During acute *Trypanosoma cruzi* infection highly susceptible mice deficient in natural killer cells are protected by a single α-galactosylceramide treatment

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Summary

The protective immune response against *Trypanosoma cruzi* is improved by treatment with the natural killer (NK) T-cell glycolipid antigen α-galactosylceramide (α-GalCer). A single α-GalCer treatment of mice before *T. cruzi* infection decreases parasitaemia and prolongs survival. This protection is dependent on CD1d-restricted NKT cells and interferon-γ (IFN-γ) suggesting that α-GalCer-activated NKT cells produce IFN-γ, which stimulates the cells of the innate and adaptive immune responses to provide protection. To learn which cells provide protection we investigate here α-GalCer treatment of mice deficient in different immune cells. Surprisingly, although NK cells provide protection against *T. cruzi*, and are a major source of IFN-γ following α-GalCer treatment, NK cells are not required for the α-GalCer-induced protection. The α-GalCer treatment of NK-cell-depleted mice controlled parasitaemia and prevented death. In contrast, phagocytes, helper T cells and cytotoxic T cells are required. Furthermore, α-GalCer treatment of MHC II$^{-/-}$ or CD8α$^{-/-}$ mice exacerbated the infection, demonstrating that α-GalCer treatment induces some responses that favour the parasite. In summary α-GalCer protection against *T. cruzi* required multiple cellular responses, but not the response of NK cells. These results provide useful information because α-GalCer is being developed as therapy for infections, autoimmune diseases, allergy and cancers.

Keywords: cellular activation; natural killer cells; natural killer T cells; parasite: protozoan; T lymphocytes

Introduction

Trypanosoma cruzi is a protozoan parasite that chronically infects many mammalian species. During the acute infection *T. cruzi* disseminates in the mammalian host and a patent parasitaemia occurs.[1] The natural acute infection is rarely lethal, and once infected individuals remain so for their lifetime. Thirty per cent of people infected develop Chagas' disease, a chronic inflammatory disease that causes significant morbidity and mortality.[1] The pathogenesis of Chagas' disease remains unclear, but previous studies argue that the severity of the chronic disease correlates with both the parasite burden and the regulation of the anti-*T. cruzi* immune response.[2,3] Safe and effective treatments for acute and chronic *T. cruzi* infection are lacking. Treatments that can lower parasite burden or improve regulation of the anti-parasite immune response are needed.

Natural killer T (NKT) cells are a distinct subset of T cells that rapidly secrete large amounts of cytokines when activated, and are known to regulate immune responses of infections, cancers and autoimmune diseases.[4] In contrast with conventional T cells, NKT cells are stimulated by glycolipid antigens presented by the major histocompatibility complex (MHC) class I-like CD1d molecular complex.[4] Most NKT cells express an invariant T-cell

Abbreviations: α-GalCer, α-galactosylceramide; ASGM1, asialo GM1; DMEM, Dulbecco's modified Eagles's medium; GPT, glutamic pyruvic transaminase; IFN-γ, interferon-γ; IL-4, interleukin-4; iNKT, invariant NKT; mAb, monoclonal antibody; MHC, major histocompatibility complex; NKT, natural killer T; PBS, phosphate-buffered saline.

receptor α-chain and are referred to as invariant NKT (iNKT) cells. α-galactosylceramide (α-GalCer) is a glycolipid antigen of iNKT cells. Treatment of mice with α-GalCer causes iNKT cells to rapidly secrete large amounts of interferon-γ (IFN-γ) and interleukin-4 (IL-4), and results in the downstream activation of NK cells, macrophages and lymphocytes.[5–10] Treatment of mice with NKT-cell-specific glycolipid antigens can improve the outcome of cancers, autoimmune diseases and infections. The safety of NKT-cell-specific glycolipid antigen treatment is currently being investigated in human clinical trials (reviewed in refs 11,12).

To investigate the pathogenesis of Chagas' disease we used the *T. cruzi* CL strain, a strain that infects humans and mice and that permits the examination of both the acute and chronic stages of the infection. We have previously reported that treatment of mice before *T. cruzi* infection with α-GalCer provides protection that is dependent on iNKT cells and IFN-γ.[13] Other investigators have also demonstrated that α-GalCer treatment provides protection against more virulent *T. cruzi* strains.[14] To determine which cells contribute to α-GalCer-induced *T. cruzi* protection we have investigated α-GalCer treatment of mice deficient in cells known to be activated downstream of iNKT cells, i.e. NK cells, phagocytes and lymphocytes. These investigations demonstrate that α-GalCer protection requires phagocytes and lymphocytes but, surprisingly, not NK cells.

Materials and methods

Mice

Age-matched and weight-matched female mice were used in each experiment. Wild-type C57BL/6 mice were obtained from Charles River (Wilmington, MA). MHC $II^{-/-}$ ($I\text{-}A\beta^{-/-}$), $CD8\alpha^{-/-}$ ($Cd8a^{tm1Mak}$) and $perforin^{-/-}$ (Pfp^{tm1Sdz}) mice, all on the C57BL/6 background, were obtained from Jackson Laboratory (Bar Harbor, ME). The Institutional Animal Care and Use Committee approved all of the animal procedures.

Trypanosoma cruzi *and parasitaemia determination*

A recently derived clone of the CL strain subclone 3 was used.[15] Trypomastigotes were obtained from culture supernatants of infected 3T3 cells grown in Dulbeccos' modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated calf serum and 50 000 U penicillin/streptomycin (all from BioWhittaker, Walkersville, MD). Mice were infected by intraperitoneal (i.p.) injection with *T. cruzi* trypomastigotes at the stated inoculum. Parasitaemia was monitored by venesection of the tail; 2 μl blood was diluted in 1·66% ammonium chloride in phosphate-buffered saline (PBS) and the trypomastigotes were counted on a haemocytometer by an investigator unaware of experimental status.

α-GalCer administration and mouse monitoring

Mice were injected i.p. with 5 μg α-GalCer (supplied by Kirin Brewery Ltd, Gunma, Japan) 1 day before *T. cruzi* infection. α-GalCer was diluted in DMEM (BioWhittaker) immediately before administration. Mice were weighed just before α-GalCer or diluent treatment and again 1 day later, before *T. cruzi* infection. For analyses, the per cent weight change of each individual mouse was calculated, and from individual per cent changes, the mean and SE per cent weight change of each group of mice were calculated. Serum glutamic pyruvic transaminase (GPT) activity was determined in blood (collected from individual mice by venesection of the tail) using a colorimetric test according to the manufacturers' instructions (GP-transaminase; Sigma, St Louis, MO).

Mouse treatments

NK cells were depleted by i.p. injection of 80 μg anti-asialo GM1 antibody [40 μl 2 mg/ml antibody in PBS (Wako Chemicals, Richmond, VA)]. Depletion of NK cells was confirmed by analyses of liver and spleen mononuclear cells stained with anti-NK1.1 phycoerythrin-conjugated monoclonal antobody (mAb) and anti-pan T-cell receptor β-chain Cy-Chrome-conjugated mAb (both from PharMingen, San Diego, CA) followed by flow cytometry as previously described.[16] Phagocyte function was inhibited by i.p. injection of 20 mg silica [200 μl 100 mg/ml in PBS (Sigma)] 2 days and 1 day before *T. cruzi* infection, and again on day 7 of infection.

Statistics

The *P*-values for cumulative parasitaemia and weight change were determined using Student's *t*-test (Microsoft Excel, Microsoft Corporation, Redmond, WA). The *P*-values for survival were calculated by determining the log rank statistic using Kaplan–Meier survival analysis (SPSS Inc., Chicago, IL).

Results

During acute *T. cruzi* infection α-GalCer treatment protects mice depleted of NK cells

We have previously demonstrated that mice treated with α-GalCer 1 day before *T. cruzi* infection are protected and that this protection is dependent on IFN-γ.[13] In this report we investigate what aspects of the immune response are activated by α-GalCer to provide protection. It is known that α-GalCer-activated iNKT cells stimulate

NK cells to secrete IFN-γ.[6,17,18] Furthermore, NK cells are critical for survival during acute *T. cruzi* infection.[19–21] Together, these data strongly suggested to us that α-GalCer-induced protection against *T. cruzi* would require iNKT cell activation of NK cells. In previous studies we have demonstrated that anti-asialo GM1 (ASGM1) antibody treatment before *T. cruzi* infection selectively depletes the NK cells and results in increased parasitaemia and more rapid death.[21] To investigate if α-GalCer-induced protection required NK cells, groups of mice were injected with PBS (control injection) or anti-asialo GM1 antibody and the spleen and liver NK cells were analysed by flow cytometry (Fig. 1a). In the spleen and liver of the mice injected with anti-asialo GM1 antibody the NK-cell populations appeared greatly diminished, whereas the NKT cell populations appeared unaffected (Fig. 1a). After confirming the selective depletion of NK cells, PBS-injected and anti-asialo GM1 antibody-injected groups of mice were treated with α-GalCer or diluent and 1 day later they were inoculated with 1×10^5 trypomastigotes. As expected, mice depleted of NK cells (group 1) experienced the highest parasitaemia and most rapid death (Fig. 1b,c). Furthermore, as we have previously shown, mice with normal NK-cell populations treated with α-GalCer (group 4), compared to mice with normal NK-cell populations treated with diluent (group 3), developed lower parasitaemia ($P = 0{\cdot}009$) and improved survival ($P = 0{\cdot}017$) confirming that α-GalCer treatment provides protection against *T. cruzi* (Fig. 1b,c).[13] Surprisingly, NK-cell-depleted, α-GalCer-treated mice (group 2), compared to NK-cell-depleted, diluent-treated mice (group 1), exhibited much lower parasitaemia ($P = 0{\cdot}002$) and strikingly improved survival ($P = 0{\cdot}002$), indicating that a normal NK-cell population is not required for α-GalCer-induced protection (Fig. 1b,c). Remarkably, NK-cell-depleted, α-GalCer-treated mice (group 2) and NK-cell normal, α-GalCer-treated mice (group 4) exhibited similar parasitaemia ($P = 0{\cdot}166$) and survival ($P = 0{\cdot}134$), which is consistent with α-GalCer treatment being able to overcome the NK-cell deficiency (Fig. 1b,c). These experiments demonstrate that NK cells are not required for α-GalCer-mediated protection against *T. cruzi* infection and that a single α-GalCer treatment before *T. cruzi* infection can prevent the rapid death experienced by NK-cell-depleted mice.

The protective effect of α-GalCer during *T. cruzi* infection requires silica-sensitive cells, and helper and cytotoxic T cells. As the α-GalCer protection against *T. cruzi* is IFN-γ-dependent, we hypothesized that IFN-γ-activated phagocytes contributed.[13] If this were correct, then inhibition of phagocytic cell activity would reduce or eliminate the α-GalCer protection. To investigate this possibility, mice were injected with silica and then treated with α-GalCer or diluent before subsequent *T. cruzi* infection.[22–24] α-GalCer treatment failed to provide protection

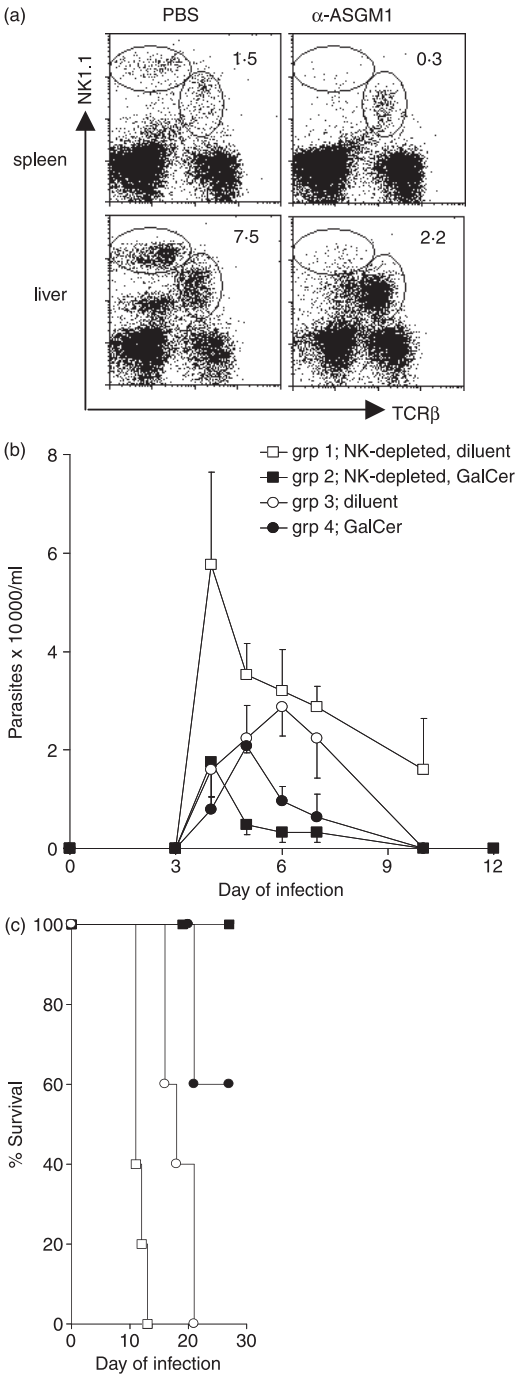

Figure 1. α-GalCer-induced *Trypanosoma cruzi* protection does not require NK cells. Mice were injected with 80 μg anti-asialo GM1 antibody or PBS, 1 day later they were treated with 5 μg α-GalCer or diluent and the next day they were inoculated with 1×10^5 trypomastigotes. (a) Spleen and liver mononuclear cells of mice treated with anti-asialo GM1 antibody or PBS were stained with anti-TCR mAb and anti-NK1.1 mAb and analysed by flow cytometry. In each flow cytometry plot the upper left ellipse indicates the NK cells and the central ellipse indicates the NKT cells. The number indicates the percentage of cells (NK cells) present in the left ellipse. (b) Mean parasitaemia and SE per group; (c) survival curves of five mice per group. Results are representative of three similar experiments.

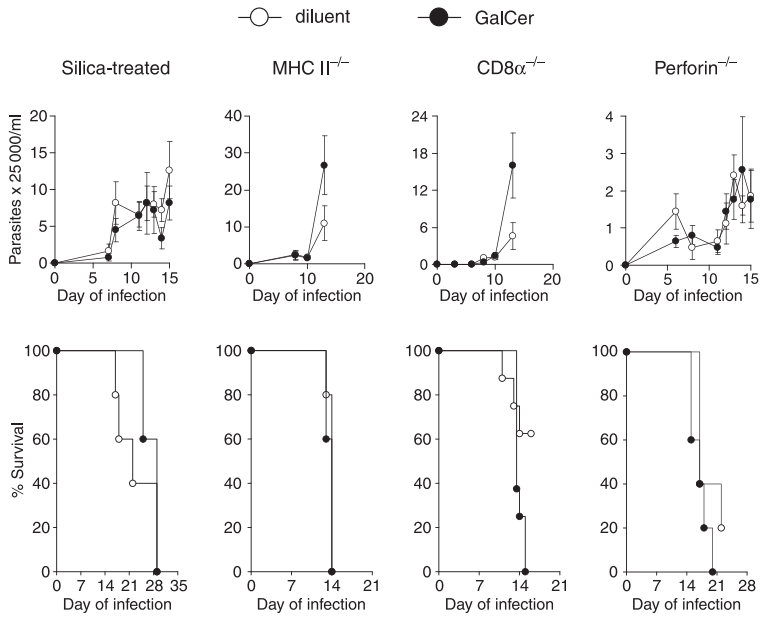

Figure 2. α-GalCer-mediated protection against *Trypanosoma cruzi* is not observed in MHC $II^{-/-}$, $CD8\alpha^{-/-}$, $perforin^{-/-}$ or silica-treated mice. Mice were treated with 5 μg α-GalCer or diluent and the next day were inoculated with 2×10^5 trypomastigotes. Five mice per group were monitored for parasitaemia (mean and SE) and five or more mice per group were monitored for survival. The results are representative of two or more experiments for each mouse strain.

because it did not reduce parasitaemia ($P = 0{\cdot}343$) or improve survival ($P = 0{\cdot}284$) (Fig. 2). These data indicate that α-GalCer-induced protection against *T. cruzi* requires phagocytic cell functions.

We next analysed the role of adaptive T cells in the α-GalCer-induced protection. First, MHC $II^{-/-}$ mice were treated with α-GalCer or diluent and 1 day later were infected with *T. cruzi*. α-GalCer treatment failed to reduce parasitaemia ($P = 0{\cdot}417$) or to prolong survival ($P = 0{\cdot}513$) (Fig. 2). In fact, the α-GalCer-treated mice appeared to develop a higher parasitaemia than the diluent-treated mice shortly before dying (Fig. 2).

Next, we investigated the role of cytotoxic T cells. β_2-$microglobulin^{-/-}$ mice were not investigated because these mice do not express CD1d, and therefore do not develop NKT cells or respond to α-GalCer.[25] Instead, $perforin^{-/-}$ and $CD8\alpha^{-/-}$ mice were analysed. As with the MHC $II^{-/-}$ mice, α-GalCer treatment of $perforin^{-/-}$ mice, compared to diluent treatment, did not reduce parasitaemia ($P = 0{\cdot}993$) or prolong survival ($P = 0{\cdot}303$) arguing that perforin-expressing, cytotoxic cells are required for the α-GalCer-induced protection (Fig. 2). Since NK cells are not required for the α-GalCer-induced protection (Fig. 1), but perforin is (Fig. 2), these results argue that cytotoxic cells other than NK cells are required. To further examine this possibility we treated $CD8\alpha^{-/-}$ mice with α-GalCer or diluent. Interestingly the α-GalCer-treated $CD8\alpha^{-/-}$ mice exhibited greater parasitaemia ($P = 0{\cdot}061$) and earlier death ($P = 0{\cdot}046$) (Fig. 2). These results argue that CD8α is required for α-GalCer-induced protection against *T. cruzi*, further suggesting that CD8 T cells are required. In addition, the data argue that α-GalCer treatment stimulates aspects of the immune response that favour the parasite.

α-GalCer treatment induces weight loss

The results argue that α-GalCer-mediated protection against *T. cruzi* requires phagocytic cells, T helper cells and cytotoxic T lymphocytes. Although protection was not observed in these experiments, other effects of the treatment were. First, surprisingly, the MHC $II^{-/-}$ mice and $CD8\alpha^{-/-}$ mice treated with α-GalCer displayed increased parasitaemia and decreased survival (Fig. 2). In addition, α-GalCer treatment is known to cause a hepatitis that is detected by measuring increased serum GPT, and significantly increased serum GPT levels were detected 24 hr after α-GalCer treatment of the MHC $II^{-/-}$ mice and $perforin^{-/-}$ mice (data not shown).[26] Furthermore, 1 day after treatment all the mice treated with α-GalCer (except for silica-treated mice) experienced significant weight loss (Fig. 3). Thus α-GalCer treatment (and silica treatment) appeared to cause weight loss (Fig. 3). This is the first report that α-GalCer treatment of mice causes weight loss. These results indicate that all the mice treated with α-GalCer, except for the silica-treated mice, were affected by the α-GalCer treatment even if protective α-GalCer effects were not observed.

Discussion

α-GalCer treatment rapidly activates iNKT cells to release large amounts of cytokines that stimulate aspects of the innate and adaptive immune responses.[27] We have previously demonstrated that α-GalCer treatment augments protection against *T. cruzi* through an IFN-γ-dependent mechanism.[13] To investigate what aspects of the innate and adaptive immune responses contribute to that protection, we examined cells known to become activated

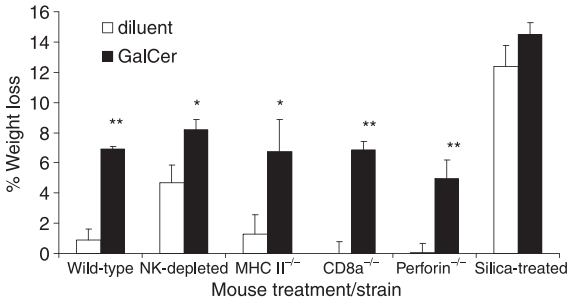

Figure 3. α-GalCer-treated mice exhibit weight loss. Wild-type, NK-cell-depleted, MHC $II^{-/-}$, $CD8\alpha^{-/-}$, $perforin^{-/-}$ and silica-treated mice were treated with 5 μg α-GalCer or diluent. Five mice per group were monitored for weight just before α-GalCer treatment and 24 hr later (just before *T. cruzi* infection) and weight change is presented as the mean and SE of the per cent weight change., The results are representative of two or more experiments for each mouse strain; $^{*}P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$, versus same strain, diluent-treated.

following α-GalCer treatment.[4,28] This study demonstrates that α-GalCer treatment can protect against *T. cruzi* despite depletion of NK cells, but not if functional phagocytes, helper T cells or cytotoxic T cells are deficient. These results suggest that phagocytes, helper T cells and cytotoxic T cells, but surprisingly not NK cells, are required for α-GalCer-induced protection against *T. cruzi*.

The α-GalCer-activated iNKT cells secrete IFN-γ, which stimulates NK cells to produce abundant IFN-γ.[6,9,17,18,29] Furthermore, NK cells and NK-cell-derived IFN-γ are critical to α-GalCer-mediated protection against many tumours.[17,30] Moreover, because IFN-γ is essential for the α-GalCer-induced protection against *T. cruzi*, and because NK cells are normally required for survival after acute *T. cruzi* infection, we speculated that NK-cell-produced IFN-γ would be required for the α-GalCer-induced protection against *T. cruzi*.[13,19,21] Surprisingly, while *T. cruzi*-infected mice depleted of NK cells and treated with diluent developed high parasitaemia and died rapidly, those treated with α-GalCer controlled parasitaemia well and survived. These results are in agreement with previous studies investigating α-GalCer protection against *Plasmodium yoelii* and indicate that α-GalCer treatment is protective against *T. cruzi* infection in mice depleted of normal numbers of NK cells.[31] Moreover, the α-GalCer protection of NK-cell-depleted mice was comparable to the α-GalCer protection of normal wild-type mice, demonstrating not only that NK cells are not required, but also that α-GalCer treatment is extremely potent because it protected NK-cell-depleted mice that died rapidly during the acute infection. This protection might be facilitated by secretion of large amounts of IFN-γ by α-GalCer-activated iNKT cells, which more than compensates for the deficiency in NK cell-produced IFN-γ.

Macrophages help to control *T. cruzi* through their own anti-trypanocidal actions and indirectly by activating other cells.[22] In addition, α-GalCer stimulation of iNKT cells results in macrophage activation.[8,9] These data argue that α-GalCer treatment will stimulate macrophages and other phagocytic cells to provide protection against *T. cruzi*. To test this possibility we inhibited phagocytic cells *in vivo* by injection of silica before α-GalCer treatment.[22–24] Since silica treatment induced weight loss and increased serum GPT levels, these parameters could not be used as evidence that the α-GalCer treatment had activated iNKT cells. Recent studies, however, indicate that in the absence of functional macrophages α-GalCer is still effectively presented by CD1d of dendritic cells to activate NKT cells.[32] Together these data argue that following silica treatment α-GalCer is presented to NKT cells by dendritic cells, and that the failure of α-GalCer to provide protection is caused by the loss of phagocytic cell functions other than α-GalCer CD1d presentation.

Our previous study indicated that α-GalCer protects against *T. cruzi* through IFN-γ, and strongly suggested that $CD4^{+}$ or $CD8^{+}$ T cells were involved.[13] In this report we analysed the role of T cells in α-GalCer treatment of *T. cruzi* infection using gene-deficient mice. MHC $II^{-/-}$ mice were not protected by α-GalCer treatment, which suggested that during *T. cruzi* infection $CD4^{+}$ T helper cells are necessary for the α-GalCer-induced protection. Having demonstrated the involvement of $CD4^{+}$ T cells and phagocytes, we next wanted to consider MHC class I-restricted T cells. β_2-microglobulin$^{-/-}$ mice could not be investigated, as these mice do not develop CD1d-restricted NKT cells. Rather, $perforin^{-/-}$ and $CD8\alpha^{-/-}$ mice were used. As expected, α-GalCer-induced treatment did not protect these mouse strains. It was surprising, however, that α-GalCer treatment of $CD8\alpha^{-/-}$ mice exacerbated parasitaemia and death similar to the effect we observed following α-GalCer treatment of IFN-$\gamma^{-/-}$ mice.[13] These results argue that in the absence of CD8α, α-GalCer-induced immune responses favour the parasite.

Our result that intraperitoneal α-GalCer treatment augments protection against *T. cruzi* infection agrees with another study.[14] That study also determined that α-GalCer treatment did not enhance protection by a *T. cruzi* DNA vaccine.[14] It remains possible that NKT-cell glycolipid antigens can be used as adjuvants for other types of vaccines against *T. cruzi*.

In human clinical trials α-GalCer treatment appears safe.[26,33] It is known that α-GalCer treatment of mice causes a transient hepatitis, and here we report that mice, following α-GalCer treatment, also suffer a transient weight loss. This weight loss appears to occur in the absence of MHC II-restricted helper T cells and MHC class I-restricted cytotoxic T cells. Although we do not know the mechanism of the weight loss, it might be caused by activated liver iNKT cells, which rapidly induce a robust inflammatory response.

The results presented here indicate that following α-GalCer injection, iNKT-cell activation triggers a complex cellular response that provides protection during *T. cruzi* infection. Surprisingly, despite a requirement of NK cells and IFN-γ to control acute *T. cruzi* infection, NK cells were not required for the α-GalCer-induced protection, arguing that α-GalCer activated other cells to overcome the absence of NK cells. These results further our understanding of NKT cells as a potential therapeutic target during infections and other diseases.

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