α -Galactosylceramide as a Therapeutic Agent for Pulmonary *Mycobacterium tuberculosis* Infection

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Rationale: Invariant natural killer T (iNKT) cells are a unique subset of T cells that recognize lipid antigens presented by CD1d molecules. Recent studies have shown that iNKT cells can protect mice against *Mycobacterium tuberculosis* (Mtb) infection. We sought to determine whether pharmacological activation of iNKT cells by α -galactosylceramide (α -GalCer) could be used to treat tuberculosis (TB).

Objectives: We hypothesized that α -GalCer, either alone or in combination with isoniazid, could be used to treat pulmonary TB.

Methods: The ability of α -GalCer-activated iNKT cells to suppress Mtb replication was evaluated using an *in vitro* coculture system. To test its potency *in vivo*, mice infected with virulent Mtb were treated with α -GalCer alone or in combination with isoniazid.

Measurements and Main Results: Quantitative colony-forming unit counts were compared for both experimental systems. Our results show that α -GalCer plus isoniazid controls bacterial growth better than α -GalCer or INH alone, and single or multiple α -GalCer administrations prolong the survival of the mice infected via the aerosol route.

Conclusions: Our results demonstrate that α -GalCer administration can improve the outcome of Mtb infection, even when transmitted by the aerosol route. However, a combination of isoniazid and α -GalCer treatment has a synergistic effect on infection control. We conclude that more efficient treatment of TB will be achieved through a combination of classic chemotherapy and modulation of the host immune response.

Keywords: CD1d; antibiotics; natural killer T cells; tuberculosis; lipids

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (Mtb) and represents an important global threat to health, especially in developing countries where new and efficient therapies are urgently needed. Although current antibiotic regimens against Mtb are effective against drug-susceptible strains, patients require long-term follow-up and can suffer severe side effects. In addition, the prevalence of multidrug- and extensively drug-resistant Mtb strains is on the rise (1). Treatment of drug-resistant bacterial

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

New drug regimens are urgently needed to improve treatment of tuberculosis. Immunomodulation of the immune system is a novel strategy to enhance host resistance to infection.

What This Study Adds to the Field

The synthetic lipid α -galactosylceramide activates NKT cells to prolong the survival of mice after infection with low-dose aerosolized *Mycobacterium tuberculosis*, and has an additive effect when combined with classical antituberculous chemotherapy.

strains is more complicated, requiring hospital supervision (2). Recent efforts to develop new therapeutic regimens are promising, but their efficiency remains to be tested in larger populations. In the current study, we investigated whether the specific activation of iNKT cells, which we have previously shown is capable of activating macrophages (M ϕ) to suppress intracellular Mtb replication, can act therapeutically against established pulmonary TB (3).

Invariant natural killer T (iNKT) cells are a sublineage of T lymphocytes that are defined by their expression of a canonical V α 14-J α 18 T-cell receptor (TCR) α -chain. The $\alpha\beta$ -TCR expressed by iNKT cells recognizes self and foreign lipid antigens presented by the antigen-presenting molecule CD1d. iNKT cells influence host immunity during infectious diseases through production of soluble mediators, such as IL-4 and IFN- γ , by reciprocal NKT-dendritic cell (DC) activation and by elaboration of chemokines that recruit inflammatory cells (4–6).

The discovery that CD1d presentation of α -galactosylceramide (α -GalCer) rapidly activates murine and human iNKT cells has greatly increased our understanding of the potential role that iNKT cells play in host immunity (7, 8). The synthetic glycosphingolipid α -GalCer is a potent iNKT cell agonist, and even though it is not a natural ligand for iNKT cells, α -GalCer and its analogs are commonly used experimentally (9). iNKT cell activation by α -GalCer *in vivo* induces downstream activation of several lymphoid and myeloid cell populations, including B and T cells, NK cells, M ϕ , and DCs, which in turn can modify multiple immune responses (10–13). For example, α -GalCer has been used to analyze the immunomodulatory properties of activated iNKT cells in mice and patients with malignancies (14–16). Administration of α -GalCer activates both the innate and adaptive immune system and has raised the possibility of using α -GalCer for therapeutic purposes (8, 17).

The number of iNKT cells in the lung is increased after infection of mice with virulent Mtb (18). CD1d, which presents antigen to iNKT cells, is up-regulated by IFN- γ and is also increased in the lungs of infected mice (19). We have previously

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shown that innate iNKT cells recognize Mtb-infected M ϕ , produce IFN- γ , and kill intracellular bacteria both *in vitro* and *in vivo* (3). Although these data suggest that iNKT cells can mediate protective immunity against TB, several laboratories have found that iNKT cells are not required for optimal immunity to Mtb (20, 21). One explanation is that iNKT cells make only a small and possibly redundant contribution to antimycobacterial immunity. Another possibility is that after infection, iNKT cells become anergic, as has been shown after BCG and viral infection (22). Given the potential ability of activated iNKT cells to suppress bacterial growth, we wished to determine whether administration of α -GalCer could enhance the antibacterial activity of iNKT cells and whether α -GalCer could serve a role in the treatment of established disease.

METHODS

Mice

Six- to 8-week-old female C3H/HeJ (C3H) (highly susceptible to Mtb) and C57BL/6 (B6) (relatively resistant to Mtb) mice were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were housed in a BSL3 facility under specific pathogen-free conditions in the Animal Biohazard Containment Suite (Dana Farber Cancer Institute, Boston, MA) and used in a protocol approved by the institution.

Bacteria and *In Vitro* Infection of Peritoneal Macrophages

Macrophages were harvested by peritoneal lavage 4 days after intraperitoneal injection of sterile 3% thioglycolate medium. M ϕ were purified by positive selection using CD11b-microbeads according to manufacturer's directions (Miltenyi Biotec, Auburn, CA). The purified cells were 95% F4/80⁺CD11b⁺, as determined by flow cytometry. Purified M ϕ (0.5×10^6 /well) were cultured in 24-well plates in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal calf serum (HyClone, Rockford, IL), penicillin/streptomycin, L-glutamine, sodium-pyruvate, 2-ME, nonessential amino acids, essential amino acids, and N-2-hydroxyethylpiperazine-N'-ethane sulfonic acid (all from Gibco, Carlsbad, CA). Virulent Mtb (H37Rv) was grown to mid-log phase in Middlebrook 7H9 medium supplemented with 10% albumin/dextrose/catalase (BD Biosciences, San Jose, CA). Bacteria were opsonized for 5 minutes using RPMI 1640 with 2% human serum (Gemini Bio-Products, West Sacramento, CA), 10% fetal bovine serum, and 0.05% Tween 80 and then washed twice with complete medium without antibiotics. Bacteria were passed through a 5- μ m syringe filter (Millipore, Billerica, MA), counted in a Petroff-Hausser chamber, and added to enriched M ϕ for 2 hours before extensive washing to remove extracellular bacteria. The length of infection was 2 hours for all experiments. Recombinant mouse IFN- γ (US Biological, Swampscott, MA), α -GalCer, and α -C-GalCer were used at the concentrations indicated.

Colony-Forming Unit Determination (*In Vitro* Model)

Bacterial growth was quantified 72 hours after coculture with naive splenocytes with or without IFN- γ and/or α -GalCer (Day 4). The cells were lysed after removing the supernatant by adding distilled water. Serial dilutions were plated on Middlebrook 7H11 agar plates. The number of bacteria contained in the supernatant was less than 10% of the cell-associated colony-forming units (CFU), independently of whether splenocytes were added. The colonies were enumerated after 3 weeks at 37°C.

Bacteria and Infections

Virulent *M. tuberculosis* (Erdman strain) were passed through mice and subsequently grown once in Middlebrook 7H9 supplemented with the oleic acid-albumin-dextrose complex and stored at 80°C. For each experiment an aliquot was thawed, sonicated twice for 10 seconds with a cup horn sonicator, and then diluted in 0.9% NaCl–0.02% Tween 80. Mice were infected via the aerosol route as described previously (23). For intravenous infections, mice were injected via the lateral tail vein

with 0.25×10^6 to 4.0×10^6 live mycobacteria. The inoculum was confirmed by plating.

Treatment of Mice with α -GalCer

α -GalCer was prepared in a vehicle of 0.5% polysorbate 20. The mice were injected with a low ($100 \mu\text{g}\cdot\text{kg}^{-1}$) or high ($500 \mu\text{g}\cdot\text{kg}^{-1}$) dose of α -GalCer, or injected with control vehicle. The following treatment regimens were tested: (1) intraperitoneal injection of $100 \mu\text{g}\cdot\text{kg}^{-1}$ α -GalCer on Days 5, 3, and 1 before aerosol infection; (2) intraperitoneal injection of $100 \mu\text{g}\cdot\text{kg}^{-1}$ α -GalCer on Days 1, 5, and 9 after aerosol infection; (3) intratracheal injection of $100 \mu\text{g}\cdot\text{kg}^{-1}$ α -GalCer on Day 1 after aerosol infection; (4) intraperitoneal injection of $500 \mu\text{g}\cdot\text{kg}^{-1}$ α -GalCer on Day 1 after aerosol infection; (5) intraperitoneal injection of $500 \mu\text{g}\cdot\text{kg}^{-1}$ α -GalCer on Day 1 after aerosol infection and then again every 30th day; (6) intraperitoneal injection of $500 \mu\text{g}\cdot\text{kg}^{-1}$ α -GalCer on Day 30 post aerosol infection and then again every 30th day.

α -GalCer and Antibiotic Treatment

Groups of C3H/HeJ mice were infected via the intravenous route and injected with a low dose of α -GalCer or vehicle on Day 1 post infection (p.i.). On Day 14 p.i., isoniazid (100 mg/L) was added to the drinking water. The drinking water was changed twice weekly during the treatment period and the mice had access to the water ad libitum. The isoniazid treatment was stopped on Day 28 p.i. and the CFU was determined in lung and spleen on Day 36 p.i.

Measurement of IFN- γ Lung Production

Before removal, lungs were perfused by injecting 5 to 10 ml of sterile PBS into the right ventricle of the heart after severing the inferior vena cava. The lungs were homogenized in 0.9% NaCl–0.02% Tween 80 with a Minibeadbeater-8 and centrifugation for 20 minutes at 3,000 rpm, then supernatants were recovered and IFN- γ was analyzed by a standard ELISA sandwich.

CFU Determination (*In Vivo* Model)

Three, 5, or 12 weeks after infection, mice were killed and their left lung and spleen were aseptically removed and individually homogenized in 0.9% NaCl–0.02% Tween 80. Viable bacteria were enumerated by plating organ homogenates onto 7H11 agar plates. Colonies were counted after 3 weeks.

Statistics

Data were expressed as mean $\pm$ SEM. Comparisons of data among experimental groups were performed using analysis of variance with Bonferroni post test. A survival analysis was performed using Kaplan-Meier method and comparisons were done with log-rank test. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

α -GalCer-Activated Splenocytes Reduce Intracellular Replication of Mtb *In Vitro*

We have previously shown that nonimmune splenocytes have innate antimycobacterial activity that is mediated by iNKT cells, which recognize Mtb infected M ϕ and restrict intracellular bacterial growth (3). Although CD1d expression by the infected M ϕ is required, which implies cognate recognition (3), it is uncertain whether CD1 presents a microbial antigen, or alternatively, whether cytokine production costimulates weak recognition of an endogenous (self) antigen (24). Because only 1 to 2% of splenocytes are iNKT cells, we wondered whether pharmacological activation of iNKT cells with α -GalCer would enhance the growth suppression of Mtb mediated by iNKT cells *in vitro*. Coculture of naive splenocytes with Mtb-infected M ϕ reduced the number of viable CFU as reported previously (Figure 1A) (3). Addition of α -GalCer led to a further reduction in CFU, as did the addition of IFN- γ , and adding both α -GalCer and IFN- γ led to an additive, and possibly coopera-

tive, effect on CFU control. Treatment with α -GalCer was associated with a dose-dependent increase in IFN- γ production (Figure 1B). Thus, although iNKT cells have the physiological potential to recognize infected M ϕ , their pharmacological activation by the synthetic lipid α -GalCer leads to a more potent mycobactericidal effect.

Systemic Therapy with α -GalCer Prolongs the Survival of Susceptible Mice after Low-Dose Aerosol Infection with *M. tuberculosis*

We next wished to determine the potential of α -GalCer to treat mice with pulmonary TB. To observe protection, we selected C3H mice, which are a susceptible mouse strain that develops extensive lung pathology and succumbs to infection earlier than B6 mice (25–27). Previously, we demonstrated that a single dose of α -GalCer ($100 \mu\text{g}\cdot\text{kg}^{-1}$) administered intraperitoneally within 5 days of Mtb infection by the intravenous route was effective in prolonging the survival of susceptible C3H mice (28). However, α -GalCer administered by the intraperitoneal route did not protect mice against low-dose aerosol (LDA) infection with virulent Mtb (Figure 2A). There are several important differences between the systemic and pulmonary routes of infection that may affect the efficacy of α -GalCer. We hypothesized that α -GalCer-activated iNKT cells acted locally. Therefore, α -GalCer ($100 \mu\text{g}\cdot\text{kg}^{-1}$) was administered intratracheally after LDA infection with Mtb. α -GalCer-treated mice had prolonged survival in two separate experiments (Figure 2A). These data indicate that locally activated iNKT cells can provide protection against LDA Mtb infection.

Ishii and colleagues reported that intraperitoneally administered α -GalCer ($10 \mu\text{g}/100 \mu\text{l}$ vehicle) has a protective effect on the lung after bleomycin treatment (29). Based on these results, we analyzed whether a high dose of α -GalCer ($500 \mu\text{g}\cdot\text{kg}^{-1}$) administered systemically protected mice challenged with LDA Mtb. Compared with mice treated with vehicle alone, α -GalCer administered intraperitoneally on Day 1 post infection prolonged the survival of susceptible C3H/HeJ mice (Figure 2B). The mean survival time (MST) of vehicle-treated mice was 60 days. In contrast, the mice that received a single injection of

α -GalCer ($500 \mu\text{g}\cdot\text{kg}^{-1}$) had a MST of 106 days. These data show that systemic administration of high-dose α -GalCer protects mice against pulmonary TB.

Having established that systemic α -GalCer could protect mice against LDA Mtb infection, we next determined whether repeated administration of α -GalCer would provide longer-term protection. Mice were given α -GalCer on Day 1 after Mtb infection and then administered α -GalCer every 30 days, a treatment regimen that significantly prolonged their survival compared with vehicle-treated control mice (MST = 124 d vs. 31 d; $P = 0.0009$). However, repeated monthly injections with α -GalCer had only an incremental effect on the survival compared with animals injected with a single dose of α -GalCer on Day 1 after LDA infection (MST = 124 d vs. 106 d; $P = \text{not significant}$) (Figure 2C). Thus, similar to the low-dose α -GalCer treatment regimen after intravenous Mtb infection

Figure 1. α -Galactosylceramide (α -GalCer)-activated splenocytes suppress *Mycobacterium tuberculosis* (Mtb) replication. (A) Infected macrophages (M ϕ) were cultured alone or with splenocytes. Some cultures included α -GalCer (100 ng/ml), IFN- γ (2.5 U/ml) or both. After 72 hours, the total colony-forming unit (CFU) was measured. Statistical testing was done using a one-way analysis of variance with Tukey posttest comparing all possible pairs. Differences between all columns are statistically significant ($*P < 0.05$). Bars, mean $\pm$ SEM of replicate cultures. (B) Treatment with α -GalCer mediates a dose-dependent increase in IFN- γ production that correlates with control of bacterial replication. Immediately before cell lysis supernatants were sampled to measure IFN- γ by ELISA. Values shown in B are mean ng/ml $\pm$ SEM of IFN- γ (open symbols) or CFU $\pm$ SEM (solid symbols).

Figure 2. Treatment with α -galactosylceramide (α -GalCer) ($100 \mu\text{g}\cdot\text{kg}^{-1}$ or $500 \mu\text{g}\cdot\text{kg}^{-1}$) significantly prolongs the survival of mice infected by the aerosol route. (A) Survival of mice after the administration of α -GalCer ($100 \mu\text{g}\cdot\text{kg}^{-1}$) by the intraperitoneal or intratracheal route ($n = 10$). Mice were divided in four groups. One group (solid diamonds) received α -GalCer group by intraperitoneal administration on Days -5, -3, and -1. A second group (solid squares) received α -GalCer group by intraperitoneal administration on Days 1, 5, and 9. A third group (closed circles) received α -GalCer group by the intratracheal route on Day 1. Mice were infected with *Mycobacterium tuberculosis* (Mtb) by low-dose aerosol (LDA) on Day 0. (B) Vehicle (open circles) or α -GalCer group ($500 \mu\text{g}\cdot\text{kg}^{-1}$) (solid circles) was administered intraperitoneally 1 day after LDA infection with Mtb. (C) Survival of mice after the administration of α -GalCer ($500 \mu\text{g}\cdot\text{kg}^{-1}$) by the intraperitoneal route ($n = 10$). No animals died in the uninfected group treated with α -GalCer. Mice received vehicle (open circles), α -GalCer on Day 1 by intraperitoneal administration (solid circle), or multiple doses of α -GalCer (solid squares) starting on Day 1 and then every 30 days. (D) Mice (solid diamonds) received multiple intraperitoneal administrations starting on Day 30 and then every 30 days. C and D are from the same experiment and the data are separated for clarity. Survival analysis was performed using Kaplan-Meier method and comparisons were done with log-rank test. $*P < 0.05$, $***P < 0.0005$.

(28), a single dose of α -GalCer injected on Day 1 after LDA Mtb infection significantly prolonged the survival of the infected mice and repeated administration did not provide any additional benefit.

Importantly, treatment with α -GalCer did not diminish the survival of uninfected mice and there were no deaths in mice repeatedly treated with α -GalCer, even after 200 days of observation (data not shown). This is consistent with Kimura and colleagues, who found that although α -GalCer treatment attenuated the development of bleomycin-induced pulmonary fibrosis, it did not appear to have any adverse effects on the pulmonary architecture (29). Three weeks after α -GalCer treatment of infected mice, no histopathological changes in the lung were observed compared with vehicle-treated control mice (data not shown). However, this does not rule out other subtle changes in the number or volume of granuloma or changes at later time points (28). These results demonstrate that systemic activation of iNKT cells can provide protection to susceptible mouse strains against LDA Mtb infection.

α -GalCer Treatment of Susceptible Mice with Established Mtb Infection Prolongs Their Survival

The ability of α -GalCer to protect mice against LDA Mtb infection suggests that it could be useful as an adjunct treatment against TB. A critical issue is whether α -GalCer had efficacy against established TB disease. To evaluate this question, treatment of mice infected with a low dose of aerosolized Mtb was postponed until Day 30 post infection. At this time point, the bacterial counts in the lung are approximately 10,000-fold greater than the initial inoculum, and the immune response has controlled bacterial replication leading to the establishment of the plateau phase of the infection. Starting on Day 30, mice were treated with α -GalCer ($500 \mu\text{g}\cdot\text{kg}^{-1}$) every 30 days. Delayed administration resulted in a significant increase in survival compared with vehicle control mice (MST = 56 d vs. 31 d; $P = 0.02$) (Figure 2D). This indicates that activated iNKT cells can alter the course of established TB infection.

α -GalCer Administration Increases Lung IFN- γ Production and Controls Bacterial Replication

Injection of α -GalCer into naive mice leads to rapid but transient activation of iNKT cells (22). Still, we observed a long-term beneficial effect after a single injection of α -GalCer in Mtb aerosol-infected mice. A dose-dependent stimulation of IFN- γ (Figure 1B) but not IL-4 (data not shown) was observed when α -GalCer is added to a coculture of splenocytes and Mtb-infected M ϕ . We hypothesized that the beneficial effect of iNKT cells is mediated in part by IFN- γ production. To determine whether iNKT cell activation induces long-term IFN- γ production in the lung, α -GalCer ($500 \mu\text{g}\cdot\text{kg}^{-1}$) or vehicle was injected intraperitoneally into resistant B6 or susceptible C3H mice 24 hours after LDA Mtb infection. Lung CFU and IFN- γ production were measured in total lung homogenates 3 weeks post infection. Although the data did not reach statistical significance, the IFN- γ concentration was elevated in lung homogenates 3 weeks after α -GalCer treatment compared with vehicle-treated control mice (Figure 3A). Importantly, the bacterial burden in the lungs of mice treated with α -GalCer was significantly reduced 3 weeks after aerosol infection compared with vehicle-treated control mice (B6 and C3H; Figures 3B and 3C). In addition, treatment of B6 mice with α -C-GalCer (30), an analog of α -GalCer that has greater Th1-inducing capability, led to a greater CFU reduction (Figure 3B).

Figure 3. Lung IFN- γ levels and bacterial burden. C57BL/6 mice ($n = 5$) were infected by the aerosol route and 24 hours after infection α -galactosylceramide (α -GalCer) ($500 \mu\text{g}\cdot\text{kg}^{-1}$) or α -C-GalCer ($500 \mu\text{g}\cdot\text{kg}^{-1}$) or saline were administered intraperitoneally. The lung inoculate was 118 colony-forming units (CFU) (B6). (A) IFN- γ levels were measured in total lung homogenate and the (B) bacterial burden was quantified at 3 weeks post infection. (C) The bacterial burden in the lungs of *Mycobacterium tuberculosis* (Mtb)-infected C3H mice 3 weeks after α -GalCer ($500 \mu\text{g}\cdot\text{kg}^{-1}$) or saline treatment. A one-way analysis of variance with Bonferroni post test was used for statistical testing. * $P < 0.05$.

α -GalCer Has an Additive Effect When Administered with Isoniazid

We have shown that α -GalCer treatment ameliorates pulmonary TB and prolongs survival of susceptible mice, even in those with established pulmonary TB. However, α -GalCer treatment did not eradicate the bacterium or fully cure infected mice, indicating that the use of α -GalCer as a single therapeutic agent is not feasible. Because immune modulation using α -GalCer and conventional antibiotics reduces bacterial growth by distinct mechanisms, we considered whether α -GalCer could have a role as an adjunct therapy. Therefore, we determined the efficacy of α -GalCer when administered with or without the first-line antibiotic isoniazid (INH). We were particularly interested in testing this combination in a different disease model, one in which there was significant systemic disease. Therefore, we infected mice by the intravenous route to rapidly disseminate the bacteria and establish a systemic infection with a high peripheral bacterial burden. Five weeks after Mtb infection, the combination of α -GalCer and INH significantly reduced bacterial burden in spleen and lung tissue. Importantly, even under conditions of disseminated disease with a high bacterial burden, the combination of α -GalCer plus INH had an additive effect in controlling bacterial growth in the lungs and in the spleen (Figure 4). Our results suggest that a combination of traditional chemotherapy and modulation of the immune response during pulmonary TB can be used for more efficient control of Mtb replication.

DISCUSSION

The promise of immunotherapy, an approach that may have important implications for drug-resistant disease (31, 32), has yet to show itself as an important part of the arsenal against TB. Although IFN- γ , IL-2, IL-12, and thalidomide have been tried as immune modulators in patients with pulmonary TB, the results of experimental studies and clinical trials have been only moderately encouraging (33–37). Although many clinical studies using IFN- γ , IL-12, and thalidomide have demonstrated the potential of these agents to enhance immune responses to infection (38, 39), a clinical response has been fleeting. Obtaining a therapeutic effect will require unraveling the mechanism of action in addition to optimizing the timing, dose, and route of administration. The complex and myriad effects of cytokines

Figure 4. Combination of α -galactosylceramide (α -GalCer) and INH has an additive effect on the reduction of the mycobacterial burden. C3H mice ($n = 6$) were inoculated intravenously with *Mycobacterium tuberculosis* (Mtb) and then treated with α -GalCer alone (Day 1 post infection) or α -GalCer (Day 1 post infection) plus isoniazid (INH) (Day 14 post infection). Five weeks later, the bacterial burden in the lung and spleen were quantified. * $P < 0.05$ by a one-way analysis of variance. All colony-forming unit (CFU) comparisons between groups were statistically significant in the lung.

most likely explain the difficulty in implementing the clinical use of these different agents.

Immune therapy with α -GalCer has also been suggested as a therapeutic option for human diseases, including multiple sclerosis, type 1 diabetes, rheumatoid arthritis, and lung cancer (16, 40, 41). Given the low frequency of these iNKT cells, it is remarkable that their activation could change the outcome of disease. Furthermore, given the complex interactions between Mtb and host immunity within the microenvironment of infected foci, simple activation of iNKT cells with α -GalCer *in vivo* is unlikely to be sufficient to elicit a curative response. A more realistic strategy would be to combine α -GalCer with conventional antibiotics.

Pharmacological activation of iNKT cells by α -GalCer modulates adaptive immunity to several different microorganisms, including Mtb, *Pseudomonas aeruginosa*, *Cryptococcus neoformans*, and several different viruses (3, 42, 43). However, it has also been shown that specific iNKT cell activation with α -GalCer can exacerbate disease caused by *Leishmania donovani* infection (44). Therefore, the use of synthetic glycolipids to potentiate the function of iNKT cells must be carefully studied. We find that α -GalCer and INH have an additive effect in reducing the bacterial burden in Mtb-infected mice, and that α -GalCer prolongs the survival of infected mice with established pulmonary TB after Mtb infection via the respiratory route.

How does α -GalCer modify the natural course of pulmonary TB? We have previously shown that the ability of α -GalCer to prolong the survival of Mtb-infected mice and to ameliorate disease requires CD1d (28) and is therefore dependent on the action of iNKT cells. We have recently shown, using an *in vitro* model of infection, that iNKT cells can kill intracellular Mtb and induce host protection after being adoptively transferred into mice infected with Mtb via the aerosol route (3). However, other actions of α -GalCer may be important. For example, Fujii and colleagues have shown that intravenous administration of α -GalCer into mice induces DC maturation. This effect is mainly mediated by TNF- α and IFN- γ , and mature DCs can induce greater T-cell responses because of more efficient antigen presentation than exposure to mycobacterial antigens alone (45). Recently, α -GalCer incorporation into BCG has been shown to enhance the conventional CD8⁺ T-cell response;

thus, it does appear that iNKT cell activation is modulating T-cell priming (46).

The ability of iNKT cells to produce IFN- γ after α -GalCer stimulation is one of their most important features to mediate host protection and may have therapeutic implications (47, 48). Given the importance of IFN- γ in controlling Mtb growth and the fact that α -GalCer administration induces IFN- γ production by iNKT cells and by NK cells after downstream activation (49), one explanation for how α -GalCer ameliorates pulmonary TB may simply be via the induction of IFN- γ . However, our *in vitro* data show that a combination α -GalCer and IFN- γ is more efficient in controlling bacterial growth than α -GalCer or IFN- γ treatment alone (Figure 1A). This suggests that α -GalCer-activated iNKT cells reduce Mtb replication via an IFN- γ independent mechanism, perhaps via direct effector functions.

In this study we wanted to determine whether α -GalCer could ameliorate TB after infection via the respiratory route and if α -GalCer could ameliorate established pulmonary TB. Therefore, we infected mice via the aerosol route to mimic the natural route of Mtb infection and we used a high dose of α -GalCer (500 μ g·kg⁻¹), which has been shown by others to influence the immune response in lung tissue (29). We found that a single dose of α -GalCer systemically or intratracheally significantly prolonged the survival of LDA Mtb-infected mice. We also observed that delayed administration of α -GalCer dramatically improved the outcome of established infection as determined by survival.

We have previously shown that repeated α -GalCer administration does not have a greater beneficial effect on survival after intravenous Mtb infection than a single α -GalCer injection. In the present study we find similar results using a model of LDA Mtb infection. The LDA Mtb-infected group that received multiple α -GalCer administrations 30 days apart lived only 18.5 more days compared with the infected group that received a single α -GalCer dose. Chiba and colleagues have previously shown using an *in vivo* model of *Mycobacterium bovis* bacille Calmette-Guérin (BCG) infection that the responsiveness of NKT cells changes during the natural course of the infection, including a transient period of anergy (22). If α -GalCer activation of NKT cells also leads to a period of anergy, the timing of repeated α -GalCer administration could be a critical variable that affects the NKT cell response.

At present, one of the most important approaches against TB infection is the design of new therapeutic options, including adjuvants and vaccines. Although current treatment is effective against susceptible Mtb strains, the emergence of multidrug- and extensively drug-resistant strains requires the use of new strategies that can enhance the immune response. We show that the use of α -GalCer as adjuvant therapy influences the immune response by enhancing iNKT cell production of IFN- γ , which presumably activates lung M ϕ to kill Mtb and induces DC maturation to improve antigen presentation to T cells. Similar effects have been observed in other infection models (50–52). Using a clever strategy to exploit the adjuvant properties of α -GalCer clinically, Venkataswamy and colleagues incorporated α -GalCer into the cell wall of BCG. A better immune response was elicited by the modified BCG (46). New and efficient antibiotics are likely to emerge on the market to help fight pulmonary TB. Unfortunately, there will be an ongoing battle between any new anti-TB drug candidate and the development of drug resistance. The inclusion of immunomodulatory agents as adjuvant therapy may prove to be beneficial in the treatment of TB. Because α -GalCer treatment in people with cancer has been proven to be safe (53), we believe that further studies with α -GalCer are warranted to determine

whether our observations can be translated into a therapeutic strategy that can aid patients with TB.

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