Natural Killer T Cell Ligand α-Galactosylceramide Enhances Protective Immunity Induced by Malaria Vaccines

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Abstract

The important role played by $CD8^+$ T lymphocytes in the control of parasitic and viral infections, as well as tumor development, has raised the need for the development of adjuvants capable of enhancing cell-mediated immunity. It is well established that protective immunity against liver stages of malaria parasites is primarily mediated by $CD8^+$ T cells in mice. Activation of natural killer T (NKT) cells by the glycolipid ligand, α-galactosylceramide (α-GalCer), causes bystander activation of NK, B, $CD4^+$, and $CD8^+$ T cells. Our study shows that coadministration of α-GalCer with suboptimal doses of irradiated sporozoites or recombinant viruses expressing a malaria antigen greatly enhances the level of protective anti-malaria immunity in mice. We also show that coadministration of α-GalCer with various different immunogens strongly enhances antigen-specific $CD8^+$ T cell responses, and to a lesser degree, Th1-type responses. The adjuvant effects of α-GalCer require CD1d molecules, Vα14 NKT cells, and interferon γ. As α-GalCer stimulates both human and murine NKT cells, these findings should contribute to the design of more effective vaccines against malaria and other intracellular pathogens, as well as tumors.

Key words: α-galactosylceramide • NKT cells • adjuvant • malaria vaccines • $CD8^+$ T cells

Introduction

During the last few years, evidence has accumulated indicating that the cellular immune response plays a critical role in defense against viral, bacterial, and parasitic infections, as well as tumor development (1–3). At present, conventional vaccine delivery systems and the adjuvants approved for human use (aluminum salts and MF59) are poor at inducing $CD8^+$ T cell responses (4). The development of adjuvants capable of eliciting a robust, specific $CD8^+$ T cell response is an active area of research (5).

The feasibility of the development of a vaccine against malaria is supported by the fact that immunization with radiation-attenuated sporozoites can elicit sterile immunity against challenge with *Plasmodium spp.* sporozoites in mice, monkeys, and humans (6, 7). A large number of studies performed in animal models and a few epidemiological studies in human subjects indicate a central role of $CD8^+$ T lymphocytes in protection against the liver stages of malaria (8–12). These studies have led us to search for a malaria vaccine based on its ability to induce a protective $CD8^+$ T cell response. Recently, recombinant attenuated viruses,

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such as adenovirus (13) and Sindbis virus (14) given as single immunizing doses, and influenza and vaccinia viruses used together in a prime-boost strategy (15), have been shown to potently induce malaria-specific $CD8^+$ T cell responses in animal models.

NKT cells comprise a lymphocyte population characterized by expression of surface markers of natural killer cells together with a semi-invariant T cell receptor (16). NKT cells have the ability to produce a large amount of IFN-γ and IL-4 promptly after their activation by in vivo administration of anti-CD3 mAb or by α-galactosylceramide (α-GalCer),* a glycolipid originally purified from a marine sponge (17, 18). The administration of α-GalCer, a ligand of NKT cells, to mice immunized with an antigen has been shown to influence the process of Th1/Th2 development. One study has provided evidence that coadministration of α-GalCer with an antigen shifts the antigen-specific T cell response toward Th1 cytokine production (19), whereas other studies support Th2 polarization by α-GalCer (20, 21). Furthermore, it has been suggested that the activation of NKT cells by α-GalCer may contribute to the generation of tumor-specific cytotoxic T cells (22), as well as induction of Th1 cells responsive to cryptococcal antigens (23). Finally, the administration of α-GalCer to mice rapidly activates NK cells (24, 25), and increases the expression of activation markers on T cells (26). Taken together, these results have led to a hypothesis that the NKT cell ligand α-GalCer could be used as an adjuvant to modulate and/or augment protective immune responses elicited by vaccines.

Materials and Methods

Parasites and Their Use for Immunization. *Plasmodium yoelii* (17X NL strain) sporozoites were obtained by dissecting the mosquito salivary glands as described (27). For immunization, sporozoites were radiation-attenuated by exposing them to 12,000 rad, and then injected intravenously into the tail vein or subcutaneously into the base of the tail of the mice. 10^4 or 10^5 irradiated sporozoites (γ-spz) were used to immunize mice for protection assay or an enzyme-linked immunospot (ELISPOT) assay, respectively.

Immunization with Recombinant Viruses. A suboptimal dose (10^7 PFU) of recombinant adenovirus expressing the entire *P. yoelii* circumsporozoite (CS) protein, AdPyCS (13), was used to immunize mice. The recombinant Sindbis virus expressing the $CD8^+$ T cell epitope (SYVPSAEQI) of *P. yoelii* CS protein, SIN(Mal), and the recombinant Sinbis virus expressing the $CD8^+$ T cell epitope (RGPGRAFVTI) of HIV p18 protein, SIN(p18), were constructed as described (14, 28), and 10^5 PFU of the viruses were inoculated subcutaneously, as a suboptimal dose.

Mice. BALB/c and B10.D2 mice were purchased from The Jackson Laboratory and maintained under standard conditions in our Departmental Animal Facility. Vα14 NKT-deficient mice ($J\alpha281^{-/-}$) were established by specific deletion of the Jα281 gene segment with homologous recombination and aggregation chimera techniques (27) and used after 3–4 backcrosses to BALB/c. CD1d-deficient mice ($CD1d^{-/-}$) were generated from embryonic stem cells of 129 origin and used after 7–8 backcrosses to BALB/c (27). IFN-γ receptor-deficient mice (IFN-γ $R^{-/-}$) were originally provided by Dr. Michel Aguet at Swiss Institute for Experimental Cancer Research (Epalinges, Switzerland), and used after three backcrosses to B10.D2 (29). Mice of either sex were used at 6–8 wk.

α-GalCer. α-GalCer, [(2S,3S,4R)-1-O-(α-D-galactopyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol], was synthesized by Kirin Brewery (17). The original solution was dissolved with 0.5% polysorbate-20 (Nikko Chemical) in 0.9% NaCl solution and diluted with PBS just before use.

Quantification of P. yoelii rRNA in the Liver of Sporozoite-inoculated Mice by Real-time PCR. Total liver RNA was isolated by the method of Chomczynski and Sacchi from mice killed 42 h after intravenous injection with 10^4 *P. yoelii* sporozoites. After reverse transcription of the extracted RNA, cDNA was generated and its amount analyzed by real-time PCR, using the ABI Prism 5700 Sequence Detection system (PE Biosystems; reference 30). Primers and fluorogenic probe with the following sequences were custom designed using the ABI Prism primer Express software (PE Biosystems), based on *P. yoelii* (17XNL) 18S rRNA sequence (30). The primers, 5′-GGGGATTGGTTTTGACGTTTTTG-CG-3′ (forward primer), and 5′-AAGCATTAAATAAAGCGA-ATACATCCTTAT-3′ (reverse primer), were obtained from Operon Technologies Inc. The specific fluorogenic probe, PyNYU, 5′-FAM-CAATTGGTTTACCTTTTGCTCTTT-TAMRA-3′, was obtained from PE Biosystems, and was generated with 5-propyne-2′-deoxyuridine (turbo Taqman probe) to achieve a proper Tm. The reaction mix contained 5 μl of 10× Taqman buffer A (PE Biosystems), 3.5 mM $MgCl_2$, 200 μM dNTP, 0.3 μM forward primer, 0.3 μM reverse primer, 50 nM turbo Taqman probe PyNYU, 1.25 U AmpliTaq Gold DNA polymerase, and water up to 50 μl final reaction volume. The temperature profile included 95°C for 10 min and 35 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. The precise amount of parasite-derived 18S cDNA molecules detected in this assay was determined by linear regression analysis using C_T values obtained from both liver samples and those obtained from a standard curve generated with known amounts of plasmid 18S cDNA.

Quantification of Epitope-specific $CD4^+$ and $CD8^+$ T Cells by ELISPOT Assay. After coating 96-well nitrocellulose plates (Millipore) with anti–mouse IFN-γ mAb, or anti–mouse IL-4 mAb overnight at room temperature, the wells were washed repeatedly and blocked with culture medium for 1 h at 37°C. The MHC-compatible target cells, A20.2J B cell lymphoma, expressing both MHC class I and II $H\text{-}2^d$ molecules, were incubated for 1 h at 37°C with the synthetic peptide representing the $CD4^+$ T cell epitope (YNRNIVNRLLGDALNGKPEEK) or $CD8^+$ T cell epitope (SYVPSAEQI) of the *P. yoelii* CS protein, or $CD8^+$ T cell epitope (RGPGRAFVTI) of the HIV p18 protein. After irradiating the peptide-pulsed target cells, the cells were washed and then added to the ELISPOT wells. Untreated target cells were used as negative controls. Serially diluted lymphocytes isolated from the spleen or lymph nodes of immunized mice were cocultured with 1.5×10^5 target cells in the ELISPOT wells. After incubating the plates 24 h for IFN-γ detection or 48 h for IL-4 detection at 37°C and 5% CO_2, the plates were treated as previously described (13), and the number of spots corresponding to IFN-γ and IL-4 secreting cells determined.

Quantification of α-GalCer–specific Cells by ELISPOT Assay. The relative numbers of IFN-γ and/or IL-4 producing α-GalCer–specific lymphocytes were determined using an ELISPOT assay. Lymphocytes were isolated from the liver of wild-type and

*Abbreviations used in this paper: α-GalCer, α-galactosylceramide; CS, circumsporozoite; ELISPOT, enzyme-linked immunospot; γ-spz, irradiated sporozoite.

IFN-γ R–deficient mice, as described (13). After 12 h incubation with 100 ng/ml of α-GalCer or vehicle at a cell density of 10^7 cells/ml, serially diluted lymphocytes, starting at 10^6 cells per well, were placed into ELISPOT wells coated with corresponding anti-cytokine antibodies. After incubating the plates for 24 h at 37°C and 5% CO_2, the plates were developed as described (13).

Flow Cytometric Analysis Using CD1d/α-GalCer Tetramers. α-GalCer–specific lymphocytes were identified using CD1d/α-GalCer tetrameric complexes, consisting of CD1d molecules and α-GalCer, as described previously (31). Freshly isolated hepatic lymphocytes were incubated first with phycoerythrin (PE)-labeled tetrameric complexes, followed by a second incubation with FITC-labeled anti-CD3 monoclonal antibody. The cells were then analyzed by a FACSCalibur™ instrument (Becton Dickinson) using CELLQuest™ software (Becton Dickinson).

Statistical Analysis. Student's *t* test was used for all comparisons. Only *P* values below 0.01 were considered significant. Data are presented as mean values ± SD.

Results

α-GalCer Enhances Protective Anti-malaria Immunity. To learn whether protective anti-malaria immune response induced by immunization with a suboptimal dose of irradiated sporozoites could be enhanced by α-GalCer, we immunized BALB/c mice intravenously with a suboptimal dose (10^4) of γ-spz together with different doses of α-GalCer (0.5, 1, 2 μg). 2 wk later, we challenged different groups of mice with 10^4 live *P. yoelii* sporozoites, and measured the levels of protective anti-malaria immunity by determining the amount of parasite-specific rRNA in the liver with a highly sensitive real-time PCR (30). We found that administration of α-GalCer significantly enhanced, in a dose-dependent manner, the level of protective immunity (percent inhibition of liver stage development) elicited by immunization with γ-spz (Fig. 1 A). The parasite load in the livers of γ-spz–immunized mice administered with 2 μg of α-GalCer was 10 times smaller than that in the livers of mice immunized with γ-spz alone.

We also determined the titers of anti-sporozoite antibodies using an immunofluorescence assay (IFA) of air-dried *P. yoelii* sporozoites, as well as the titers of antibody against the CS protein, the major surface antigen of sporozoites, using ELISA. The antibody titers were identical among the groups of γ-spz–immunized mice regardless of whether or not they received α-GalCer (Fig. 1 A). Furthermore, we determined the immunoglobulin isotype of the anti-CS antibodies. No significant differences in IgE, IgG_1, IG_{2a} or IgM isotype profiles of anti-CS antibodies were detected between α-GalCer–treated and untreated mice (data not shown). These results indicate that the anti-malarial humoral response is not affected by α-GalCer treatment.

We then examined the kinetics of this adjuvant activity displayed by α-GalCer by administering 2 μg of the glycolipid to BALB/c mice on the same day, 2 d before or 2 d after intravenous immunization with 10^4 γ-spz. The highest level of protective anti-malaria immunity was elicited when we administered α-GalCer on the same day as γ-spz (Fig. 1 B). Administration of α-GalCer 2 d after γ-spz immunization did not significantly enhance the level of protective immunity induced by vaccination. Interestingly, when we administered α-GalCer 2 d before γ-spz immunization, protective immunity was completely abolished. It is possible that α-GalCer, administered 2 d earlier, might

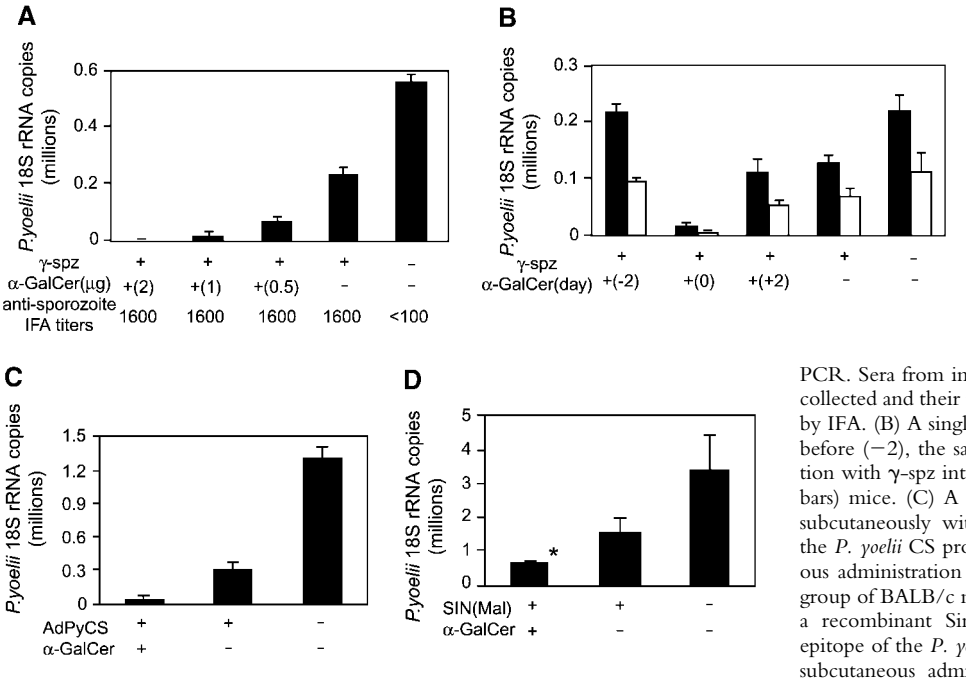

Figure 1. α-GalCer enhances protective anti-malaria immunity induced by irradiated sporozoites and recombinant viruses expressing a plasmodial antigen. (A) Groups of BALB/c mice were coinjected intraperitoneally with different doses of α-GalCer (0.5, 1, or 2 μg) or vehicle (−), together with intravenous immunization with *P. yoelii* irradiated sporozoites (γ-spz). 2 wk later all groups of mice were challenged with infective sporozoites, and the amount of parasite ribosomal RNA in the livers was measured by real-time PCR. Sera from immunized and nonimmunized mice were collected and their titers of anti-sporozoite antibodies assayed by IFA. (B) A single dose of α-GalCer was administered 2 d before (−2), the same day (0) or 2 d after (+2) immunization with γ-spz into BALB/c (black bars) or B10.D2 (white bars) mice. (C) A group of BALB/c mice was immunized subcutaneously with a recombinant adenovirus expressing the *P. yoelii* CS protein, AdPyCS, together with subcutaneous administration of α-GalCer (+) or vehicle (−). (D) A group of BALB/c mice was immunized subcutaneously with a recombinant Sindbis virus expressing a $CD8^+$ T cell epitope of the *P. yoelii* CS protein, SIN(Mal), together with subcutaneous administration of α-GalCer (+) or vehicle (−). Asterisk (*) indicates a significant ($P < 0.01$) difference between the two values using an unpaired *t* test. In B–D, all groups of mice were infected with live *P. yoelii* sporozoites 2 wk later, and the parasite burden in the liver was determined, as described in panel A. Results are expressed as the mean values ± SD of five mice.

have eliminated the sporozoites before they could be processed and presented by antigen-presenting cells, thereby preventing the induction of a malaria-specific immune response. We have previously shown that α-GalCer administered 2 d before challenge with live sporozoites completely eliminates the parasites from the liver in a manner dependent on NKT cells and IFN-γ (27). Similar kinetics of the adjuvant activity of α-GalCer were observed in B10.D2 mice (Fig. 1 B).

To determine whether or not α-GalCer's enhancement of the protective immune response against malaria was a particular phenomenon related to γ-spz immunization, or a more general phenomenon independent of the immunogen administered, we administered α-GalCer to BALB/c mice on the same day as subcutaneous immunization with a suboptimal dose of recombinant adenovirus expressing the whole *P. yoelii* CS protein, AdPyCS (13), or recombinant sindbis virus expressing the $CD8^+$ T cell epitope of the CS protein, SIN(Mal) (14). As shown in Fig. 1, C and D, α-GalCer significantly enhances the protective immune response induced by immunization with a suboptimal dose of the two different recombinant viruses. In the case of AdPyCS, the protection was augmented almost 10 times to that of control, and in the case of SIN(Mal), the protection after coadministration with α-GalCer was enhanced 3 times.

To further assess the adjuvant activity of α-GalCer coadministered with vaccines, we monitored parasitemia, i.e., the presence of parasites in the blood, daily by microscopic examination of thin blood smears. Briefly, we immunized BALB/c mice either intravenously with 10^4 γ-spz or subcutaneously with 10^7 PFU of AdPyCS, doses which otherwise fail to confer protection against malaria, with or without α-GalCer treatment. 2 wk later, we challenged all mice with 50 viable *P. yoelii* sporozoites, and determined the occurrence of blood infection by monitoring parasitemia. We found that 28 out of 30 α-GalCer–treated, γ-spz–immunized mice were protected, while most of the α-GalCer–untreated, γ-spz–immunized mice developed malaria infection (Table I). Similarly, administration of α-GalCer together with AdPyCS strongly enhanced the protective effect induced by a suboptimal dose of the virus. On the other hand, administration of α-GalCer alone failed to protect the challenged mice. Overall, these results corroborate the liver stage data (Fig. 1), and together indicate that α-GalCer administration increases the efficacy of a suboptimal immunizing dose of both γ-spz and recombinant viruses, revealing a profound adjuvant effect.

α-GalCer Enhances T Cell Responses Elicited by Various Vaccines. To determine which components of the malaria-specific T cell response, i.e., $CD4^+$ and/or $CD8^+$ T cells, are enhanced by coinjection of α-GalCer with γ-spz, we compared these immune parameters in γ-spz–immunized mice treated with or without the glycolipid. For this purpose, we immunized BALB/c mice with 10^5 γ-spz, together with vehicle or α-GalCer. 2 or 6 wk later, we isolated splenic lymphocytes and determined the numbers of CS-specific, IFN-γ– and IL-4–secreting $CD8^+$ and $CD4^+$ T cells by an ELISPOT assay (13). As shown in Fig. 2 A,

Table I. *α-GalCer Enhances Protective Immunity Induced by Malaria Immunogens*

Immunogen	No. of mice protected /no. challenged	Percent protection (no parasitemia)
γ-spz[a]	6/30	20
γ-spz + α-GalCer	**28/30**	**93**
AdPγCS[a]	2/30	7
AdPγCS + α-GalCer	**24/30**	**80**
α-GalCer	0/30	0
None	0/30	0

[a]BALB/c mice were immunized either intravenously with 10^4 γ-spz or subcutaneously with 10^7 PFU of AdPγCS.

α-GalCer treatment strikingly enhanced the level of CS-specific T cell responses elicited by γ-spz at 2 wk after immunization. Specifically, α-GalCer increased the number of IFN-γ–secreting CS-specific $CD8^+$ T cells approximately sevenfold compared with that induced by γ-spz immunization alone. The number of IFN-γ secreting CS-specific $CD4^+$ T cells was also significantly increased, albeit to a lesser degree. More importantly, the administration of α-GalCer not only enhanced the level of CS-specific $CD8^+$ T cell response but also prolonged the duration of the response (Fig. 2 A). We did not observe this strong enhancement of the T cell responses by α-GalCer treatment when we administered α-GalCer 2 d prior or 2 d after γ-spz immunization (data not shown). We found no difference in the numbers of CS-specific $CD4^+$ or $CD8^+$ T cells secreting IL-4 (data not shown), indicating that α-GalCer treatment primarily enhances antigen-specific Th1-type responses in our experimental system. Because we obtained similar results in both BALB/c and B10.D2 mice, we conclude that the adjuvant effect of α-GalCer is not influenced by the different genetic backgrounds of these mice (data not shown).

To determine whether α-GalCer also enhances CS-specific T cell responses upon immunization with recombinant viruses, we administered α-GalCer to BALB/c mice at the same time as subcutaneous immunization with a suboptimal dose of AdPyCS. 10 d later, we obtained splenocytes from these mice and determined the number of CS-specific T cells secreting IFN-γ or IL-4 by an ELISPOT assay. The number of both CS-specific $CD4^+$ and $CD8^+$ T cells secreting IFN-γ elicited in α-GalCer–treated, AdPyCS-immunized mice was more than 10-fold higher than that of T cells from a group of mice immunized with the virus alone (Fig. 2 B). When we used SIN(Mal), we found that α-GalCer treatment also significantly increased the number of CS-specific $CD8^+$ T cells secreting IFN-γ (Fig. 2 C). We next determined if the adjuvant activity of α-GalCer is a phenomenon related specifically to the H-$2K^d$–restricted $CD8^+$ T cell epitope of the CS, or can be applied to different epitopes. For this purpose we immunized BALB/c mice with a recombinant

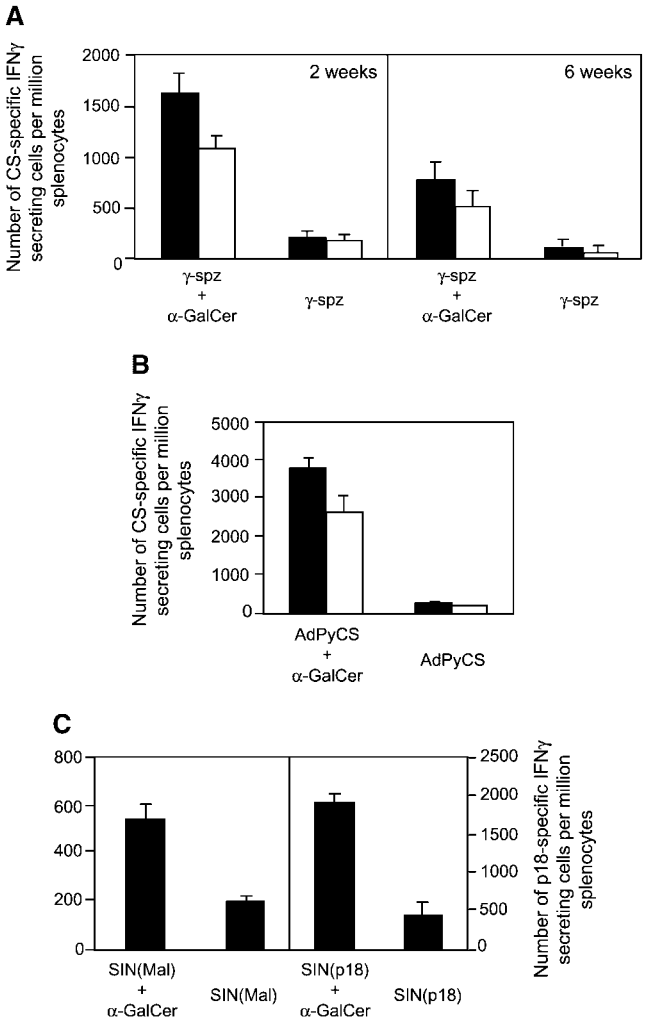

Figure 2. α-GalCer increases the level of antigen-specific T cell responses elicited by various vaccines. (A) A group of BALB/c mice was immunized subcutaneously with γ-spz together with or without administration of α-GalCer by the same route, and 2 or 6 wk later splenic lymphocytes were isolated and the number of IFN-γ–secreting CS-specific $CD8^+$ (black bars) and $CD4^+$ (white) T cells was determined by an ELISPOT assay. (B) A group of BALB/c mice was immunized subcutaneously with AdPyCS together with or without subcutaneous administration of α-GalCer (+) or vehicle (−). 2 wk later the number of IFN-γ secreting CS-specific $CD8^+$ (black bars) and $CD4^+$ (white bars) T cells was determined by an ELISPOT assay. (C) A group of BALB/c mice was immunized subcutaneously with SIN(Mal) or SIN(p18) together with or without subcutaneous administration of α-GalCer. 2 wk later the number of IFN-γ secreting CS-specific and p18-specific $CD8^+$ T cells was determined by an ELISPOT assay. The data represent one of two experiments with similar results and are expressed as the mean values ± SD of three mice.

Sindbis virus expressing a H-$2D^d$–restricted $CD8^+$ T cell epitope from the p18 protein of HIV (28). α-GalCer coadministration increased the number of p18-specific IFN-γ–secreting $CD8^+$ T cells induced by immunization with SIN(p18) fourfold (Fig. 2 C). Taken together these results indicate that the enhancement of the cellular immune response by treatment with α-GalCer is independent of the antigen delivery system (attenuated pathogen or recombinant virus) and the epitope.

The Adjuvant Activity of α-GalCer Requires CD1d Molecules, Vα14 NKT Cells, and IFN-γ. Next, we investigated the cellular mechanism underlying α-GalCer's adjuvant activity, using mice lacking CD1d molecules, and those deficient in T cells expressing the canonical NKT cell receptor. Briefly, we immunized these knockout mice, along with wild-type controls, with a suboptimal dose of γ-spz with or without α-GalCer treatment. 2 wk later, we challenged these immunized mice, as well as nonimmunized controls, with live sporozoites, and determined the levels of protective anti-malaria immunity. Administration of α-GalCer, which increased the level of γ-spz–induced protective immunity in wild-type mice, failed to enhance protective immunity in CD1d-deficient mice, as well as in Jα281-deficient mice, which lack Vα14 NKT cells (Fig. 3 A). These results indicate that the adjuvant activity of α-GalCer is dependent on both CD1d molecules and Vα14 NKT cells.

To further demonstrate the importance of CD1d molecules and Vα14 NKT cells for the adjuvant activity of α-GalCer, we measured the number of CS-specific $CD8^+$ T cells in γ-spz-immunized, α-GalCer–treated or untreated mice deficient in either CD1d or Vα14 NKT cells. As shown in Fig. 3 B, α-GalCer treatment failed to increase the number of CS-specific $CD8^+$ T cells induced by γ-spz immunization in CD1d-deficient mice compared with that of untreated mice, indicating that α-GalCer requires CD1d to enhance the CS-specific $CD8^+$ T cell response. Interestingly, in γ-spz–immunized and α-GalCer–treated, Jα281-deficient mice, the number of CS-specific $CD8^+$ T cells was slightly but significantly increased compared with that of untreated mice (Fig. 3 B). However, this increase did not reach the level of α-GalCer–treated, γ-spz–immunized wild-type mice (Fig. 3 B), and did not enhance the level of protective anti-malaria immunity (Fig. 3 A). These findings, therefore, demonstrate the importance of Vα14 NKT cells in mediating the adjuvant effect of α-GalCer.

Lastly, in order to gain insight into the molecular mechanism underlying α-GalCer's adjuvant activity, we immunized mice lacking the IFN-γ receptor (IFN-γ $R^{-/-}$) with γ-spz with or without α-GalCer cotreatment, and 10 d later, we analyzed the numbers of CS-specific IFN-γ-secreting $CD8^+$ and $CD4^+$ T cells using an ELISPOT assay. α-GalCer coadministration failed to augment the number of CS-specific IFN-γ–secreting $CD8^+$ and $CD4^+$ T cells in the γ-spz–immunized knockout mice (Fig. 4 A). It has been reported that mice deficient in different molecules such as GM-CSF receptor β-chain (32) and Fas (33) are also partially deficient in NKT cells. To exclude the possibility that the absence of the IFN-γ receptor results in a decreased number and/or defective function of NKT cells, we analyzed the presence and the function of NKT cells in these IFN-γ $R^{-/-}$ mice by CD1d/α-GalCer tetramer staining and ELISPOT assay. Flow cytometric analysis using CD1d/α-GalCer tetramers revealed that the percentage of α-GalCer–specific NKT cells among hepatic lymphocytes in IFN-γ $R^{-/-}$ mice is similar to that in wild-type mice (Fig. 4 B). In addition, the number of α-GalCer–specific cells secreting IFN-γ in the liver (Fig. 4 C) and spleen

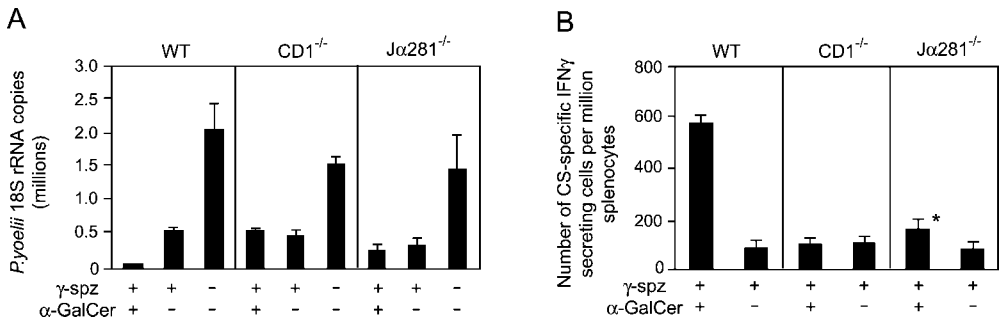

Figure 3. The adjuvant activity of α-GalCer requires CD1d molecules and Vα14 NKT cells. (A) Groups of CD1d-deficient ($CD1^{-/-}$), Vα14 NKT ($J\alpha281^{-/-}$) deficient and wild-type (WT) mice on a BALB/c background were immunized intravenously with γ-spz together with intraperitoneal administration of α-GalCer (+) or vehicle (−). 2 wk later these and nonimmunized mice were challenged with viable sporozoites, and the parasite burden in the liver was measured as described in Fig. 1. (B) Identical groups of mice as described in panel A were immunized with γ-spz with intraperitoneal injection of α-GalCer (+) or vehicle (−). 2 wk later the number of IFN-γ secreting CS-specific $CD8^+$ T cells in the spleens was determined by an ELISPOT assay. Asterisk (*) indicates a significant ($P < 0.01$) difference between the two values using an unpaired *t* test. The results reflect two experiments with similar results and are expressed as the mean values ± SD of five (A) or three (B) mice.

(data not shown) of wild-type and IFN-γ $R^{-/-}$ mice is similar, eliminating the possibility that the lack of adjuvant activity was due to a defect in the NKT cell population. Collectively, these results indicate that α-GalCer's adjuvant activity is dependent on IFN-γ production.

Discussion

Our current study addresses the ability of the NKT cell ligand α-GalCer to modulate acquired anti-malaria immunity. We found that α-GalCer administered to mice immunized with a suboptimal dose of irradiated *P. yoelii* sporozoites or with suboptimal doses of recombinant viruses expressing malarial antigens greatly enhances protective anti-malaria immunity.

The main immune components affected by α-GalCer administration are malaria-specific $CD8^+$ and $CD4^+$ T cells that secrete IFN-γ. The levels of the humoral response and the Th2 response are unaltered by this treatment. Administration of α-GalCer increased the number of IFN-γ–secreting CS-specific $CD8^+$ and $CD4^+$ T cells induced by γ-spz immunization approximately sevenfold and fivefold, respectively. Furthermore, the level of CS-specific T cell responses remains much higher at 6 wk after γ-spz immunization in α-GalCer–treated mice compared with that in nontreated mice. As protective immunity against the liver stages of malaria is primarily mediated by $CD8^+$ T cells, as well as $CD4^+$ T cells, and requires production of IFN-γ (8, 9, 34), it is not surprising that the level of anti-malaria protection was increased by α-GalCer treatment. This adjuvant effect of α-GalCer was also observed in α-GalCer–treated mice immunized with recombinant viruses expressing either the *P. yoelii* CS protein or the H-$2K^d$–restricted $CD8^+$ T cell epitope of this protein. These results confirm and extend the data obtained by γ-spz immunization, indi-

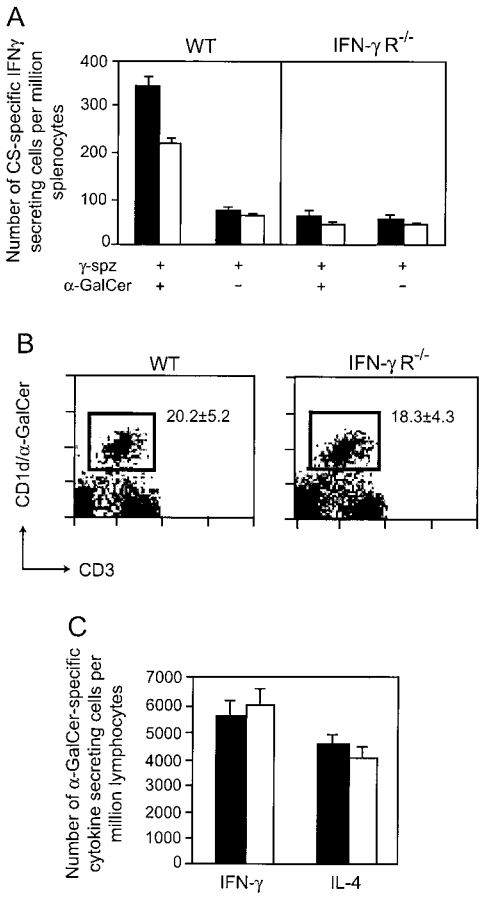

Figure 4. The adjuvant activity of α-GalCer is abolished in IFN-γ receptor-deficient mice. (A) Groups of IFN-γ receptor-deficient (IFN-γ $R^{-/-}$) and wild-type (WT) mice on a B10.D2 background were immunized intravenously with γ-spz together with intraperitoneal administration of α-GalCer (+) or vehicle (−). 2 wk later splenic lymphocytes were obtained and the number of IFN-γ secreting CS-specific $CD8^+$ (black bars) and CD4+ (white bars) T cells were determined by an ELISPOT assay. (B) Hepatic lymphocytes were obtained from IFN-γ $R^{-/-}$ and wild-type mice and stained with PE-labeled CD1d/α-GalCer tetramer and FITC-labeled anti-CD3 antibody, and the percentage of α-GalCer–specific T cells was determined by flow cytometric analysis. The number indicated in the upper right corners represents the percentage of double-positive cells among the liver lymphoid cell population. (C) Hepatic lymphocytes were obtained from IFN-γ $R^{-/-}$ (black bars) or WT (white bars) mice, and the number of IFN-γ or IL-4 secreting α-GalCer–specific cells were determined by an ELISPOT assay. Results are expressed as the mean values ± SD of five mice.

cating that malaria-specific $CD8^+$ and $CD4^+$ T cell responses are enhanced by α-GalCer administration regardless of the type of immunogen used, parasite or recombinant virus. We also demonstrated that the $CD8^+$ T cell response enhanced by α-GalCer administration is independent of the $CD8^+$ T cell epitope used, as the immune response induced by a recombinant Sindbis virus expressing a H-$2D^d$–restricted T cell epitope of HIV was also enhanced.

α-GalCer's ability to augment the level of protective anti-malaria immunity induced by γ-spz immunization requires both CD1d molecules and Vα14 NKT cells. Without these components, α-GalCer was unable to increase the protection elicited by a suboptimal dose of the immunogen. Although both CD1d molecules and Vα14 NKT cells were needed for α-GalCer's ability to augment protective anti-malaria immunity, we detected a noticeable increase in the number of CS-specific $CD8^+$ T cells in Jα281-deficient mice after α-GalCer treatment. It is possible that the high degree of genetic heterogeneity in these mice affects the T cell response and causes this moderate increase. Alternatively, CD1d-reactive, non-Vα14 NKT cells may exist in Jα281–deficient mice. Further investigation is required to explain this result.

While the precise molecular mechanism of the adjuvant effect of α-GalCer remains to be fully clarified, our finding that these activities of α-GalCer are eliminated in mice lacking IFN-γ receptor indicates that IFN-γ is important in mediating the adjuvant effect of α-GalCer. Studies by a number of different investigators clearly show that IFN-γ is secreted by both NKT and NK cells after α-GalCer treatment (22, 24, 25). It is possible that IFN-γ secreted by NKT and/or NK cells acts on antigen-presenting cells, by up-regulating the MHC class I processing machinery, e.g., TAP, proteasome subunits, and class I heavy chains. Alternatively, IFN-γ may enhance the acquired cell-mediated immune response by directly acting on antigen-specific $CD8^+$ T cells. Nevertheless, the exact role of IFN-γ and other critical molecules requires further investigation.

Our kinetic studies show that α-GalCer displays a maximal adjuvant effect only when the glycolipid is coadministered with γ-spz. Administration of α-GalCer 2 d before or after immunization with γ-spz fails to elicit adjuvant activity. A recent study on the in vivo kinetics of NKT cells after α-GalCer administration using CD1d/α-GalCer tetramers indicates that murine NKT cells, especially those in the liver where they constitute 20–30% of the lymphocyte population, are promptly activated, secrete large amounts of IFN-γ and IL-4, and readily disappear 5 h after stimulation. Interestingly, this acute disappearance of α-GalCer–activated NKT cells was also confirmed by phenotypic analysis of the peripheral blood of cancer patients treated with α-GalCer (unpublished data).

As previously shown by various investigators, NKT cell activation not only causes activation of NK cells but also proliferation of memory $CD4^+$ and $CD8^+$ T cells (26), or induction of the early activation marker CD69 on the surface of T cells and B cells (22), suggesting a role for activated NKT cells in initiating T cell and B cell responses. It is pertinent to note that a recent study has shown that administration of α-GalCer to mice immunized with a T cell lymphoma enhances the generation of tumor-specific cytotoxic T cells (22). In this regard, our study indicates for the first time that α-GalCer–activated NKT cells play a role in the induction of "protective immunity" where specific $CD8^+$ T cells are the primary effectors.

In conclusion, by showing that α-GalCer–activated NKT cells enhance protective anti-malaria immune responses, our studies present evidence for a role of NKT cells in bridging innate and adaptive immunity. Our current findings on the adjuvant activity of α-GalCer might be applicable to various other intracellular microbial pathogens in addition to malaria, as well as to tumor models. Finally, as it has been demonstrated that α-GalCer can also stimulate human NKT cells (35, 36), these findings are expected to contribute to the understanding of the role of human NKT cells, and the design of novel, more effective vaccines.

We thank Ruth S. Nussenzweig for her continuing support. We also thank Sandy Nosseir, Ivette Caro, and Wendy Chen for their technical assistance.

This work was supported by research grants CA-52511 (M. Kronenberg), AI-33314 (C.C. Bergmann), AI-01682 (M. Tsuji), AI-40656 (M. Tsuji), and AI-47840 (M. Tsuji) from the National Institutes of Health.

Submitted: 14 November 2001
Revised: 21 December 2001
Accepted: 22 January 2002

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