Activation of Natural Killer (NK) T Cells during Murine Cytomegalovirus Infection Enhances the Antiviral Response Mediated by NK Cells

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NK1.1⁺ T (NKT) cells are efficient regulators of early host responses which have been shown to play a role in tumor surveillance. The relevance of NKT cells in immune surveillance of viral infections, however, is not well understood. In this study, we investigated the functional relevance of NKT cells in controlling herpesvirus infections by using challenge with murine cytomegalovirus (MCMV) as the study model. This model has proven to be one of the best systems for evaluating the role of NK cells during virus infection. Using gene-targeted mice and α -galactosylceramide (α -GalCer) as an exogenous stimulator of NKT cells, we have analyzed the role of these cells in the immune surveillance of MCMV infection. Our studies in NKT-cell-deficient, T-cell receptor J α 281 gene-targeted mice have established that classical NKT cells do not play a critical role in the early clearance of MCMV infection. Importantly, however, activation of NKT cells by α -GalCer resulted in reduced viral replication in visceral organs. Depletion studies, coupled with analysis of gene-targeted mice lacking perforin and gamma interferon (IFN- γ), have revealed that the antiviral effects of α -GalCer involve NK cells and have clearly demonstrated that the antiviral activity of α -GalCer, unlike the antitumor one, is critically dependent on both perforin and IFN- γ .

Innate immune responses are critical in the process of immune surveillance, with natural killer (NK) and NK1.1⁺ T (NKT) cells being among the main mediators of early host responses. The role of NK cells in these processes has been the subject of intensive investigation, and it is clear that they play a crucial role as effectors in tumor control (38, 41, 46) as well as in limiting the spread of viral infections (4, 5, 42). In particular, infection of mice by the herpesvirus murine cytomegalovirus (MCMV) has proven to be one of the best systems for evaluating the role of NK cells following a viral challenge (29, 43). In contrast, NKT cells have been shown to play a central role in early innate responses to tumors (35, 37), but their relevance in response to challenges with viral pathogens is still largely unclear (5). Here, we have examined the relevance of classical, T-cell receptor (TCR)-restricted NKT cells in controlling MCMV infection. Because of the similarity in structure and biology between MCMV and human cytomegalovirus, the former provides a unique model of human disease and, importantly, it permits the study of *in vivo* infection in the natural host (1).

Classical murine NKT cells are a specialized subset of T lymphocytes that express a number of NK cell markers, including NK1.1 (3, 14), together with a restricted TCR repertoire, consisting of a single invariant TCR α chain encoded by V α 14J α 281 in association with a V β 8.2, V β 7, or V β 2 TCR β chain. The resultant semi-invariant TCR $\alpha\beta$ is specific for

CD1d, a nonclassical class I molecule (3) and recognizes glycolipid antigens, the nature of which is currently unclear. A marine sponge-derived glycosphingolipid, α -galactosylceramide (α -GalCer), has been identified as a ligand that specifically binds the semi-invariant TCR of NKT cells in association with CD1d (8, 21). Following the association of the α -GalCer/CD1d complex with the V α 14 TCR, NKT cells become activated (8, 21), rapidly release immunoregulatory cytokines, such as gamma interferon (IFN- γ) or interleukin 4 (IL-4), and increase their cytotoxic capacity (3, 21, 22).

Rapid release of cytokines by NKT cells suggests their role in the regulation of immune responses and highlights their potential as targets for immunotherapy. Indeed, α -GalCer-stimulated NKT cells have been shown to drive immune responses with both Th1 and Th2 biases. For example, repeated α -GalCer administration can enhance Th2-mediated immune responses (9, 24, 33), which have been shown to protect against experimentally induced Th1-type colitis (31) and type I diabetes (17, 32). Conversely, α -GalCer has also been shown to protect against malaria (15) and to suppress the ability of the B16 melanoma to form liver and lung metastases by a Th1- and IFN- γ -dependent mechanism (22, 27, 36, 45). Importantly, it is now appreciated that the antitumor effects of α -GalCer are mediated in a collaborative manner by both NK and NKT cells (26, 34, 36).

The induction of antitumor immune responses following α -GalCer therapy has been proposed to occur in a defined sequence of events (35). Briefly, production of IFN- γ by α -GalCer-stimulated NKT cells induces IL-12 production by CD11c⁺ dendritic cells (DC) in a CD40/CD40L-dependent manner,

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thus establishing a positive feedback loop for IFN- γ production (23, 44). IFN- γ and IL-12, resulting from activation of NKT cells, then selectively induce NK cell proliferation, IFN- γ secretion, and cytotoxicity (12, 35). NKT cells activated by α -GalCer can subsequently promote bystander activation of NK cells, B cells, CD4 $^{+}$ T cells, CD8 $^{+}$ cytotoxic T lymphocytes, and DC (9, 10, 12, 23, 24, 28).

The relevance of α -GalCer-activated classical NKT cells in the control of herpesvirus infections is unknown. Here, we have utilized the MCMV model of viral infection to determine the relevance of NKT cells in the early immune responses that limit the spread of this herpesvirus infection in visceral organs. The cytotoxic function of NK cells is known to control MCMV replication in visceral organs during acute-phase infection (7, 43) and limit viral spread and replication prior to the development of adaptive CD8 $^{+}$ -cytotoxic-T-lymphocyte responses which resolve productive virus infection (25, 30). In this report, we show that although NKT cells do not play a role in the natural control of MCMV infection, their activation, following α -GalCer therapy, results in improved viral clearance through the activation of bystander NK cells.

MATERIALS AND METHODS

Mice. Wild-type (WT) inbred C57BL/6J (B6) and BALB/c mice were purchased from the Animal Resources Centre (Perth, Australia) or from the Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia). B6 TCRJ α 281-deficient (B6.J α 281 $^{-/-}$) mice (originally provided by M. Taniguchi, Chiba University Graduate School of Medicine, Chiba, Japan), BALB/c perforin-deficient (BALB/c.pfp $^{-/-}$) mice, BALB/c IFN- γ -deficient (BALB/c.IFN- γ $^{-/-}$) mice (Genentech, San Francisco, Calif.), and BALB/c mice deficient for both perforin and IFN- γ (BALB/c.pfp $^{-/-}$.IFN- γ $^{-/-}$) were bred at the Peter MacCallum Cancer Institute (Melbourne, Australia). Adult female mice (8 to 12 weeks old) were used in all experiments. All animal experimentation was performed with the approval of the Animal Ethics and Experimentation Committee of the University of Western Australia and according to the guidelines of the National Health and Medical Research Council of Australia.

Cells. Primary mouse embryo fibroblasts were cultured in minimal essential medium (Gibco BRL, Grand Island, N.Y.) supplemented with 10% neonatal calf serum (Gibco BRL) and antibiotics (100 μ g of penicillin [CSL]/ml and 40 μ g of gentamicin [Pharmacia and Upjohn, Sydney, Australia]/ml).

Treatment of mice. For the pathogenesis studies, mice were infected intraperitoneally (i.p.) with 10^4 PFU of salivary gland-propagated stocks of the virulent MCMV strain K181-Perth. Control animals were mock infected with mouse osmolarity-buffered saline (MOBS)-5% fetal calf serum (FCS). Mice were sacrificed at various times postinfection, and tissues were collected (see below). For α -GalCer therapy, mice received 2 μ g of α -GalCer diluted in MOBS-5% FCS by i.p. injection on days 0 and 4 relative to virus inoculation. On day 0, mice were inoculated with α -GalCer 1 h after virus inoculation. Control mice were injected with an identical volume of vehicle solution (0.025% polysorbate) diluted in MOBS-5% FCS. α -GalCer was kindly provided by Kirin Brewery Pharmaceutical Research Laboratory, Gunma, Japan. To deplete NK cells, mice were inoculated i.p. with 100 μ l of MOBS-0.5% FCS containing 200 μ g of anti-asialoGM1 antibody (Wako Pure Chemical Industries Ltd., Osaka, Japan), as described previously (34), on days -1, 0, and 6 relative to virus inoculations.

In vivo growth of MCMV. Mice were inoculated with the virulent MCMV strain K181-Perth, as described above. At designated times postinoculation (p.i.), mice were sacrificed and spleens, livers, lungs, and salivary glands were removed. All organs were individually weighed, homogenized in cold minimal essential medium-2% neonatal calf serum, and centrifuged at $3,000 \times g$ for 15 min at 4°C. The supernatants were stored at -80°C, and virus titers were subsequently quantified on mouse embryo fibroblasts by standard plaque assay (2). Virus titers are expressed as log means $\pm$ standard errors of the mean (SEM). Statistical analyses were performed by using the nonparametric Mann-Whitney test.

FIG. 1. α -GalCer therapy reduces MCMV replication in BALB/c mice. MCMV-susceptible BALB/c mice were inoculated i.p. with 10^4 PFU of MCMV and treated with 2 μ g of α -GalCer on days 0 and 4. Virus titers in the spleen, liver, lung, and salivary gland are shown. The solid line represents α -GalCer-treated mice, and the dashed line represents vehicle-treated mice. The dashed line across the graph indicates the limit of detection of the assay. Results are presented as log means $\pm$ SEM from 15 mice per group and were derived by combining data from 5 independent experiments, each utilizing 3 mice per group. **, $P < 0.001$; *, $P \leq 0.05$.

RESULTS

α -GalCer therapy improves clearance during acute-phase MCMV infection. To determine the relevance of activated NKT cells in anti-MCMV responses, we studied the effect of α -GalCer therapy on the ability of MCMV to replicate in visceral organs and salivary glands. MCMV replicates to high titers in the visceral organs of susceptible mice during the acute stage of infection (days 2 to 6). Treatment with α -GalCer significantly ($P < 0.001$) reduced virus titers in the spleens of susceptible BALB/c WT mice, at days 2, 4, and 6 postinfection (Fig. 1). At these times, MCMV titers were reduced by >1.0 log (day 2), >2.0 log (day 4), and >0.5 log (day 6) when treated and untreated mice were compared. In the liver, the therapeutic effect of α -GalCer was also significant at days 2 and 4 p.i. ($P < 0.001$) and day 6 p.i. ($P \leq 0.05$) but less pronounced with an ~ 1.0 log reduction in virus titers observed at day 2 p.i. and a ≤ 0.5 log reduction at days 4 and 6 p.i. in α -GalCer-treated mice compared to controls (Fig. 1). In the lungs and salivary glands, sites of viral persistence for MCMV, viral replication is not detectable until at least days 4 and 6 p.i., respectively. At these early times, when virus infection is first established in these organs, α -GalCer significantly reduced viral replication (Fig. 1). However, no therapeutic effects of α -GalCer were

FIG. 2. MCMV replication in B6 mice treated with α -GalCer. MCMV-resistant B6 mice were inoculated i.p. with 10^5 PFU of MCMV and treated with $2 \mu\text{g}$ of α -GalCer on days 0 and 4. Virus titers in the spleen and liver are shown. Solid lines represent α -GalCer-treated mice, and dashed lines represent vehicle-treated mice. The dashed line across the graph indicates the limit of detection of the assay. Results are presented as log means $\pm$ SEM from 6 mice per group and were derived by combining data from duplicate independent experiments, each utilizing 3 mice per group. **, $P < 0.005$; *, $P < 0.01$.

observed during the persistent phase of virus infection in either the lungs or the salivary glands (Fig. 1).

A therapeutic effect of α -GalCer was also observed in B6 WT mice infected with 10^5 PFU of MCMV. At this virus dose, MCMV replicates effectively in MCMV-resistant B6 WT mice. As observed in BALB/c WT mice, α -GalCer treatment resulted in reduced viral replication in visceral organs (Fig. 2). In the spleens of α -GalCer-treated B6 mice, a significant >1.0 log reduction ($P < 0.005$) in virus titer was observed at day 2 p.i. (Fig. 2). In the liver, virus titers were significantly ($P < 0.005$) reduced at days 2 and 4 (>1.0 log reduction) (Fig. 2). A transient increase in virus titer was reproducibly observed at day 6 p.i. in the livers of α -GalCer-treated B6 mice. However, by day 10 p.i., virus titers in visceral organs were equivalent in α -GalCer-treated B6 and untreated control B6 mice. Titers from salivary glands were comparable at day 10 p.i. in α -GalCer-treated mice and vehicle-treated control mice (data not shown).

The above results indicate that α -GalCer-activated NKT cells play a significant role in limiting MCMV replication during early acute-phase infection of visceral organs. The phenotype observed in MCMV-infected mice after α -GalCer treatment is consistent with the timing of an NK cell response, which is induced shortly after MCMV infection (2, 7, 43).

Role of NKT cells in the antiviral effects of α -GalCer. Having shown that α -GalCer has an antiviral effect in vivo during the acute stages of MCMV infection, we wanted to determine whether the observed therapeutic effects are mediated directly by activated, cytolytic NKT cells. To address the specific involvement of NKT cells in antiviral responses, we analyzed MCMV pathogenesis in B6.J α 281 $^{-/-}$ mice that are deficient in classical NKT cells (11). It must be noted that CD1d $^{-/-}$ mice, also deficient in NKT cells, were not used in these experiments as it would not be possible to discriminate between a role of (i) NKT cells themselves, (ii) the CD1d molecule, and/or (iii) polyclonal CD1d-reactive T cells. Since the therapeutic effects

FIG. 3. Replication of MCMV in NKT-cell-deficient B6.J α 281 $^{-/-}$ mice. B6.J α 281 $^{-/-}$ and B6 mice were inoculated i.p. with 10^4 PFU of MCMV. Virus titers in the spleen and liver are shown. Solid squares represent B6.J α 281 $^{-/-}$ mice, and open squares represent B6 WT mice. The dashed line across the graph indicates the limit of detection of the assay. Results are presented as log means $\pm$ SEM from 12 mice per group and were derived by combining data from 4 independent experiments, each utilizing 3 mice per group. **, $P < 0.005$.

of α -GalCer were observed in both BALB/c and B6 mice, the specific role of classical NKT cells was analyzed by using J α 281 $^{-/-}$ mice on the B6 genetic background. In B6 mice, the loss of innate effectors required for effective MCMV clearance would result in increased viral pathogenesis, with viral replication expected to be equivalent to that observed in susceptible mice, which have inefficient innate immune responses and are thus unable to clear MCMV infection effectively (7). For these experiments, B6.J α 281 $^{-/-}$ and B6 WT control mice were infected with 10^4 PFU of MCMV. This virus dose was chosen so that, in the event that NKT cells were required for MCMV clearance, viral replication and disease would at most be equivalent to those observed in susceptible mice but would not lead to death. If NKT cells were found to be necessary for effective MCMV clearance, a higher dose of virus (10^5 PFU) would result in mortality. Following MCMV infection, NKT-cell-deficient B6.J α 281 $^{-/-}$ mice showed viral replication which was overall equivalent to that observed in B6 WT mice (Fig. 3). The loss of NKT cells did not result in significant differences in MCMV titers in the spleen or liver at either day 2, day 4, or day 6 p.i. (Fig. 3), indicating that NKT cells per se do not play a critical role in the natural clearance of virus from visceral organs during acute-phase MCMV infection. A consistent increase in virus titer was observed in the livers of B6.J α 281 $^{-/-}$ mice compared to those of B6 WT controls at day 10 p.i. This difference, although significant ($P < 0.005$), was transient and disappeared by day 12 p.i. (Fig. 3). No differences in virus titers were observed in the salivary glands and lungs of B6.J α 281 $^{-/-}$ mice compared to those of B6 WT mice at days 6, 10, and 12 p.i. (data not shown).

The antiviral effects of α -GalCer therapy were then tested in NKT-cell-deficient B6.J α 281 $^{-/-}$ mice. As expected, in B6.J α 281 $^{-/-}$ mice, viral replication was not altered by α -GalCer therapy and virus titers were equivalent in treated and untreated mice (Fig. 4). The effects of α -GalCer therapy were tested in B6.J α 281 $^{-/-}$ mice infected with both 10^4 and 10^5 PFU of MCMV (Fig. 4A and B, respectively). In B6 WT mice

FIG. 4. Replication of MCMV in NKT-cell-deficient B6.Jα281^{-/-} mice with and without α-GalCer therapy. (A) B6.Jα281^{-/-} mice were inoculated i.p. with 10⁴ PFU of MCMV and treated with 2 μg of α-GalCer on days 0 and 4. Virus titers in the spleen and liver are shown. Solid squares represent α-GalCer-treated mice, and open squares represent vehicle-treated mice. The dashed line across the graph indicates the limit of detection of the assay. (B) B6.Jα281^{-/-} mice were inoculated i.p. with 10⁵ PFU of MCMV and treated with 2 μg of α-GalCer on days 0 and 4. Virus titers in the spleen and liver at day 2 p.i. are shown. Results are presented as log means ± SEM from 6 mice per group and were derived by combining data from duplicate independent experiments, each utilizing 3 mice per group.

infected with 10⁵ PFU of MCMV, the therapeutic effects of α-GalCer in both the spleen and the liver are maximal at day 2 p.i. (Fig. 2). At the same virus dose and same time point, virus titers in the spleen and liver were equivalent in untreated B6.Jα281^{-/-} mice and B6.Jα281^{-/-} mice that received α-GalCer (Fig. 4B).

Based on these results, we conclude that NKT cells are involved in inducing the antiviral effects of α-GalCer, despite the fact that they do not appear to possess direct antiviral activity during a natural infection with MCMV.

Role of NK cells in the antiviral effects of α-GalCer. Since it appears that NKT cells play an indirect role in restricting viral replication after α-GalCer treatment, possibly by activating NK cells, we specifically assessed the role of NK cells in the observed antiviral effect of α-GalCer therapy. Viral pathogenesis was assessed in α-GalCer-treated WT mice that were selectively depleted of NK cells, but not NKT cells, by anti-asialoGM1 administration, as described previously (34). MCMV-susceptible BALB/c WT mice were used in this experiment, resulting in naturally high virus titers in visceral organs

at early times postinfection. Depletion of NK cells by anti-asialoGM1 treatment resulted in a 0.5 log elevation of MCMV titers in the liver at day 6 p.i. ($P < 0.05$) (Fig. 5), whereas virus titers in the spleen (Fig. 5) and salivary gland (data not shown) remained equivalent to those of untreated control animals.

The therapeutic effect of α-GalCer was markedly diminished after depletion of NK cells by anti-asialoGM1 treatment. A marginal (<1 log) reduction in virus titer was observed in the spleens of anti-asialoGM1-treated animals that received α-GalCer at day 4 p.i. ($P < 0.005$) in comparison to the >2 log reduction ($P < 0.0005$) observed at the same time point in animals that received α-GalCer without NK cell depletion (Fig. 5). As expected, significant reductions in virus titers in the spleen were observed after α-GalCer treatment on days 2 ($P < 0.0005$) and 6 ($P < 0.005$) post-MCMV infection. In contrast, at day 2 and 6 p.i., α-GalCer did not lead to any reduction in virus titers in the spleens of mice that were also depleted of NK cells (Fig. 5). Similar to the observations made for the spleen, administration of α-GalCer in NK-cell-depleted BALB/c WT mice resulted in a marginal reduction of virus titers in the liver at day 4 p.i. only ($P < 0.005$). This reduction was only to levels equivalent to those observed in control, vehicle-treated mice (Fig. 5). In contrast, and as expected, significant reductions in viral replication in the liver were observed on days 2 ($P < 0.005$), 4 ($P < 0.005$), and 6 ($P < 0.05$) p.i., when mice received α-GalCer without anti-asialoGM1 to deplete NK cells.

The replication of MCMV in salivary glands was not altered by α-GalCer therapy in NK-cell-depleted mice compared with that in non-NK-cell-depleted mice (data not shown). These results indicated that in MCMV-susceptible BALB/c mice, NK cells are the primary mediators of the antiviral activity of α-GalCer in visceral organs. The minimal residual antiviral effect of α-GalCer in NK-cell-depleted mice may be mediated by other bystander cells or may represent an effect of the few NK cells (<10% as assessed by fluorescence-activated cell sorter analysis) (data not shown) not completely depleted by anti-asialoGM1 treatment.

Role of perforin and IFN-γ in the antiviral effects of α-GalCer. Having shown that NK cells are the primary effector population mediating the antiviral effects of α-GalCer, we analyzed the specific roles of perforin and IFN-γ, these being the two major effector molecules used by NK cells to clear MCMV infection (29, 43). In addition, IFN-γ released by activated NKT cells activates NK cells to release more IFN-γ and up-regulates their perforin-mediated cytotoxic activity (12, 16, 36). To test the specific importance of perforin and IFN-γ in the enhanced antiviral host responses induced by α-GalCer, we investigated the efficacy of α-GalCer therapy in mice deficient in perforin (BALB/c.pfp^{-/-}), IFN-γ (BALB/c.IFN-γ^{-/-}), or perforin and IFN-γ (BALB/c.pfp^{-/-}.IFN-γ^{-/-}) (Fig. 6).

In BALB/c.pfp^{-/-} mice, α-GalCer did not significantly reduce virus titers (Fig. 6). In spleens of α-GalCer-treated BALB/c.pfp^{-/-} mice, virus titers were only slightly, and not significantly, reduced at day 4 p.i. (Fig. 6). In the liver and lung, virus titers in BALB/c.pfp^{-/-} mice were not altered by α-GalCer therapy (Fig. 6). In BALB/c.IFN-γ^{-/-} mice, a significant ($P < 0.05$) 1 log reduction in virus titer was observed at day 2 p.i. in the livers of α-GalCer-treated mice (Fig. 6). In other organs of BALB/c.IFN-γ^{-/-} mice, virus titers were not reduced by α-GalCer therapy (Fig. 6) and, indeed, administra-

FIG. 5. NK cells are the major effectors in improved MCMV clearance mediated by α -GalCer therapy. BALB/c mice were depleted of NK cells by the administration of three doses of anti-asialoGM1 (α -aGM1) on days -1, 0, and 6 relative to MCMV inoculation. Mice were then inoculated i.p. with 10^4 PFU of MCMV and treated with $2 \mu\text{g}$ of α -GalCer on days 0 and 4. Virus titers in the spleen and liver are shown. The dashed line across the graph indicates the limit of detection of the assay. Results are presented as log means $\pm$ SEM from 12 mice per group and were derived by combining data from 4 independent experiments, each utilizing 3 mice per group. , $P < 0.005$ when groups 1 and 2 are compared; $\S$, $P < 0.005$ when groups 2 and 4 are compared; ***, $P < 0.0005$ when groups 3 and 4 are compared; **, $P < 0.005$ when groups 3 and 4 are compared; *, $P < 0.05$ when groups 3 and 4 are compared.

tion of α -GalCer increased virus titers at day 6 p.i. in the liver and lung ($P < 0.05$) (Fig. 6).

In BALB/c.pfp $^{-/-}$.IFN- γ $^{-/-}$ mice, α -GalCer did not alter virus titers in the spleen at any time postinfection (Fig. 6). Similarly, α -GalCer therapy did not reduce virus titers in either the liver or the lung of BALB/c.pfp $^{-/-}$.IFN- γ $^{-/-}$ mice (Fig. 6). In fact, in BALB/c.pfp $^{-/-}$.IFN- γ $^{-/-}$ mice, α -GalCer therapy resulted in a transient, but significant ($P < 0.05$), increase in virus titer in the lung at day 4 p.i. (Fig. 6).

Although overall virus titers (except for those of the lung) were only marginally increased in the untreated gene-targeted mice examined compared to those in BALB/c WT mice, MCMV already replicates in the spleens and livers of BALB/c WT mice at the maximal load sustainable in these visceral organs. In fact, all of the gene-targeted mice tested were more susceptible to MCMV infection, and unlike BALB/c WT, none of which died as a result of MCMV infection, all of the gene-targeted mice succumbed to viral infection by day 8 or 9 p.i. (data not shown).

DISCUSSION

The contribution of NK cells in innate immune responses to viral infections is well established. In contrast, the involvement of NKT cells in viral immune surveillance remains poorly understood (5). In this study, we investigated the relevance of NKT cells during infection with the herpesvirus MCMV. The role of NKT cells was defined both in the context of a natural infection and after activation with α -GalCer. Treatment of mice with α -GalCer induced a significant antiviral response resulting in decreased virus titers in the visceral organs of MCMV-susceptible BALB/c WT mice. Similar results were obtained in B6 WT mice. Although normally resistant to MCMV infection, B6 WT mice show susceptibility to escalating doses of virus (2). At a dose of 10^5 PFU, which caused

pathogenesis in B6 WT vehicle-treated mice, B6 WT mice receiving α -GalCer demonstrated reduced viral replication, with lower titers in both the spleen and liver, similar to the effects observed with BALB/c WT mice.

The effects of α -GalCer therapy are normally mediated by a potent and selective activation of NKT cells via association of the α -GalCer/CD1d complex with the V α 14 TCR (6, 8, 21). In MCMV infection, we have shown that NKT cells are necessary to induce the α -GalCer-mediated antiviral therapy, since the effects of α -GalCer were abolished in NKT-cell-deficient B6.J α 281 $^{-/-}$ mice. Importantly, although activation of NKT cells results in improved viral clearance, these cells do not play a major role in controlling the early acute phase of natural infection with MCMV. Indeed, at early times postinfection, NKT-cell-deficient B6.J α 281 $^{-/-}$ mice did not show increased susceptibility to MCMV, since viral replication in the visceral organs of these mice was equivalent to that observed in B6 WT mice. Similarly, NKT cells have been shown to be dispensable for the control of lymphocytic choriomeningitis virus infection (39). Although NKT cells do not appear to play a major role in restricting MCMV replication during the acute stage of infection, virus titers were transiently and slightly increased (<1.0 log) in the livers of B6.J α 281 $^{-/-}$ mice 10 days after infection, suggesting that NKT cells may play a role in helping to activate adaptive immune responses that control viral replication during the later stages of acute-phase infection. This conclusion was corroborated by the finding that the effects observed in B6.J α 281 $^{-/-}$ mice at day 10 p.i. were transient, with equivalent virus titers in B6.J α 281 $^{-/-}$ and B6 WT mice by day 12 p.i.

Previous studies have reported on the antiviral effects of α -GalCer-activated NKT cells, with improved viral clearance or prevention of virus-induced disease described for hepatitis B virus (20), diabetogenic encephalomyocarditis virus (13), and respiratory syncytial virus (18). In the case of diabetogenic

FIG. 6. Role of perforin and IFN- γ in the improved MCMV clearance mediated by α -GalCer therapy. Mice were inoculated i.p. with 10^4 PFU of MCMV and treated with 2 μ g of α -GalCer on days 0 and 4. Virus titers in the spleen, liver, and lung are shown. Squares represent α -GalCer-treated mice, and triangles represent vehicle-treated mice. Results are presented as means $\pm$ SEM of at least 3 mice per group and are representative of duplicate independent experiments. **, $P < 0.005$; *, $P < 0.05$.

encephalomyocarditis virus (13) and respiratory syncytial virus (18) infections, the therapeutic effects of α -GalCer were accompanied by the demonstration that NKT cells also play a critical role in viral clearance without α -GalCer activation. Although it is not possible to conclusively ascribe the reported effects directly to classical NKT cells, as the studies were undertaken in CD1d^{-/-} mice, it appears that there may be differences in the role played by NKT cells and α -GalCer-activated NKT cells in different viral infections. The studies reported here may be more analogous to those of Kakimi and colleagues, which demonstrated that the α -GalCer-induced inhibition of hepatitis B virus replication was principally mediated by the rapid induction of IFN- α/β and IFN- γ in the liver (20).

Thus, having shown that NKT cells are dispensable for the control of MCMV replication during a natural infection but essential for the antiviral efficacy of α -GalCer therapy, we wanted to establish whether the observed antiviral response was mediated directly through NKT cells or indirectly through activation of other immune effectors. It is known that activated

NKT cells exhibit increased perforin-mediated cytotoxicity against tumor cell lines in vitro (10, 12) while also rapidly releasing immunoregulatory cytokines such as IL-4 and IFN- γ (3). Cytokines produced by activated NKT cells can in turn trigger bystander activation of NK cells, which involves proliferation, further IFN- γ production, and cytotoxicity (10, 12).

Depletion of NK cells prior to α -GalCer therapy confirmed the involvement of these effectors in the antiviral response induced by α -GalCer, with the efficacy of the therapy almost completely abolished in mice depleted of NK cells but possessing an intact NKT cell population. Having shown that the therapeutic effects of α -GalCer in MCMV infection were mediated by the activation of NK cells, we further examined the involvement of specific effector mechanisms, namely perforin and IFN- γ , in the antiviral response by utilizing gene-targeted mice. These studies demonstrated that the α -GalCer-induced antiviral response was dependent upon both perforin-mediated cytotoxicity and IFN- γ release, as mice deficient for one or both of these molecular effector systems were unable to mount a complete α -GalCer-induced antiviral response.

Recent studies in a B16 melanoma metastasis model demonstrated that the antitumor activity of α -GalCer requires the sequential production of IFN- γ by both NKT and NK cells, but it occurs independently of perforin release (36). This contrasts with our present findings, which clearly demonstrate that perforin is the major contributor to the α -GalCer-induced antiviral response. Although treatment of BALB/c.IFN- $\gamma^{-/-}$ mice with α -GalCer demonstrated that the production of IFN- γ was integral to the α -GalCer-mediated antiviral effect in the spleen, the available data cannot distinguish between the relative importance of IFN- γ produced by NKT cells and NK cells. The present studies have revealed that activated NKT cells are key mediators of the antiviral effects of α -GalCer, with NK cells, IFN- γ , and perforin all playing crucial roles. Interestingly, although it has been postulated that IFN- γ is the most important anti-MCMV mechanism in the liver (43), α -GalCer still showed an antiviral effect in these organs in IFN- γ -deficient mice. In contrast, α -GalCer therapy did not reduce viral replication in the livers of perforin-deficient mice, suggesting that perforin plays a crucial antiviral role at this site in α -GalCer-treated mice.

Activation of NKT cells by α -GalCer can lead to both IFN- γ and IL-4 secretion (9, 24, 33). IL-4 is involved in the induction of a Th2-mediated humoral immune response, which does not play a role in the clearance of MCMV infection (19). In BALB/c.IFN- $\gamma^{-/-}$ mice, α -GalCer therapy had detrimental effects in the liver and lung at day 6 postinfection. Due to the targeted deletion of the IFN- γ gene in these mice, α -GalCer-stimulated NKT cells only secrete IL-4. Consequently, in these animals the balance of the immune response induced by activated NKT cells will be biased towards the Th2 pathway, resulting in less-efficient clearance of MCMV, as observed.

It is important to note that our previous studies on the effects of α -GalCer in mediating tumor suppression, together with our present analysis of α -GalCer antiviral activity, have revealed that activated NKT cells can regulate different effector immune responses. Thus, the antitumor effects of α -GalCer are dependent on NK cells and IFN- γ , whereas the α -GalCer antiviral activity requires NK cells, IFN- γ , and perforin. These differences are most likely due to differences in the key factors associated with tumor suppression versus viral clearance. Thus, a requirement for perforin-mediated cytotoxicity may be unnecessary for tumor suppression when the antiangiogenic effects of IFN- γ alone may be sufficient to prevent tumor growth and metastasis (40). In contrast, perforin is likely to be essential in mediating the α -GalCer antiviral effects due to a requirement for direct cytolysis of virus-infected targets. Consistent with this hypothesis is the finding that the antiviral response induced by α -GalCer appears to be most potent during acute-phase MCMV infection, controlling virus replication most effectively in the spleen and liver. In both these organs, the early control of MCMV infection is highly reliant on innate effector mechanisms (7, 43). Thus, in α -GalCer therapy, the timing of the antiviral response elicited and the organs in which it occurs concur with our conclusion that the therapy specifically and potently enhances innate immune responses mediated principally by activated NK cells.

In this report we have provided the first evidence that, although TCR V α 14J α 281 NKT cells do not play a critical role in restricting early acute-phase natural infection with MCMV,

activating them with α -GalCer induces a potent and specific antiviral response. We have shown that the antiviral response elicited by α -GalCer is dependent on V α 14J α 281 NKT cells, that the effectors are activated NK cells, and that both perforin and IFN- γ are critical molecular effector mechanisms. These studies have not only elucidated the role of different innate immune effectors in viral immune surveillance, but they provide the basis for the design of targeted antiviral therapies.

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