provide insights into the chronic inflammatory process of Chagas disease and how chronic inflammatory diseases can be prevented and treated.

References