Activation of Natural Killer T Cells by α-Galactosylceramide Impairs DNA Vaccine-Induced Protective Immunity against Trypanosoma cruzi

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Innate immunity as a first defense is indispensable for host survival against infectious agents. We examined the roles of natural killer (NK) T cells in defense against Trypanosoma cruzi infection. The T. cruzi parasitemia and survival of CD1d-deficient mice exhibited no differences compared to wild-type littermates. NK T-cell activation induced by administering α-galactosylceramide (α-GalCer) to T. cruzi-infected mice significantly changed the parasitemia only in the late phase of infection and slightly improved survival when mice were infected intraperitoneally. The combined usage of α-GalCer and benznidazole, a commercially available drug for Chagas’ disease, did not enhance the therapeutic efficacy of benznidazole. These results suggest that NK T cells do not play a pivotal role in resistance to T. cruzi infection. In addition, we found that the coadministration of α-GalCer with DNA vaccine impaired the induction of epitope-specific CD8+ T cells and undermined the DNA vaccine-induced protective immunity against T. cruzi. Our results, in contrast to previous reports demonstrating the protective roles of NK T cells against other infectious agents, suggest that these cells might even exhibit adverse effects on vaccine-mediated protective immunity.

Trypanosoma cruzi is the etiological agent that causes Chagas’ disease in Central and South America (8, 22, 23, 36). As it invades and replicates inside essentially all types of cells in mammalian hosts, various immune effector cells must join to contain the devastating infection and to suppress the formation of systemic pathologies. Particularly in the acute phase, both innate immunity, including neutrophils, macrophages, and natural killer (NK) cells (3, 5, 9, 10), and acquired immunity, including CD8+ and CD4+ T cells (10, 31, 38, 39, 45, 46, 47), are orchestrated to mount the protective immune responses of the host.

NK T cells are an immune cell population that was first identified relatively recently (2, 41). They exhibit several characteristic features, including unique localization, i.e., they localize mainly in the liver, spleen, and thymus (2, 41). The discovery of a specific ligand for NK T-cell activation, α-galactosylceramide (α-GalCer), facilitated the analyses of the immunological function of NK T cells in vivo (21, 24, 25). They secrete vast amounts of cytokines in a CD1-restricted manner (15, 42) and effectively exert protective immune responses against tumors (21, 34) and infectious diseases (13, 18, 20). Both gamma interferon (IFN-γ) and interleukin-4 (IL-4) cytokines secreted by NK T cells could accelerate the induction and maintenance of effective CD8+ T-cell responses (1, 7). In addition to the strong protective immune responses exerted by the direct activation of NK T cells, their potent adjuvant effect in enhancing CD8+ T-cell-mediated immunity was demonstrated when immunogens were coadministered together with α-GalCer (14). This effect could revolutionize vaccine strategy, since CD8+ T cells are one of the most important effector cell populations for containing intracellular infectious agents (19, 30, 31, 33, 35).

However, the immunological roles of NK T cells in defense against infectious diseases were mostly elucidated in diseases that primarily cause pathologies in restricted organs, such as liver, where NK T cells are intensely localized. There are few reports concerning their function in infections resulting in systemic diseases affecting all organs. In addition, there are conflicting reports on the roles of NK T cells with regard to CD8+ T-cell induction with NK T cells reported either to enhance (14) or suppress (32, 48) CD8+ T-cell responses.

We therefore decided to try to answer these questions by elucidating the immunological function of NK T cells in systemic T. cruzi infection. It was recently demonstrated that NK T cells can limit parasitemia and augment antibody response (11, 12); however, immunological function affecting T-cell-mediated immunity has not yet been characterized. We report here that NK T cells might play only minimal roles in defense against T. cruzi infection. On the contrary, we found that they can impair the induction of CD8+ T-cell responses and abolish vaccine-induced protective immunity. Our results are in contrast to those of previous reports demonstrating the potent protective roles of NK T cells in infections (13, 18, 20) and tumors (21, 34), as well as the potent adjuvant effect of α-GalCer in enhancing T-cell-mediated immunity (14).
MATERIALS AND METHODS

Animals and parasite. Female C57BL/6 (H-2b) and BALB/c (H-2d) mice, 5 to 8 weeks of age, were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). CD1-deficient mice, either from a BALB/c background or from a B6 background (CD1d−/−), were obtained from Luc Van Kaer at the Department of Microbiology and Immunology, Howard Hughes Medical Institute, Vanderbilt University School of Medicine (Nashville, Tenn.). Blood-form trypomastigotes of T. cruzi Tulahuen strain (28) were maintained in outbred CD1 or inbred BALB/c mice by intramuscular (i.m.) inoculation of 5,000 trypomastigotes into naïve mice every 2 weeks.

Reagents. α-GalCer was supplied by Takashi Mise and Kazuhiro Motoki at the Kirin Brewery Co. and was produced as described previously (25). The stock solution (200 μg/ml) of α-GalCer was diluted with phosphate-buffered saline to make a working solution of 10 μg/ml just before inoculation. Two hundred microliters of the working solution (2 μg of α-GalCer) was administered intraperitoneally (i.p.) at the times indicated in the figure legends. We decided to use 2 μg of α-GalCer per dose for all of our studies based on either our own experiences in tumor immunology (15, 24, 34, 42), in which the same dose had been reproducibly effective in various assays, or on the report of Dutchie and Kahn (12), who demonstrated the doses of 0.5, 1, and 5 μg to be effective in assays with T. cruzi nonviral strain for infection. The intact biological activity of α-GalCer, after it was administered into mice, was occasionally monitored and confirmed by measuring both the IFN-γ and the IL-4 content in serum as described by Hayakawa et al. (15). Benzimidazole (BNZ) was purchased from the Hoffmann-La Roche, Inc. (Sao Paulo, Brazil). The doses for BNZ administration were 100 mg/kg/day for an optimal dose and 25 mg/kg/day for a suboptimal dose. BNZ was given per os (p.o.) for seven consecutive days beginning on the day of challenge infection as described previously (27).

Cells and culture. The C57BL/6-derived thyroma cell line EL-4 was used for antigen-presenting cells for the CD8+ T-cell cultures and assays. These cells were cultured in high-glucose Dulbecco modified Eagle medium (DMEM; Life Technologies/Gibco-BRL, Rockville, Md.) supplemented with 10% fetal bovine serum, 2.6 g of sodium bicarbonate (Sigma, St. Louis, Mo.)/liter, 200 mg of 1-glutamine (Life Technologies/Gibco-BRL)/liter, 36 mg of l-arginine hydrochloride (Life Technologies/Gibco-BRL)/liter, 2.6 g of HEPES (Sigma)/liter, 5 × 10−3 M 2-mercaptoethanol (Sigma), and antibiotics (complete DMEM). The medium used for the enzyme-linked immunosorbent (ELISPOT) assay, and the culture of lymphocytes was supplemented with phorbol myristate acetate-stimulated EL-4 cell culture supernatant as a source of 30 U of IL-2 (complete DMEM–IL-2)/ml.

Plasmid DNA and peptide. pCMV-Tag epitope tagging mammalian expression vector (pCMV; Stratagene) was used to construct a T. cruzi trans-sialidase surface antigen (TSSA) gene-expressing plasmid DNA and was designated ptSSA (19). A CD8+ T-cell-inducing, H-2Kb-restricted peptide, ANYNFTLV, derived from TSSA (19) was used for immunological assay.

Routes of immunization, schedules, dosages, and challenge infection. We used several immunization and infection protocols as described in the figure legends. For immunization of immune T cells by DNA immunization, mice were injected i.m. with 100 μg of ptSSA or control pCMV vector suspended in 50 μl of sterile phosphate-buffered saline into the right-hind-leg quadriceps once or twice at approximately 10-day intervals, depending on the immunization schedule. α-GalCer was administered i.p., and BNZ was given orally. The mice were challenged with an appropriate number of Tulahuen strain T. cruzi blood-form trypanosomes between 11 and 14 days after the last immunization. The challenge infection was administered intravenously (i.v.), i.m., or i.p. Blood from all infected animals was obtained from the tail vein periodically, and the numbers of parasites in 5 μl of blood (parasitemia) were determined microscopically. Survival was monitored daily.

Quantification of antigen-specific T cells by ELISPOT assay. The frequency of antigen-specific T cells was determined by ELISPOT assay for IFN-γ-secreting cells essentially as described previously (6, 29, 30, 33). Briefly, serial dilutions of spleenocytes or T cells (1 × 104 to 100 × 104) were cocultured with irradiated EL-4 cells that had been pulsed with 1 μM peptide in anti-IFN-γ monoclonal antibody-coated plates for 24 to 28 h. The spots formed by IFN-γ-secreting cells were detected by using biotinylated anti-IFN-γ monoclonal antibody, followed by the addition of peroxidase-labeled streptavidin and diaminobenzidine. The developed spots were counted under a microscope and were expressed as the number of spots per 106 cells.

Statistical analysis. Statistical analyses were performed by using the unpaired Student t test for parasitemia counts and ELISPOT assays and Dunnett one-tailed t test for the counts of parasitemia in Fig. 4. The unpaired Mann-Whitney U test or the Fisher exact test were used to determine significant differences in the survival data. P values of <0.05 were considered significant.

RESULTS AND DISCUSSION

NK T cells are ineffective for controlling T. cruzi infection in the quiescent state. We first infected CD1d-deficient mice, which lack CD1d-restricted Vα14 NK T cells, that had either a B6 or a BALB/c background, with 10 or 20 T. cruzi blood-form trypanosomes in i.m. from either a B6 background (Fig. 1A and B) or 10 (BALB/c mice [C and D]) Tulahuen strain of T. cruzi blood-form trypanosomes. The number of parasites in 5 μl of peripheral blood (parasitemia) was counted periodically (A and C); survival was monitored daily (B and D).

FIG. 1. A deficiency of the CD1 molecule does not alter the course of T. cruzi infection in mice. Four CD1d-deficient mice (●) and four wild-type littersmates (□) were infected i.m. with 20 (C57BL/6 mice [A and B]) or 10 (BALB/c mice [C and D]) Tulahuen strain of T. cruzi blood-form trypanosomes. The number of parasites in 5 μl of peripheral blood (parasitemia) was counted periodically (A and C); survival was monitored daily (B and D).
1 x 10^5 per mouse was used. Alternatively, as for the route of infection, we infected mice i.m., whereas Duthie et al. (11) chose i.p. infection. Since we found that i.m. infection causes a more virulent course of disease in T. cruzi infection than i.p. or i.v. infection, the difference in the route of infection might have affected the disease outcome. Another possible interpretation for the discrepancy between the data of Duthie et al. and the present study might be that the CD1d deficiency is not enough to delete the NK cell activity completely, thus resulting in our inability to detect the alterations of T. cruzi infection in the CD1d^-/- mice when we used the virulent strain of T. cruzi for infection. Duthie et al. (11) used Jc281^-/- mice, which are deficient in NK T-cell activity more thoroughly; this difference might possibly have allowed us to detect the alterations of T. cruzi Tulahuen strain infections.

Duthie et al. analyzed the dynamics of NK T-cell populations both in the liver and in the spleen during the infection of T. cruzi CL strain (11). These authors found that the percentage of NK T-cell populations in the organs varied depending on the number of days postinfection. Duthie et al. observed that the intensity of NK1.1 staining of the NK T cells observed between 21 and 45 days postinfection was decreased, leading them to speculate that the NK T cells, during the infection, might gain an altered phenotype which might affect their function. Although we did not perform similar analyses for our studies during the infection of T. cruzi Tulahuen strain, there might be different dynamics for the NK T-cell populations that could be distinct from the ones noted during the T. cruzi CL infection. The similar analyses might possibly explain why we did not detect the significant differences in parasitemia and percent survival when CD1d^-/- mice were infected with a more virulent strain of T. cruzi.

Since NK T cells localize primarily in restricted organs such as the liver, spleen, and thymus (2, 41), it is possible that NK T cells might not be able to exert their immunological function at distant organs where the intracellular stage form of T. cruzi replicates and proliferates. We assumed that liver infection might be exacerbated in CD1d-deficient mice due to their lack of NK T cells. To examine this possibility, we scanned 200 fields of each stained live pathological section, which was derived from either three CD1d^-/- mice or three CD1d^-/- mice, and then counted the number of T. cruzi-infected hepatocytes. However, we observed no statistically significant difference in the number of infected hepatocytes as determined by the unpaired Student t test (data not shown), suggesting that the role of NK T cells in the quiescent state might not be strong enough to alter disease progression both systemically and locally in natural T. cruzi infection.

α-GalCer-activated NK T cells slightly improve the survival of mice with lethal T. cruzi infection. We next tested whether the activation of NK T cells by α-GalCer (21) administration confers protection against T. cruzi infection. Considering the immunological effect of cytokines, including IFN-γ and IL-4, which NK T cells secrete vastly upon α-GalCer activation (15, 42), we expected that the compound would have a therapeutic efficacy against T. cruzi infection. In accordance with this expectation, there have been several studies demonstrating the potent immunological effect of α-GalCer conferring resistance against tumors (21, 34) or infectious agents (13, 18, 20). We injected 2 μg of α-GalCer into mice four times at 4-day intervals before and after lethal T. cruzi inoculation. After mice were infected i.m. with 5,000 T. cruzi blood-form trypomastigotes 2 days after the first α-GalCer inoculation, the number of parasites in 5 μl of peripheral blood (parasitemia) was counted periodically (A and B), i.p. (C and D), or i.v. (E and F) with 5,000 Tulahuen strain T. cruzi blood-form trypomastigotes 2 days after the first α-GalCer inoculation. The number of parasites in 5 μl of peripheral blood (parasitemia) was counted periodically (A, C, and E); survival was monitored daily (B, D, and F). * P < 0.05 (in comparisons between groups of α-GalCer-treated mice and vehicle-treated mice). The percent survival of α-GalCer-administered mice was significantly different from that of vehicle-administered control mice when infection was achieved i.p. (P < 0.05) (D).
leading to the formation of a localized tumor-like pathology. If the primarily infected foci where intracellular *T. cruzi* are proliferating are far from the place where the NK T cells are present, the secretion of large amounts of cytokines, which are effective against intracellular amastigotes but not against extracellular trypomastigotes (17, 37), might be useless in reducing the proliferating parasite burden. We therefore decided to change the route of infection by infecting the mice i.p. or i.v., through which *T. cruzi* might swiftly spread to the whole body without forming locally restricted intracellular *T. cruzi* replication sites. In the case of i.p. infection, mice given α-GalCer mice survived significantly longer than the vehicle-administered mice (*P* < 0.05), although parasitemia in the groups differed significantly only in the late stage of infection (Fig. 2C and D). Although NK T cells are reported to be capable of killing cells directly (26), secreted cytokines, such as IFN-γ, might be the main effector mechanism in the acute phase for controlling infections (13, 17, 18, 37) and might not be enough to suppress effectively the rising parasitemia. The importance of IFN-γ, secreted by activated NK T cells, in containing infectious agents has also been suggested for *T. cruzi* infection as reported elsewhere (12). Infection of mice i.v. with 5,000 parasites was no longer lethal (Fig. 2F); however, the activation of NK T cells had little impact on the improvement of parasitemia (Fig. 2E). Considering reports that have demonstrated the strong immunological capacity of NK T cells against other infections, the present results are rather surprising. One obvious interpretation for the results would be that NK T cells, even if they are activated by α-GalCer, are not so effective against systemic *T. cruzi* parasitemia. An alternative interpretation of the results would be that the routes of infection and α-GalCer administration may have affected the disease outcome. Since α-GalCer was administered i.p. in all experiments, the proximity of parasite inoculation and α-GalCer administration in *T. cruzi* i.p. infection might effectively mount NK T-cell responses in order to suppress local parasite proliferation (Fig. 2C and D). Although we do not have any data to reject the second interpretation, we think that it is not likely because of the restricted localization of NK T-cell populations, mostly in liver, spleen, or thymus. Third, we considered the possibility that repeated injection of α-GalCer biased the host immune responses toward the Th2 type as reported elsewhere (4), consequently suppressing IFN-γ production. We therefore tested the efficacy of a one-time inoculation of α-GalCer on the day of i.m. infection; however, we observed similar results as in mice receiving repeated administration of α-GalCer, demonstrating no improvement in either parasitemia or percent survival (data not shown). Duttie et al. observed that the multiple α-GalCer administration prior to *T. cruzi* CL strain infection exacerbated the infection, whereas a single α-GalCer administration at day −1 led to the optimal protection (12). We were not aware of the similar phenomena during the virulent *T. cruzi* Tulahuen strain infection in mice, partly because the infection dose of 5,000 blood-form trypomastigotes, which was employed for our studies, has already reached to the dose for inducing the deadly infection course for host and was lethal to all naive B6 mice. In addition, we never performed a single α-GalCer administration at day −1 for our studies; therefore, it is possible that this type of treatment could result in optimal protection even when the virulent strain of *T. cruzi* was used in these studies. The absence of a drastic effect of NK T-cell activation on *T. cruzi* infection is in contrast to other infectious agents such as hepatitis B virus (18) and malaria (13) infection, against which NK T-cell activation contains the liver-restricted pathogens and suppresses the formation of pathologies.

α-GalCer-activated NK T cells do not augment the therapeutic efficacy of BNZ against Chagas’ disease. As α-GalCer administered alone was not effective in suppressing the disease progression, we decided to combine it with a conventional therapy. BNZ is one of the few commercially available therapeutic drugs against *T. cruzi*; however, its clinical efficacy has frequently been questioned, especially for the chronic stage of infection. Although the biochemical mechanisms of its drug action have not been fully elucidated, the involvement of cytokines in its efficacy has not yet been demonstrated (27). Despite this situation, the combined usage of BNZ with IL-12 administration demonstrates a cumulative therapeutic effect against *T. cruzi* (27), prompting us to test the combined usage of α-GalCer together with BNZ in curing the Chagas’ disease. As shown in Fig. 3, however, we saw no significant suppression of parasitemia in mice treated with a suboptimal dose of BNZ (25 mg/kg/day) p.o. (△) for seven consecutive days beginning on the day of *T. cruzi* infection. As controls, mice were treated with BNZ (25 mg/kg/day) p.o. and vehicle solution (●), whereas others were treated with an optimal dose of BNZ (100 mg/kg/day) p.o. for seven consecutive days (□). The number of parasites in 5 μl of peripheral blood (parasitemia) was counted periodically (A); survival was monitored daily (B). BZ, BNZ.
The DNA constructs of pCMV and pTSSA are described in Materials and Methods and in reference 19. A total of 100 μg of DNA was injected on one i.m. into the right hind-leg quadriceps.

α-GalCer (2 μg per mouse) or 200 μl of vehicle solution was administered i.p. once on the day of DNA immunization.

α-GalCer (2 μg per mouse) or 200 μl of vehicle solution was administered i.p. four times at 4-day intervals starting 2 days before T. cruzi infection.

As CD8+ T cells are important for controlling the acute phase of T. cruzi infection, we evaluated the induction of epitope-specific CD8+ T cells in mice coadministered DNA and α-GalCer. Mice, with four mice in each group, were immunized twice with DNA with or without α-GalCer and were subjected to ELISPOT assay 12 days after the last immunization (Fig. 5). Appropriate control groups of mice were included in the experiment as shown in Fig. 5. The induction of ANNYFTLV-specific CD8+ T cells (19) in mice immunized with the pTSSA–α-GalCer solution was significantly lower than in mice coimmunized with pTSSA-vehicle (P < 0.05) (Fig. 5). Since α-GalCer would influence several cell populations, as well as T cells, via NK T-cell activation, as evidenced by the enormous spleen enlargement, we assumed that the relative number of T cells in the whole immune cell population was reduced, therefore undermining the CD8+ T-cell responses. Another possible explanation for the reduced CD8+ T-cell responses in DNA–α-GalCer-administered mice is that the effect of α-GalCer on T cells could be slow progressing; therefore, the CD8+ T-cell responses might not be fully enhanced only 12 days after the last α-GalCer administration. To exclude these possibilities, we performed the ELISPOT assay when the immune cells had reverted to the quiescent state more days after the final α-GalCer administration. However, the number of epitope-specific CD8+ T-cell responses in pTSSA–α-GalCer solution-immunized mice was still significantly lower than in the pTSSA-vehicle-immunized mice (P < 0.01) at 21 days postimmunization (data not shown), thus confirming the impaired induction of ANNYFTLV-specific CD8+ T cells in pTSSA–α-GalCer-immunized mice. We assume that the inability to control the parasite burden at the end of acute T. cruzi infection in pTSSA–α-GalCer-coadministered mice was partly, if not totally, due to the impaired induction of protective CD8+ T-cell responses. Duthie et al. reported that the antibody responses in NK T cell-deficient mice were quite different from the ones in wild-type mice. Although we could not reject a possibility that antibody responses to the TSSA could be negatively affected by α-GalCer administration resulting in the impairment of DNA-vaccine-induced protective immunity, our previous findings, which demonstrated the importance of T-cell-mediated protective immunity in pTSSA vaccination (19), suggested that the failure of protection in the pTSSA–α-GalCer-immunized mice were mainly due to the impairment of T-cell-mediated immunity rather than the alterations of antibody responses.

The results are rather surprising in one aspect, as they are obviously contradictory to previous reports demonstrating the potent adjuvant effect of α-GalCer coadministration in enhancing CD8+ T-cell-mediated protective immunity against malaria (14) and tumors (34). This contradiction might be partly due to the immunogens used for coadministering with α-GalCer. Gonzalez-Aseguinolaza et al. used recombinant viruses (14) for immunization, whereas we used DNA for vaccination. The immunological effects induced by vaccination in various situations vary depending on the immunogens used. For example, although immunization of CD28-deficient mice with viruses can induce epitope-specific CD8+ T-cell responses as robust as those in wild-type mice (44), immunization of mice with DNA vaccine failed to induce this response (16). We therefore assume that it is perhaps not surprising to

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α-GalCer (2 μg per mouse) or 200 μl of vehicle solution was administered i.p. once on the day of DNA immunization.

α-GalCer (2 μg per mouse) or 200 μl of vehicle solution was administered i.p. four times at 4-day intervals starting 2 days before T. cruzi infection.
VI. The standard deviations of parasitemia of mice in groups III, IV, V, and VI were counted, and the data are indicated as the means ± SD of four mice in each group. The number of parasitemia of mice in each group. The P values comparing parasitemia between mice in group I and mice in group III were significant and are indicated in the figure. (B) Parasitemia at 20 days postinfection was measured, and the data are indicated as the means ± SD of parasitemia of mice in each group. The P values comparing parasitemia between mice in group I and mice in group III were significant and are indicated in the figure. (C) The survival curves reveal that α-GalCer administration either at the time of DNA vaccination or at the time of T. cruzi infection eventually abolished the pTSSA vaccine-induced protective immunity against T. cruzi infection. The symbols represent the survival of mice immunized with pCMV-vehicle (△) (group I), pCMV-α-GalCer (●) (group II), pTSSA-vehicle (○) (group III), pTSSA-α-GalCer (○) (group IV), pTSSA-α-GalCer (○) (group V), and pTSSA-α-GalCer (○) (group VI). It was determined by using the Fisher exact test that the survival of mice in group III was significantly better (P < 0.05) than that of mice in group I. However, the survival of mice in groups II, IV, V, or VI was not significantly different compared to that of mice in group I. The data are representative one of two independent experiments.
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