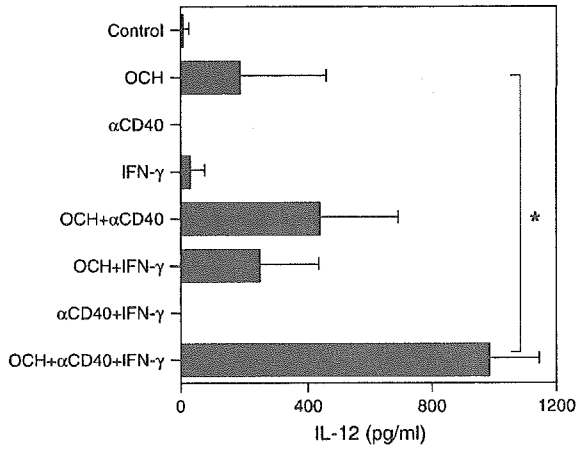

Fig. 5. Co-administration of IFN-γ and stimulatory anti-CD40 mAb augments IL-12 production after OCH administration *in vivo*. B6 mice were treated intra-peritoneally with 2 μg per mouse of glycolipids in combination with murine IFN-γ (1 μg per mouse) and/or stimulatory anti-CD40 mAb (100 μg per mouse) and serum samples were collected 2 h after treatment. The level of IL-12 production was determined by ELISA. These data represent one of two experiments with similar results. $*P < 0.05$.

mouse of CpG ODN plus OCH) and induced moderate IL-4 production (2.6-fold induction with 10 μg per mouse of CpG ODN plus OCH and 2.1-fold induction with 100 μg per mouse of CpG ODN plus OCH). Accordingly, co-administration of OCH and 10 μg per mouse of CpG ODN exhibited strong induction of IL-12 production (Fig. 7B, left panel), suggesting the synergic effect of OCH and CpG ODN for preferential up-regulation of IL-12. These results suggested that the PAMPs could be a considerable determinant for the cytokine profile following *in vivo* administration of OCH through regulating the availability of pro-inflammatory cytokines such as IL-12.

Discussion

In this study, we clarified the effect of OCH on bystander cell activation including the sequential IFN-γ production by NK cells and the functional conditioning of DCs. *In vivo* administration of OCH induced much lower IFN-γ production from both NKT and NK cells compared with that induced by αGC administration. NKT cell-derived IFN-γ was partially involved in inducing IFN-γ production by NK cells after αGC administration, implying that an IFN-γ-independent mechanism is also important for indirect up-regulation of IFN-γ production by NK cells after αGC administration *in vivo*. OCH administration induced lower CD40L expression by NKT cells compared with αGC administration, resulting in the lower production of IL-12 by DCs. Co-injection of stimulatory CD40 mAb and IFN-γ with OCH augmented the OCH-induced IL-12 production. Likewise, co-injection of IL-12 with OCH enhanced the production of IFN-γ by OCH administration alone. Furthermore, administration of OCH and CpG ODN into mice selectively induced IFN-γ production *in vivo*.

Consistent with the previous reports (9, 29), we here demonstrated that OCH administration induced less amount of IFN-γ than that of αGC in iNKT cells. Supporting these observation is another report in which truncation of the phytosphingosine lipid chain of αGC increases the relative amounts of IL-4 release by human NKT cells (30).

The functional relevance between NKT cells and NK cells was demonstrated in which NK-sensitive tumor incidence was higher and the time of tumor development was earlier in NKT cell-deficient mice compared with B6 mice (31). Considering that NKT cell-deficient mice still possess NK cells (32), NKT cells might serve as a modulator of NK cell function in tumor immunity, though the molecular mechanisms of how NKT cells modulate NK cells has not been clarified yet. Recently, β-anomeric galactosylceramide has been reported to have a capacity to reduce numbers of NKT cells without inducing typical NK cell-mediated responses (29, 33). We demonstrated in this study that OCH-induced IFN-γ production by NK cells was lower compared with that induced by αGC. This is at least partly due to the lower induction of IFN-γ by OCH-stimulated NKT cells and the lower induction of IL-12 by DCs, leading to weak activation of NK cells. There is a report showing that OCH and αGC can induce comparable amount of IFN-γ by NK cells 8 and 24 h after stimulation (29), even though serum levels of IFN-γ induced by OCH treatment were significantly lower than that by αGC treatment 6 or 24 h after stimulation. Since the major producer of IFN-γ *in vivo* after treatment with glycolipids at the later time points were demonstrated to be NK cells (3, 10), it is not clear whether cells other than NKT cells or NK cells could be the IFN-γ producer after αGC stimulation in their experimental condition. Although the basis for the discrepancy is not clear, it may be related to the difference in the synthetic methods of those glycolipids. Nevertheless, we reproducibly confirmed the *in vivo* ameliorating effects of OCH in various autoimmune mouse models including EAE, CIA and inflammatory bowel disease (7, 8, 34) through the differential induction of various cytokines.

The CD40 pathway plays an intrinsic role in physiological conditions by eliciting IL-12 production by DCs (35, 36). However, cross-linking of CD40 alone has been shown to be incapable of inducing IL-12 production by DCs. Schulz *et al.* (23) has demonstrated that effective production of bioactive IL-12 by DCs through T cell activation should be initiated by innate signals such as microbial stimuli. Activated T cell-mediated IL-12 production by DCs through CD40 signaling requires another signal, for example, IFN-γ (24–26), which is also shown to be required for uncommitted immature DCs to develop the capacity to produce high levels of IL-12 upon subsequent contact with naive T cells (25). Consistent with the observation, IFN-γ enhances gene transcription encoding both the p40 and p35 components of IL-12, resulting in a particularly marked production of the heterodimeric IL-12 (37, 38). Intriguingly, αGC-induced expression of IL-12R on NKT cells requires the production of IFN-γ by NKT cells and the production of IL-12 by DCs (21). In addition, IL-12 itself has been shown to act directly on DCs to promote IL-12 production (39). αGC provides dual signals to DCs by up-regulating CD40L on NKT cells and by inducing IFN-γ production by NKT cells, resulting in a large amount of IL-12 production by DCs. Our reconstitution experiment clearly showed that signals through CD40 and IFN-γ provided by OCH lead to small

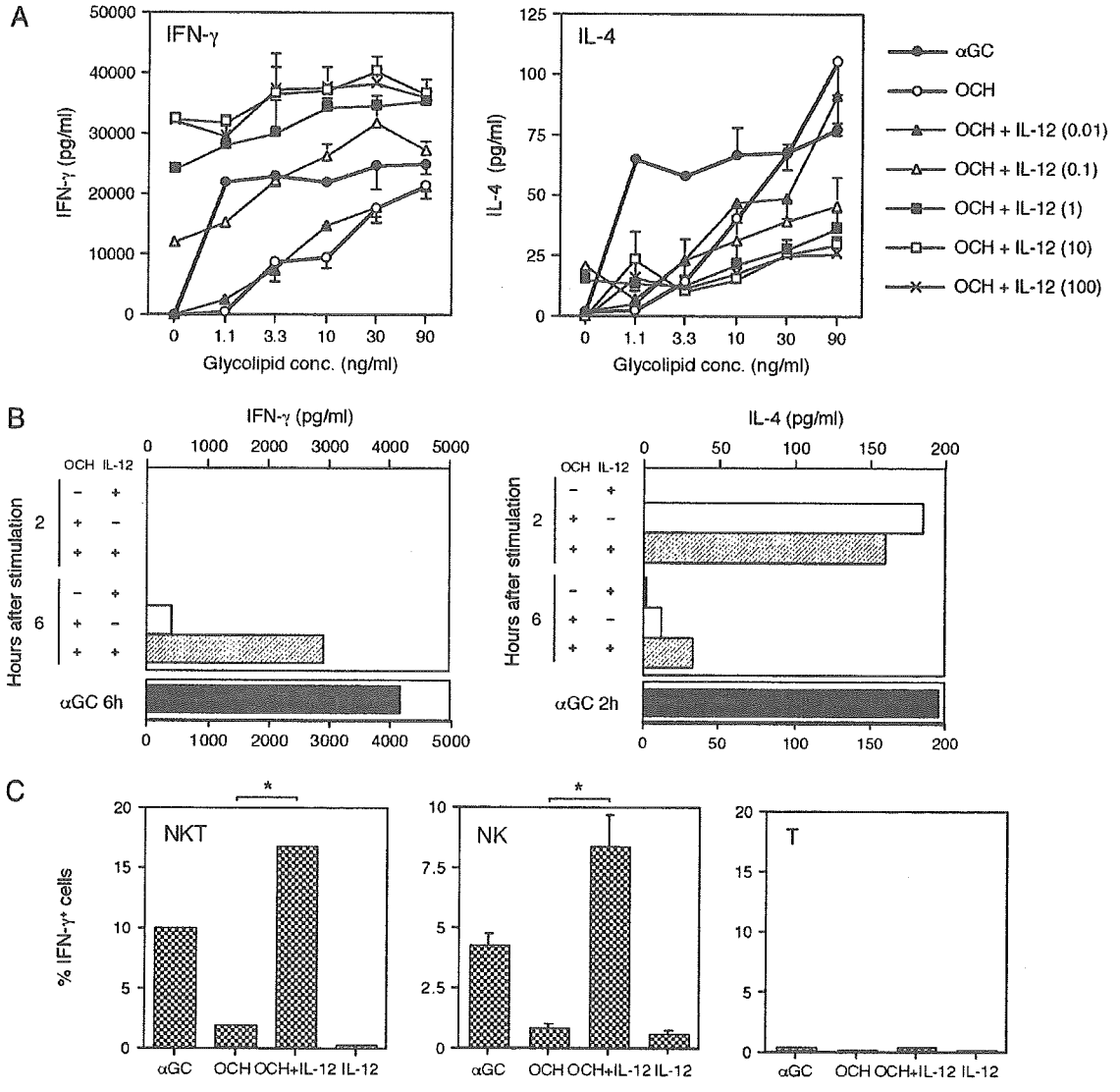

Fig. 6. Co-administration of IL-12 augments IFN-γ production by OCH. (A) Effects of IL-12 on cytokine production of splenocytes stimulated with glycolipids *in vitro*. Splenocytes were stimulated with various concentration of αGC or OCH in the presence or absence of IL-12 (with concentrations from 0.01 to 100 ng ml^{-1}) for 72 h and the levels of IFN-γ (left) or IL-4 (right) in the supernatants were measured by ELISA. Data are expressed as mean ± SD for triplicate wells. This figure represents one of two experiments with similar results. (B) Effects of IL-12 on cytokine production after glycolipid administration *in vivo*. B6 mice were treated with 10 ng per mouse of IL-12, 2 μg per mouse of OCH or OCH plus IL-12 and serum samples were collected at indicated times after injection. Serum levels of IFN-γ (left) and IL-4 (right) were determined by ELISA. This figure represents one of three experiments with similar results. (C) B6 mice were treated with 100 ng per mouse of IL-12 alone or in combination with 2 μg per mouse of OCH and spleen cells were harvested at various time points after glycolipid administration and subjected to intracellular cytokine staining as described in Methods. NKT cells, NK cells and T cells were analyzed for the presence of intracellular IFN-γ as described in Fig. 1. Similar results were obtained by analyzing liver mononuclear cells after glycolipid administration (data not shown). Data are expressed as mean ± SD for triplicate wells and represent one of two experiments with similar results. $*P < 0.05$.

amount of IL-12 production from DCs that is unable to trigger the IFN-γ burst by NKT cells and NK cells.

Treatment of mice with OCH together with sub-optimal doses of IL-12 resulted in significantly augmented IFN-γ production *in vivo*, indicating that the impaired IL-12 production by OCH is likely to be one of the major causes for less effective IFN-γ production *in vivo*. Similar observations were reported previously, in which treatment of mice with sub-optimal doses of αGC together with sub-optimal doses of IL-12 resulted in strongly enhanced natural killing activity and IFN-γ production (21). These results indicate an important role for DC-derived IL-12 for glycolipid-induced activation of NKT cells and suggest that NKT cells may be able to condition DCs for subsequent immune responses. To further clarify the

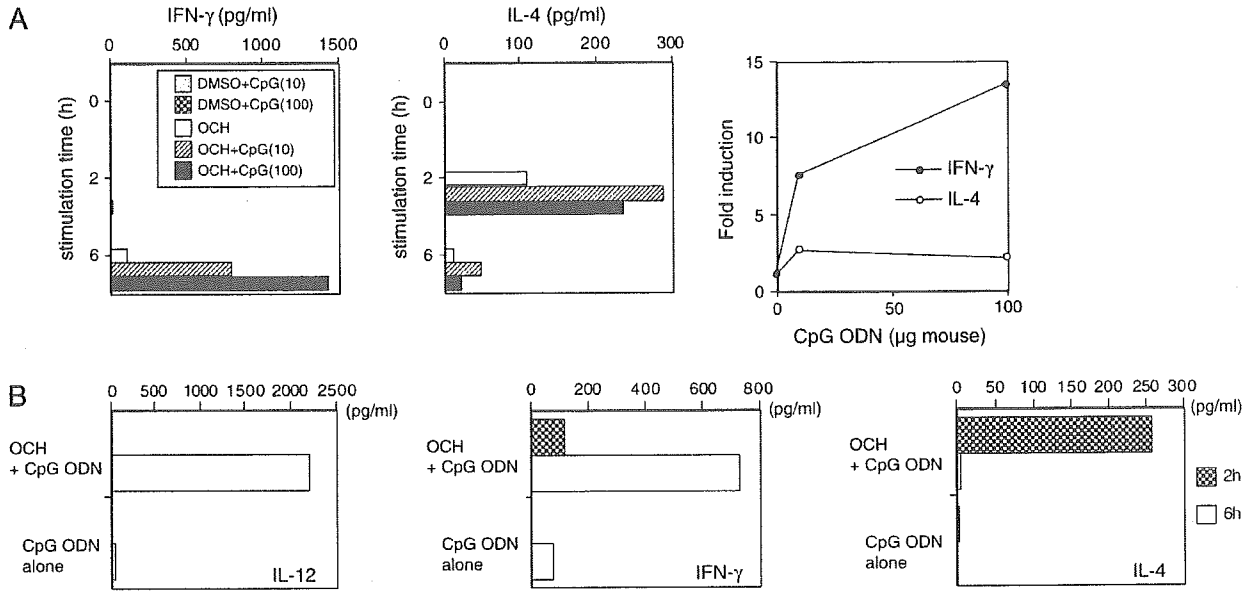

Fig. 7. Co-administration of CpG ODN augments IFN-γ production by OCH stimulation *in vivo*. (A) B6 mice were injected with 10 μg per mouse or 100 μg per mouse of CpG ODN alone or in combination with 2 μg per mouse of OCH and serum samples were collected at indicated times after injection. Serum levels of IFN-γ (left) or IL-4 (center) were determined by ELISA. The ratio of cytokine production was plotted in the right panel as fold induction for IFN-γ (at 6 h after injection) and IL-4 (at 2 h after injection). This figure represents one of two experiments with similar results. (B) B6 mice were injected with 10 μg per mouse of CpG ODN alone or in combination with 2 μg per mouse of OCH and serum samples were collected at indicated times after injection. Serum levels of IL-12 (left), IFN-γ (center) or IL-4 (right) were determined by ELISA. This figure represents one of three experiments with similar results.

cooperative roles of IL-12 for effective IFN-γ production by glycolipid-stimulated NKT cells, CpG ODN (27) was co-administered with OCH, in which IFN-γ production was preferentially augmented in response to IL-12 expression. CpG ODN induces innate immune responses similar to bacterial DNA, and is one of the PAMPs expressed by a diverse group of microorganisms. Taken together, a variety of glycolipid antigens elicit differential effects, not only on NKT cells but also on bystander cells such as NK cells and DCs, which may modulate subsequent immune responses. Recently, Brigl *et al.* demonstrated that a bacterial infection can induce a predominantly T_h1 cytokine responses from self-antigen-primed NKT cells. In this instance, microbial products were recognized not by NKT cells directly, but by DCs, resulting in IL-12 secretion and subsequent potent IFN-γ production (17). Following the exposure of immune cells to exogenous antigens or infection, IL-12 is produced by DCs in response to CD40 signals or microbial products, and co-stimulates the responses of NKT cells to self-antigens, resulting in a significant augmentation of IFN-γ production but no detectable IL-4 production (40). It is noteworthy to point out that the behavior of OCH in response to IL-12 is analogous to that of the putative self-antigen for NKT cells (Fig. 6). Therefore, NKT cells also respond to OCH in a diverse manner according to the availability of IL-12, which can be induced by a wide variety of pathogens, and thus OCH may be a useful tool to evaluate the physiological responses of NKT cells to various innate immune conditions.

Regarding the predominant effect of OCH on T_h2 polarization by NKT cells, several molecules have been identified that positively regulate T_h2 polarization, such as thymus-specific lymphopoietin (TSLP), OX40 ligand (OX40L) or prostaglandin (PG) E_2. In the microarray analysis of glycolipid-stimulated NKT cells and DCs, no inducible transcription of TSLP and OX40L in NKT cells was observed 1.5 or 12 h after OCH treatment. Furthermore, synthetic pathway for PGs seems quiescent because the expression of PG H synthetase (or cyclooxygenase 2), a key enzyme initiating PG synthesis, was not induced in either NKT cells or DCs after treatment with OCH. Considering that all of these molecules are regulated transcriptionally upon stimulation, the involvement of these molecules for OCH-mediated T_h2 polarization seems minimum. Taken together, the results demonstrated in this study suggest that OCH induces T_h2 predominance by a default pathway.

In summary, we have demonstrated here that OCH-mediated dominant T_h2 polarization is accomplished not only by the preferential IL-4 induction by NKT cells but also by the evasion of the secondary IFN-γ burst. This effect of OCH is due to the ineffective induction of IFN-γ and CD40L by NKT cells and the subsequent reduction of IL-12 secretion. These results demonstrate the cellular mechanisms involved in altered glycolipid ligand (OCH)-induced T_h2 polarization and immune regulation *in vivo*. Therefore, proper assessment of the effects of the innate immune system on the host's response should be taken into consideration when modulating NKT responses *in vivo* by glycolipids, such as OCH.

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Abbreviations

APC	allophycoerythrin
CD40L	CD40 ligand
CIA	collagen-induced arthritis
DC	dendritic cell
EAE	experimental autoimmune encephalomyelitis
Flt3L	Flt3-ligand
αGC	α-Galactosylceramide
iNKT	invariant NKT
NF-κB	nuclear factor-κB
ODN	oligodeoxynucleotide
OX40L	OX40 ligand
PAMP	pathogen-associated molecular pattern
PG	prostaglandin
TSLP	thymus-specific lymphopoietin

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Stimulation of Host NKT Cells by Synthetic Glycolipid Regulates Acute Graft-versus-Host Disease by Inducing Th2 Polarization of Donor T Cells[1]

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NKT cells are a unique immunoregulatory T cell population that produces large amounts of cytokines. We have investigated whether stimulation of host NKT cells could modulate acute graft-vs-host disease (GVHD) in mice. Injection of the synthetic NKT cell ligand α-galactosylceramide (α-GalCer) to recipient mice on day 0 following allogeneic bone marrow transplantation promoted Th2 polarization of donor T cells and a dramatic reduction of serum TNF-α, a critical mediator of GVHD. A single injection of α-GalCer to recipient mice significantly reduced morbidity and mortality of GVHD. However, the same treatment was unable to confer protection against GVHD in NKT cell-deficient CD1d knockout ($CD1d^{-/-}$) or $IL\text{-}4^{-/-}$ recipient mice or when $STAT6^{-/-}$ mice were used as donors, indicating the critical role of host NKT cells, host production of IL-4, and Th2 cytokine responses mediated by donor T cells on the protective effects of α-GalCer against GVHD. Thus, stimulation of host NKT cells through administration of NKT ligand can regulate acute GVHD by inducing Th2 polarization of donor T cells via STAT6-dependent mechanisms and might represent a novel strategy for prevention of acute GVHD. *The Journal of Immunology,* **2005, 174: 551–556.**

Allogeneic hemopoietic stem cell transplantation (HSCT)[3] cures various hematologic malignant tumors, bone marrow (BM) failures, and congenital metabolic disorders. Emerging evidence suggests that allogeneic HSCT is also useful for treatment of other diseases, including solid tumors and autoimmune diseases (1, 2). However, graft-vs-host disease (GVHD) is a major obstacle that precludes wider application of allogeneic HSCT. The pathophysiology of acute GVHD is complex, involving 1) donor T cell responses to the host alloantigens expressed by host APCs activated by conditioning regimens (i.e., irradiation and/or chemotherapy), and 2) dysregulation of inflammatory cytokine cascades, leading to further T cell expansion and induction of cytotoxic T cell responses (3).

$CD4^+$ helper T cells can be divided into two distinct subpopulations: Th1 and Th2 cells (4). Th1 cells produce IFN-γ and IL-2, whereas Th2 cells produce IL-4, IL-5, and IL-13. Although the role of Th1 and Th2 cytokines in the pathophysiology of acute GVHD is complex and controversial (5–8), Th1 polarization of donor T cells predominantly plays a role in inducing the "cytokine storm" that is seen in several models of acute GVHD (3, 9), whereas Th2 polarization mostly suppresses inflammatory cascades and reduces acute GVHD (10–12). Many properties of dendritic cells (DCs), including the type of signal, the duration of activation, the ratio of DCs to T cells, and the DC subset that presents the Ag, influence the differentiation of naive $CD4^+$ T cells into Th1 or Th2 cells (13). The cytokines that are present during the initiation of the immune responses at the time when the TCR engages with MHC/peptide Ags are critically important for Th cell differentiation (14).

NKT cells are a distinct subset of lymphocytes characterized by expression of surface markers of NK cells together with a TCR. Although the NKT cell population exhibits considerable heterogeneity with regard to phenotypic characteristics and functions (15), the major subset of murine NKT cells expresses a semi-invariant TCR, Vα14-Jα18, in combination with a highly skewed set of Vβs, mainly Vβ8 (16). NKT cells can be activated via their TCR by glycolipid Ags presented by the nonpolymorphic MHC class I-like molecule CD1d expressed by APCs (17). Stimulation of NKT cells rapidly induces secretion of large amounts of IFN-γ and IL-4, thereby influencing the Th1/Th2 balance of conventional $CD4^+$ T cell responses (18). In particular, NKT cells are considered an important early source of IL-4 for the initiation of Th2 responses (19, 20), although these cells are not absolutely required for the induction of Th2 responses (21–23). NKT cells are absent in CD1d knockout ($CD1d^{-/-}$) mice because of defects in their thymic positive selection, which requires CD1d expression on hemopoietic cells, probably double-positive thymocytes (24, 25).

Considering the critical role of cytokines in the development of acute GVHD, we investigated the role of host NKT cells in an experimental model of GVHD, using synthetic NKT cell ligands.

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[3] Abbreviations used in this paper: HSCT, hemopoietic stem cell transplantation; BM, bone marrow; GVHD, graft-vs-host disease; DC, dendritic cell; α-GalCer, α-galactosylceramide; BMT, bone marrow transplantation; TBI, total body irradiation; TCD, T cell depletion; LN, lymph node; WT, wild type.

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α-galactosylceramide (α-GalCer) (26), a glycolipid originally purified from a marine sponge, and its analog, OCH (27). Our findings indicate that stimulation of host NKT cells with NKT ligands can modulate acute GVHD.

Materials and Methods

Mice

Female C57BL/6 (B6, H-2^b) and BALB/c (H-2^d) mice were purchased from Charles River Japan. IL-$4^{-/-}$ B6 and STAT$6^{-/-}$ BALB/c mice were purchased from The Jackson Laboratory. CD1$d^{-/-}$ B6 mice were established by specific deletion of the CD1d1 gene segment (22). Mice, between 8 and 16 wk of age, were maintained in a specific pathogen-free environment and received normal chow and hyperchlorinated drinking water for the first 3 wk post-bone marrow transplantation (BMT). All experiments involving animals were performed under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources, Okayama University Advanced Science Research Center.

Bone marrow transplantation

Mice were transplanted according to a standard protocol described previously (28). Briefly, B6 mice received lethal total body irradiation (TBI; x-ray), split into two doses separated by 6.5 h to minimize gastrointestinal toxicity. Recipient mice were injected with 5×10^6 BM cells plus 5×10^6 spleen cells from either syngeneic (B6) or allogeneic (BALB/c) donors. T cell depletion (TCD) of donor BM cells was performed using anti-CD90 MicroBeads and the AutoMACS system (Miltenyi Biotec) according to the manufacturer's instructions. Donor cells were resuspended in 0.25 ml of HBSS (Invitrogen Life Technologies) and injected i.v. into recipients on day 0. Survival was monitored daily. The degree of systemic acute GVHD was assessed weekly by a scoring system incorporating five clinical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity, as described (29).

Glycolipids

α-GalCer, (2S,3S,4R)-1-*O*-(α-D-galactopyranosyl)-2-(*N*-hexacosanoylamino)-1,3,4-octadecanetriol (KRN7000), was synthesized and provided by Kirin Brewery Company (30). A homologue of α-GalCer, OCH, was selected from a panel of synthesized α-GalCer analogues by replacing the sugar moiety and/or truncating the aliphatic chains, because of its ability to stimulate enhanced IL-4 and reduced IFN-γ production by NKT cells, as previously described (27, 31). BMT recipient mice were injected i.p. with α-GalCer or OCH (100 μg/kg) immediately after BMT on day 0. Mice from the control groups received the diluent only.

Flow cytometric analysis

mAbs used were FITC- or PE-conjugated anti-mouse CD4, H-$2K^b$, and H-$2K^d$ (BD Pharmingen). Cells were preincubated with 2.4G2 mAb (rat anti-mouse FcγR) for 10 min at 4°C to block nonspecific binding of labeled Abs, and then were incubated with the relevant mAbs for 15 min on ice. Finally, cells were washed twice with 0.2% BSA in PBS and fixed. After lysis of RBCs with FACS lysing solution (BD Pharmingen), cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). 7-Amino-actinomycin D (BD Pharmingen)-positive cells (i.e., dead cells) were excluded from the analysis. Fluorochrome-conjugated irrelevant IgG were used as negative controls. At least 5000 live events were acquired for analysis.

Cell cultures

Mesenteric lymph nodes (LNs) and spleens were removed from animals 6 days after BMT and four to six mesenteric LNs or spleens from each experimental group were combined. Numbers of cells were normalized for T cells and were cultured in complete DMEM (Invitrogen Life Technologies) supplemented with 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.02 mM 2-ME, and 10 mM HEPES in wells of a 96-well flat-bottom plate, at a concentration of 5×10^4 T cells/well with 1×10^5 irradiated (20 Gy) peritoneal cells harvested from naive B6 (allogeneic) animals, or with 5 μg/ml plate-bound anti-CD3ε mAbs (BD Pharmingen) and 2 μg/ml anti-CD28 mAbs (BD Pharmingen). Forty-eight hours after the initiation of culture, supernatants were collected for the measurement of cytokine levels.

ELISA

ELISA was performed according to the manufacturer's protocols (R&D Systems) for measurement of IFN-γ, IL-4, and TNF-α levels, as described previously (32). Samples were obtained from culture supernatant and blood from retro-orbital plexus, diluted appropriately, and run in duplicate. Plates were read at 450 nm using a microplate reader (Bio-Rad). The sensitivity of the assays was 31.25 pg/ml for IFN-γ, 7.6 pg/ml for IL-4, and 23.4 pg/ml for TNF-α.

Histology

Formalin-preserved livers and small and large bowels were embedded in paraffin, cut into 5-μm-thick sections, and stained with H&E for histological examination. Slides were coded without reference to prior treatment and examined in a blinded fashion by a pathologist (C. Liu). A semiquantitative scoring system was used to assess the following abnormalities known to be associated with GVHD, as previously described (33): 0, normal; 0.5, focal and rare; 1.0, focal and mild; 2.0, diffuse and mild; 3.0, diffuse and moderate; and 4.0, diffuse and severe. Scores were added to provide a total score for each specimen. After scoring, the codes were broken and data were compiled. Pathological GVHD scores of intestine are the sum of scores for small bowel and colon.

Statistical analysis

Mann-Whitney *U* test was applied for the analysis of cytokine data and clinical scores. We used the Kaplan-Meier product limit method to obtain survival probability, and the log-rank test was applied for comparing survival curves. Differences in pathological scores between the α-GalCer-treated group and the diluent-treated group were examined by two-way ANOVA. We defined $p < 0.05$ as statistically significant.

Results

Administration of α-GalCer stimulates lethally irradiated mice to produce IFN-γ and IL-4

We first determined whether administration of synthetic NKT ligands such as α-GalCer and OCH can stimulate heavily irradiated mice to produce cytokines. B6 mice were given 13 Gy TBI and were injected i.p. with α-GalCer, OCH, or diluent 2 h after TBI. Six hours later, blood samples were obtained, and serum samples were prepared for measurement of IFN-γ and IL-4. TBI alone or BMT itself did not stimulate diluent-treated mice to produce IFN-γ or IL-4 (Fig. 1). Administration of α-GalCer increased serum levels of IFN-γ and IL-4, even in mice receiving TBI. However, serum levels of IFN-γ were much less in irradiated mice than in unirradiated mice. By contrast, the ability of irradiated mice to produce IL-4 to α-GalCer was maintained for 48 h after irradiation. Serum levels of IFN-γ and IL-4 in response to α-GalCer were not altered when irradiated wild-type (WT) mice were injected with 5×10^6 BM cells and 5×10^6 spleen cells isolated from allogeneic (BALB/c) donors. Furthermore, these cytokine responses were not observed when α-GalCer was injected into irradiated NKT cell-deficient CD1$d^{-/-}$ mice with or without BMT. These results suggest that host NKT cells that survive for at least 48 h after irradiation, rather than from infused donor cells, are critically involved in the production of these cytokines in response to glycolipids. Irradiation appears to impair the ability of mice to produce IFN-γ while preserving IL-4 production in response to α-GalCer. Similar cytokine profiles were observed when OCH was administered (data not shown).

Administration of α-GalCer to recipients polarizes donor T cells toward Th2 cytokine production after allogeneic BMT

Induction of GVHD fundamentally depends upon donor T cell responses to host alloantigens. We next evaluated the effect of glycolipid administration on donor T cell responses after allogeneic BMT in a well-characterized murine model of acute GVHD (BALB/c→ B6) directed against both MHC and multiple minor histocompatibility Ags. Lethally irradiated B6 mice were transplanted with 5×10^6 BM cells and 5×10^6 spleen cells from

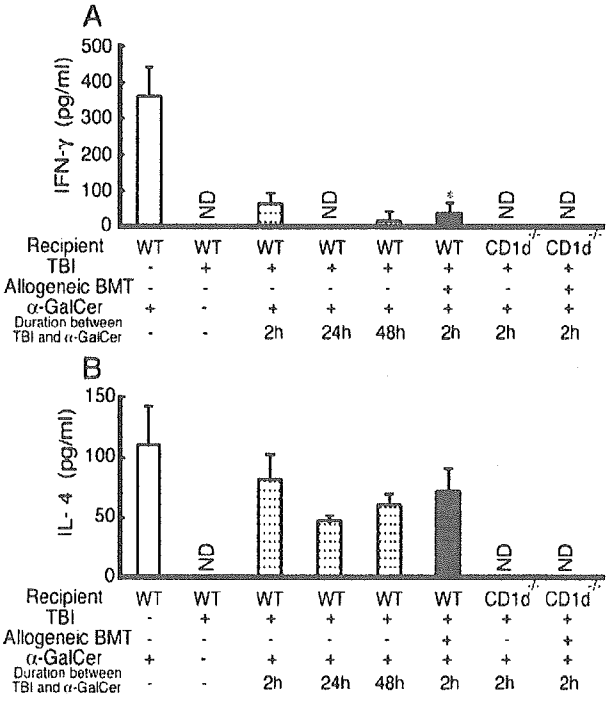

FIGURE 1. Cytokine responses to α-GalCer in lethally irradiated mice with or without BMT. WT and CD1d$^{-/-}$ B6 mice received 13 Gy TBI. Two, 24, or 48 h later, mice were injected i.p. with α-GalCer (100 μg/kg) or diluent. A cohort of animals were transplanted with allogeneic BM cells (5×10^6) and spleen cells (5×10^6) from WT BALB/c donors immediately after TBI, followed by injection of α-GalCer 2 h after TBI. Six hours after the administration of α-GalCer, serum samples were collected, and levels of IFN-γ (*A*) and IL-4 (*B*) were measured. α-GalCer-treated control mice without TBI (□), recipients of TBI plus α-GalCer (▢), and recipients of TBI, allogeneic BMT, and α-GalCer (■) are shown ($n = 3$ per group). Results represent one of three similar experiments and are shown as mean ± SD. *, $p < 0.05$ vs nonirradiated controls. ND, Not detected.

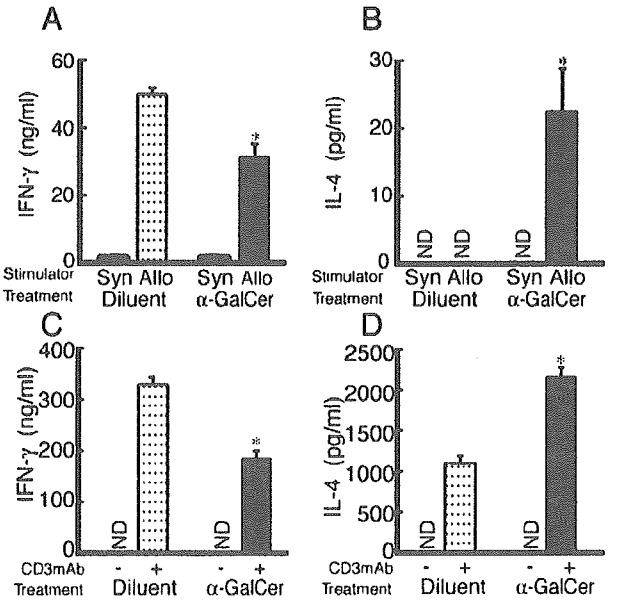

FIGURE 2. Administration of α-GalCer to recipients of allogeneic BMT polarizes donor T cells toward Th2 cytokine secretion. Lethally irradiated (13 Gy) B6 mice were transplanted with BM cells (5×10^6) and spleen cells (5×10^6) isolated from BALB/c mice, followed by injection of either α-GalCer or control diluent. Mesenteric LN cells obtained from diluent-treated recipients (□) and α-GalCer-treated recipients (■) 6 days after BMT were standardized for numbers of CD4$^+$ T cells as 5×10^4/well and were stimulated with 1×10^5/well of allogeneic or syngeneic peritoneal cells (*A* and *B*) or with CD3 (*C* and *D*). After 48 h, cytokine levels in the supernatant were measured by ELISA. Results shown are mean ± SD. *, $p < 0.05$ vs diluent-treated group. ND, Not detected; Syn, syngeneic; Allo, allogeneic.

either syngeneic (B6) or allogeneic (BALB/c) donors. Immediately after BMT, B6 recipients were injected i.p. with either α-GalCer or diluent. Six days after BMT, T cells isolated from mesenteric LN of recipient mice were cultured with irradiated B6 peritoneal cells or with anti-CD3ε mAbs and anti-CD28 mAbs for 48 h, and cytokine levels in the supernatant were determined. Flow cytometric analysis showed that >97% of LN T cells from both control recipients and α-GalCer-treated recipients were donor derived, as assessed by H-2^d vs H-2^b expression. T cells from α-GalCer-treated mice secreted significantly less IFN-γ, but more IL-4, in response to host alloantigens (Fig. 2, *A* and *B*) or to CD3 stimulation (Fig. 2, *C* and *D*) compared with those from controls. Similar results were obtained when T cells isolated from spleens were stimulated by anti-CD3ε and anti-CD28 mAbs. T cells from α-GalCer-treated mice secreted significantly less IFN-γ (18 ± 2 vs 164 ± 6 ng/ml), but more IL-4 (1022 ± 114 vs 356 ± 243 pg/ml), compared with controls. These results demonstrate that a single injection of α-GalCer to BMT recipients polarizes donor T cells toward Th2 responses after allogeneic BMT.

In α-GalCer-treated mice, serum levels of IFN-γ were dramatically reduced on day 6 compared with controls (Fig. 3*A*), and IL-4, which is usually hardly detectable in serum in this model, failed to be detected in the serum of mice of either group (data not shown). This impaired Th1 response of donor T cells was associated with a marked reduction of TNF-α levels in α-GalCer-treated mice (Fig. 3*B*).

Administration of α-GalCer or OCH to BMT recipients modulates acute GVHD

We next examined whether immune deviation mediated by administration of glycolipids can modulate acute GVHD. BMT was performed as above and α-GalCer was injected immediately after BMT on day 0. GVHD was severe in allogeneic controls, with 27% survival at day 50. A single injection of α-GalCer significantly improved survival to 86% ($p < 0.05$) (Fig. 4*A*). Allogeneic control mice developed significantly more severe clinical GVHD compared with syngeneic controls, as assessed by clinical GVHD scores (Fig. 4*B*). Clinical GVHD scores were significantly reduced in α-GalCer-treated recipients compared with allogeneic controls, but were greater than in syngeneic controls. Histological analysis showed that administration of α-GalCer significantly suppressed GVHD pathological scores in the intestine ($p < 0.05$). Analysis of donor cell engraftment at day 60 after BMT in spleens showed complete donor engraftment in α-GalCer-treated recipients (>99% H-2K^{d+}/H-2K^{b-} donor chimerism), ruling out rejection or mixed chimerism as a potential cause of GVHD suppression.

Similar protective effects against GVHD were observed in mice treated with OCH, further confirming the protective effects of NKT ligands (Fig. 4*C*). We performed BMT from B6 donors to BALB/c recipients to rule out strain artifacts. Again, a single injection of α-GalCer to BALB/c recipients reduced GVHD and significantly improved survival of animals (Fig. 4*D*).

Host NKT cells and host production of IL-4 are required for suppression of GVHD by α-GalCer

We examined the requirement of host NKT cells in this protective effect of α-GalCer, using NKT cell-deficient CD1d$^{-/-}$ mice as

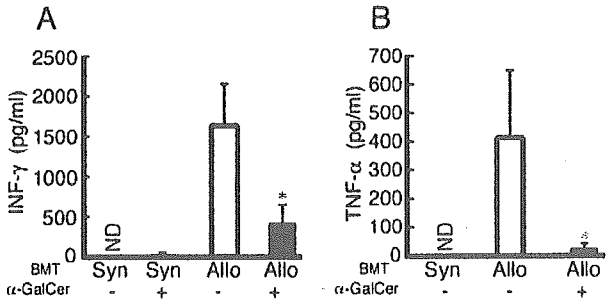

FIGURE 3. A single injection of α-GalCer to recipients of allogeneic BMT markedly reduces serum levels of IFN-γ and TNF-α. WT B6 mice were transplanted as in Fig. 2. Sera (n = 3–10/group) were obtained from diluent-treated (□) and α-GalCer-treated (■) recipients on day 6 after BMT, and serum levels of IFN-γ (*A*) and TNF-α (*B*) were determined. Results from three similar experiments are combined and shown as mean ± SD. *, $p < 0.05$ vs allogeneic, diluent-treated group. ND, Not detected; Syn, syngeneic; Allo, allogeneic.

BMT recipients. Lethally irradiated CD1d$^{-/-}$ mice were transplanted with BM cells and spleen cells from WT BALB/c donors, followed by administration of α-GalCer immediately after BMT on day 0. Protective effects of α-GalCer administration were not observed when CD1d$^{-/-}$ B6 mice were used as recipients, confirming the requirement for host NKT cells (Fig. 5*A*). We next examined the requirement of IL-4 production by host cells in this

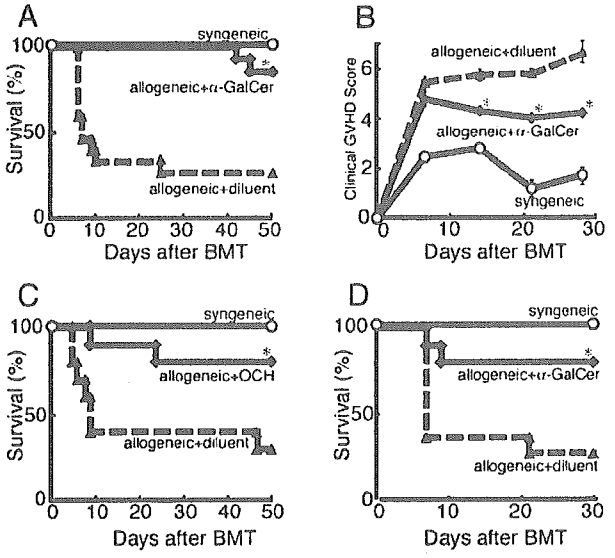

FIGURE 4. A single injection of NKT ligands to BMT recipients modulates acute GVHD. BMT was performed as in Fig. 2. *A*, Survival curves of syngeneic control group (○, solid line; n = 9); allogeneic, diluent-treated recipients (▲, dotted line; n = 15); and allogeneic, α-GalCer-treated recipients (◆, solid line; n = 14) are shown. Data from three similar experiments were combined. *B*, Clinical scores of syngeneic control group (○, solid line); allogeneic, diluent-treated recipients (▲, dotted line); and allogeneic, α-GalCer-treated recipients (◆, solid line) are shown as the mean ± SE. *C*, Survival curves of syngeneic control group (○, solid line; n = 6); allogeneic, diluent-treated recipients (▲, dotted line; n = 10); and allogeneic, OCH-treated recipients (◆, solid line; n = 10) are shown. Data from two similar experiments were combined. *D*, Lethally irradiated (9 Gy) BALB/c mice were transplanted from B6 donors. Survival curves of the syngeneic control group (○, solid line; n = 6); allogeneic, diluent-treated recipients (▲, dotted line; n = 10); and allogeneic, α-GalCer-treated recipients (◆, solid line; n = 10) are shown. Data from two similar experiments were combined. *, $p < 0.05$ vs diluent-treated group.

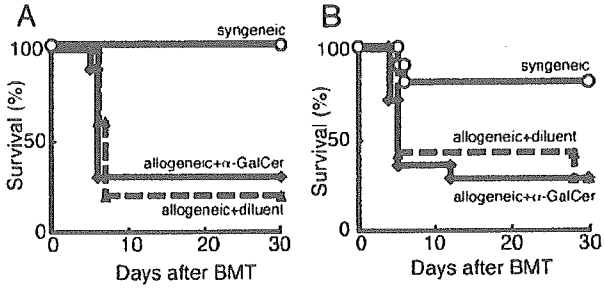

FIGURE 5. Host NKT cells and host IL-4 production are required for suppression of GVHD by α-GalCer. *A*, Lethally irradiated CD1d$^{-/-}$ B6 mice were transplanted as in Fig. 2. Survival curves of syngeneic control group (○, solid line; n = 6); allogeneic, diluent-treated recipients (▲, dotted line; n = 10); and allogeneic, α-GalCer-treated recipients (◆, solid line; n = 10) are shown. Data from two similar experiments were combined. *B*, Lethally irradiated IL-4$^{-/-}$ B6 mice were transplanted as in Fig. 2. Survival curves of syngeneic control group (○, solid line; n = 11); allogeneic, diluent-treated recipients (▲, dotted line; n = 14); and allogeneic, α-GalCer-treated recipients (◆, solid line; n = 14) are shown. Data from three similar experiments were combined.

protective effect. Lethally irradiated IL-4$^{-/-}$ B6 mice were transplanted from WT BALB/c donors and administered α-GalCer as above. α-GalCer did not confer protection against GVHD in IL-4$^{-/-}$ recipients (Fig. 5*B*). Taken together, these results indicate that protective effects of α-GalCer are dependent upon host NKT cells and host production of IL-4.

STAT6 signaling in donor T cells is required for modulation of GVHD by α-GalCer

To determine whether IL-4-induced signaling in donor T cells is critical for the protective effect of glycolipids on GVHD, we used donor spleen cells that lack STAT6 and have impaired IL-4 responses (34, 35). Spleen cells from STAT6$^{-/-}$ BALB/c mice and TCD BM cells from WT BALB/c mice were transplanted after lethal TBI, followed by a single injection of α-GalCer. α-GalCer treatment failed to reduce morbidity and mortality of acute GVHD when STAT6$^{-/-}$ BALB/c donors were used (Fig. 6), demonstrating that STAT6 signaling in donor cells is critical for the protective effect of α-GalCer against GVHD.

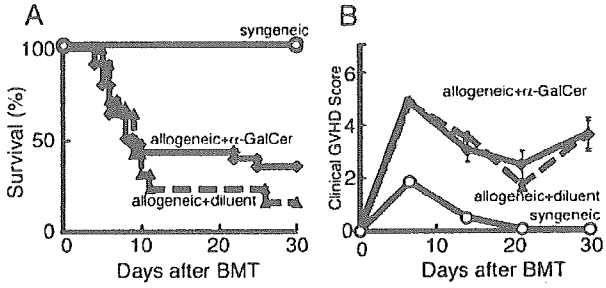

FIGURE 6. The protective effects of α-GalCer against GVHD are dependent upon the STAT6 pathway of donor T cells. Lethally irradiated B6 mice were transplanted with TCD-BM cells (4 × 10^6) from WT BALB/c mice and spleen cells (5 × 10^6) from STAT6$^{-/-}$ BALB/c mice. *A*, Survival curves of the syngeneic control group (○, solid line; n = 15); allogeneic, diluent-treated recipients (▲, dotted line; n = 25); and allogeneic, α-GalCer-treated recipients (◆, solid line; n = 25) are shown. Data from five similar experiments were combined. *B*, Clinical GVHD scores of syngeneic control group (○, solid line); allogeneic, diluent-treated recipients (▲, dotted line); and allogeneic, α-GalCer-treated recipients (◆, solid line) are shown as the mean ± SE.

Discussion

NKT cells are critically involved in the development and suppression of various autoimmune diseases. In experimental models, their regulatory mechanisms mostly depend on IL-4 production and subsequent inhibition of Th1 differentiation of autoreactive $CD4^+$ T cells (18). Previous studies have demonstrated that donor NKT cells regulate acute GVHD in an IL-4-dependent manner when administered together with donor inoculum (36). Considering these immunomodulating functions of NKT cells, we evaluated whether stimulation of host NKT cells could modulate GVHD in a mouse model of this disease.

Administration of α-GalCer stimulates NKT cells to produce both IFN-γ and IL-4 in naive mice, which can promote Th1 and Th2 immunity, respectively (18). We first determined whether administration of synthetic NKT ligands such as α-GalCer and OCH can stimulate heavily irradiated mice to produce cytokines. Surprisingly, irradiation of mice dramatically reduced IFN-γ production in response to α-GalCer, while preserving IL-4 production. This result may account for Th2, but not Th1, polarization of donor T cells by α-GalCer, even in conditions such as allogeneic BMT, which preferentially promotes Th1 polarization. Although mechanisms of selective suppression of IFN-γ production induced by irradiation need to be elucidated, irradiation may modulate the cytokine production profile of NKT cells or neighboring NK cells. Although OCH stimulates NKT cells to predominantly produce IL-4 compared with α-GalCer, resulting in potent Th2 responses (27, 31), both OCH and α-GalCer equally stimulate IL-4 production in irradiated mice and exert equivalent protection against acute GVHD.

Stimulation of host NKT cells by injecting α-GalCer or OCH polarized donor T cells toward Th2 cytokine secretion, resulting in marked reduction of serum IFN-γ levels after BMT. Th2 cytokine responses subsequently inhibited inflammatory cytokine cascades and reduced morbidity and mortality of acute GVHD, as previously described (10–12). Inflammatory cytokines have been shown to be important effector molecules of acute GVHD (37). α-GalCer treatment failed to confer protection against acute GVHD when $STAT6^{-/-}$ BALB/c donors were used, demonstrating that Th2 polarization via STAT6 signaling is critical for this protective effect of α-GalCer, although STAT6-independent Th2 induction has been reported (38, 39).

α-GalCer did not confer protection against GVHD in $CD1d^{-/-}$ or $IL\text{-}4^{-/-}$ recipients. Therefore, the protective effect of α-GalCer against GVHD is dependent upon host NKT cells and host production of IL-4. Sublethal total lymphoid irradiation enriches NKT cells in host lymphoid tissues, and these NKT cells induce Th2 polarization of conventional T cells by IL-4 production, resulting in reduced GVHD (40–42). These findings are consistent with our observation that IL-4 production is critical for the protective effects of NKT cells against acute GVHD. It should be noted, however, that systemic administration of IL-4 is either ineffective or toxic (6). Because the cytokine environment during the initial interaction between naive T cells and APCs is critically important for induction of Th1 or Th2 differentiation (14), local IL-4 production in the secondary lymphoid organs where donor T cells encounter host APCs might be necessary to cause effective Th1→Th2 immune deviation after allogeneic HSCT (43).

Current strategies for prophylaxis and treatment of GVHD primarily target depletion or suppression of donor T cells. These interventions suppress donor T cell activation and are associated with increased risk of infection and relapses of malignant diseases. Th1→Th2 deviation of donor T cells represents a promising strategy to reduce acute GVHD while preserving cytolytic cellular effector functions against tumors and infectious agents (33, 44–47). To achieve Th1→Th2 immune deviation of donor T cells, cytokines have been administered to either donors or recipients in animal models of GVHD. Donor treatment with cytokines such as IL-18 and G-CSF, and recipient treatment with IL-11, induces Th2 polarization of donor T cells and reduces acute GVHD (33, 44, 48). The present study reveals an alternative strategy to induce Th2 polarization of donor T cells by injecting NKT ligands into recipients to activate recipient NKT cells.

Prior studies (36, 40–42, 49) and the current study suggest that both donor and host NKT cells can regulate acute GVHD through their unique properties to secrete large amounts of cytokines and subsequent modulation of adaptive immunity. These studies reveal that there are several ways by which the NKT cell system can be exploited to suppress GVHD. First, administration of donor NKT cells expanded in vitro by repeated stimulation with glycolipid (50) can suppress GVHD (36). Second, total lymphoid irradiation enriches host NKT cells in lymphoid organs and thereby skews donor T cells toward Th2 cytokine production (40–42). Third, as shown here, administration of glycolipid to recipients stimulates host NKT cells to suppress GVHD. A recent phase I trial for patients with various solid tumors demonstrated that administration of α-GalCer was well tolerated with minimal side effects, which included temporal fever, headache, vomiting, chills, and malaise (51). Therefore, α-GalCer treatment may provide an effective and relatively safe option for preventing GVHD.

Cells belonging to the innate arm of the immune system, such as monocytes/macrophages, NKT cells, and NK cells, can produce large amounts of cytokines quickly upon stimulation. Innate immunity can thereby augment donor T cell responses to alloantigens in allogeneic HSCT (3). Our findings reveal a novel role for host NKT cells in regulating GVHD and indicate that stimulation of host innate immunity may serve as an effective adjunct to clinical regimens of GVHD prophylaxis.

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NKT Cells Are Critical for the Initiation of an Inflammatory Bowel Response against *Toxoplasma gondii*[1]

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We demonstrated in this study the critical role of NKT cells in the lethal ileitis induced in C57BL/6 mice after infection with *Toxoplasma gondii*. This intestinal inflammation is caused by overproduction of IFN-γ in the lamina propria. The implication of NKT cells was confirmed by the observation that NKT cell-deficient mice (Jα281$^{-/-}$) are more resistant than C57BL/6 mice to the development of lethal ileitis. Jα281$^{-/-}$ mice failed to overexpress IFN-γ in the intestine early after infection. This detrimental effect of NKT cells is blocked by treatment with α-galactosylceramide, which prevents death in C57BL/6, but not in Jα281$^{-/-}$, mice. This protective effect is characterized by a shift in cytokine production by NKT cells toward a Th2 profile and correlates with an increased number of mesenteric Foxp3 lymphocytes. Using chimeric mice in which only NKT cells are deficient in the IL-10 gene and mice treated with anti-CD25 mAb, we identified regulatory T cells as the source of the IL-10 required for manifestation of the protective effect of α-galactosylceramide treatment. Our results highlight the participation of NKT cells in the parasite clearance by shifting the cytokine profile toward a Th1 pattern and simultaneously to immunopathological manifestation when this Th1 immune response remains uncontrolled. *The Journal of Immunology*, **2005, 175: 899–908.**

Natural killer T cells represent a minor subset of T lymphocytes that share receptor structures with conventional T cells and NK cells (1, 2). Murine NKT cells express intermediate levels of a TCR using a semi-invariant Vα14-Jα281 TCR α-chain paired with Vβ8, -7, or -2 TCR β-chain together with NK cell receptors (NKR-P1, Ly-49, and NK1.1 in C57BL/6 mice) (3, 4). These cells are located mainly in the liver, spleen, thymus, and bone marrow and recognize Ag in the context of the monomorphic CD1d Ag-presenting molecule (5, 6). CD1d and the invariant TCR α-chain are essential for the normal development of NKT cells (7). CD1 molecules present hydrophobic lipid Ags (8), and the marine sponge derived glycolipid, commonly referred to as α-galactosylceramide (α-GalCer),[4] was identified as a potent stimulatory factor for NKT cells (9).

A potential role of NKT cells in the regulation of immune responses has been hypothesized because of their capacity to rapidly release large amounts of IL-4 and IFN-γ upon activation (10). NKT cells play crucial roles in various immune responses, including antitumor, autoimmune, and antimicrobial immune responses (1, 11). Within hours of TCR engagement, CD1d-reactive T cells produce Th1 and/or Th2 cytokines (9, 11, 12) by a mechanism not yet identified that can influence other immune cells, such as conventional T (13–15), NK cells (16), and dendritic cells (DC) (17). NKT cell-derived Th1 cytokines (such as IFN-γ) are important in the initiation of the antitumor immune response, whereas NKT cell-derived Th2 cytokines (IL-4 and IL-10) are involved in down-regulation of the autoimmune response (18). When stimulated with α-GalCer, NKT cells exhibit the ability to proliferate and to produce both Th1 and Th2 cytokines (9, 19). However administration of α-GalCer at the time of priming of mice with Ag results in the generation of only Ag-specific Th2 cells. Thus, α-GalCer might be useful for modulating the immune response toward a Th2 phenotype (12).

Recent evidence suggests that NKT cells are important in the host/pathogen immune response, including cytotoxicity, Ab production, and regulation of Th1/Th2 differentiation. NKT cells have been shown to participate in the immune response to a range of different infectious agents, including *Listeria*, *Mycobacteria*, *Salmonella*, *Plasmodium*, viral hepatitis (20, 21), HIV (22), and even *Toxoplasma gondii* (23). *T. gondii* is an obligate intracellular parasite acquired by oral ingestion of tissue cysts containing either bradyzoites or sporozoites from contaminated soil. It has been observed that after oral infection with tissue cysts, the intestinal epithelial and lamina propria cells are invaded by the parasites. Parasite infection induces a strongly biased Th1 response in the gut that displays a dual effect. IFN-γ produced by the CD4 T cells from the lamina propria (24) limits parasite replication, conferring resistance in mice in certain inbred strains. However, in C57BL/6 (B6) mice, an overwhelming IFN-γ production leads to a lethal acute ileitis within 10 days after oral infection. This *Toxoplasma*-induced intestinal disease shares histological and immunological similarities with human inflammatory bowel disease, such as

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[4] Abbreviations used in this paper: α-GalCer, α-galactosylceramide; DC, dendritic cell; IEL, intraepithelial lymphocyte; LPL, lamina propria lymphocyte; MLN, mesenteric lymph node; SAG1, surface Ag-1.

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Crohn's disease. The regulation of this inflammatory process requires a delicate homeostatic balance that is influenced by either a Th1 or Th2 response.

In this report the role of NKT cells in the initiation of the inflammatory process in response to oral infection with *T. gondii* was evaluated. Our findings suggest a potentially critical role for these early responder cells in the initiation and regulation of the lethal inflammatory process.

Materials and Methods

Mice and parasites

Female, 8- to 10-wk-old, inbred B6 mice and CBA were obtained from IFFA-Credo. Mice were housed under approved conditions of the Animal Research Facility at Institut Pasteur. IL-$10^{-/-}$ mice were supplied by Dr. Bandeira (Institut Pasteur, Paris, France). We were provided with Jα281$^{-/-}$ mice by Dr. M. Taniguchi (Riken Research Center for Allergy and Immunology, Yokohama, Japan) (9). Vα14Tg mice by Dr. A. Lehuen (Institut National de la Santé et de la Recherche Médicale, Paris, France) (25), actin-GFP mice by Dr. M. Okabe (Genome Information Research Center, Osaka University, Osaka, Japan) (26), and CD1$^{-/-}$ mice by Dr. L. Van Kaer (Vanderbilt University School of Medicine, Nashville, TN) (7). All the genetically modified strains were on a B6 genetic background. 76K strain cysts isolated from the brains of chronically infected CBA mice were used for in vivo studies. Mice were infected orally by intragastric gavage of 35 cysts, a lethal condition for B6 wild-type mice as described previously (27). After infection, mortality was evaluated, and morbidity was estimated by the percentage of weight loss compared with the initial weight.

Treatment with α-GalCer, anti-CD25, or anti IL-4 Abs

α-GalCer was kept dissolved in PBS buffer containing 20% DMSO at 220 μg/ml as a stock solution. Mice received a single i.p. injection of 5 mg of α-GalCer the day before infection by *T. gondii*. Control mice received an i.p. injection of PBS/20% DMSO, which has no influence on the course of *T. gondii* infection.

Neutralization of IL-4 was conducted by injecting i.p. 1 mg of anti-IL-4 (11B11; provided by Dr. P. Launois, World Health Organization Immunology Research and Training Center, Institute of Biochemistry, Epalinges, Switzerland) mAb 24 h before α-GalCer treatment and 48 h before infection. Control mice were treated with rat IgG Abs (Sigma-Aldrich).

Mice were depleted of CD25$^+$ cells by i.p. administration of 0.5 mg of anti-CD25 (PC61; provided by Dr. R. J. Noelle, Dartmouth Medical School, Lebanon, NH) mAb. Three days after the treatment, the efficiency of CD25$^+$ cell depletion was controlled in peripheral blood by FACS analysis. The CD25$^+$ cell depletion remained stable over 15 days. Control mice were treated with a mouse IgD1 isotype Ab (MOPC31C k; BD Pharmingen).

Cell purification

Lamina propria. The method used to isolate intestinal lamina propria lymphocytes (LPLs) was modified as described previously (24). After dissection and removal of Peyer's patches, the sectioned intestines were incubated in PBS-3 mM EDTA at 37°C and 5% CO_2 (four times, 20 min each time). Then intestinal pieces were incubated at 37°C in RPMI 1640-5% FCS with Liberase (0.14 Wunch units/ml; Roche) and DNase (10 U/ml; Sigma-Aldrich). After 45 min, the digested suspension containing LPLs was filtered on a cell strainer and washed twice, and the pellet was submitted to a Percoll gradient to isolate the lymphocytes. Total cells were resuspended in a 80% isotonic Percoll solution (Pharmacia Biotech) and overlaid with a 40% isotonic Percoll solution. Centrifugation for 30 min at 3000 rpm resulted in concentration of mononuclear cells at the 40–80% interface. The collected cells were washed once with PBS supplemented with 2% FCS. The purity of the LPL population was assessed by the relative percentage of B cells (>50%), CD4 T cells (~ 20%), CD8 T (<3%) cells, and enterocytes (<5%).

Intraepithelial lymphocytes (IELs). IELs were isolated as previously described (28). Briefly, the small intestine was flushed with PBS and divided longitudinally after removal of Peyers's patches. The mucosae were scraped, dissociated by mechanical disruption, in RPMI 1640 containing 4% FCS and 1 mmol/L DTT. After passage over a glass-wool column, the lymphocytes were separated by Percoll as described for LPLs. The purity of IEL population was assessed by the relative percentages of B cells (<2%), CD4 T cells (<10%), CD8 T cells (>80%), and enterocytes (<5%).

Mesenteric lymph node (MLN) and spleen. MLN and spleen were dissociated and freed of connective tissue by filtration (70 μm). Unless otherwise stated, each mouse was analyzed individually.

Liver. Single-cell suspensions were obtained from liver as described previously by us (29).

Cytometric analysis

FACS analysis of NKT cells. Single-cell suspensions were first incubated 10 min with an anti-FcγRII/III mAb (Fcblock, 2.4G2; BD Pharmingen), followed by a 1-h exposure to CD1d/α-GalCer tetramer-allophycocyanin under agitation at 4°C. CD1d/α-GalCer tetramers were prepared as described by Matsuda et al. (30). After two washes, other cell surface stainings were performed with the following Abs: anti-TCRβ (H57-597), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-NK1.1 (PK136), anti-CD25 (C363 16A), anti-CD45RB (7D4), and anti-CD5 (BD Biosciences). PerCP-streptavidin and CyChrome-streptavidin were purchased from BD Biosciences. Cells were analyzed in PBS containing 2% FCS using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

Cell sorting. NKT cells stained with the tetramer were magnetically sorted. After tetramer CD1d/α-GalCer-allophycocyanin staining, cells suspensions were incubated for 15 min in PBS/2% FCS/2 mM EDTA at 4°C with anti-allophycocyanin beads as described by the provider (Miltenyi Biotec). After washing and filtration, samples were run on AutoMACS (Miltenyi Biotec). Purity was controlled by cytometric analysis, and the sorted cells were frozen until molecular biology analysis.

For the reconstitution experiment, NKT from the liver and the spleen of actin-GFP mice were sorted with both anti-CD5 biotin (53-7.3), and anti-NK1.1-PE (PK136) mAbs and streptavidin-allophycocyanin using a MoFlo (DakoCytomation). Purified NKT-GFP$^+$ cells were collected in RPMI 1640 supplemented with 10% FCS. The purity of the sorted NKT-GFP cells was found to exceed 97%.

Adoptive transfer of NKT-GFP$^+$ cells. Highly purified NKT cells (1×10^6) were injected i.v. into Jα281$^{-/-}$ mice. At the same time these mice were treated with 5 μg of α-GalCer i.p. One day later, NKT cells were transferred, and α-GalCer-treated mice were infected.

Histological examination

Histopathology and morphometric analysis. Intestines were immediately fixed in buffered 10% formalin after dissection. Then they were embedded, sectioned, and stained with H&E for histological examination. Inflammation was scored by the ratio of the length/thickness of the villi (mean of 20 measures for a total of four different fields).

Confocal microscopic examination. Intestinal and hepatic samples from NKT-GFP-transferred mice were microscopically examined. On day 7 after infection mice were scarified, and samples from intestines and livers were incubated for 24 h in paraformaldehyde (4%) and saccharose (30%). Then tissues were frozen in liquid nitrogen using OCT embedding compound (Sakura). Frozen sections (10 μm) were cut on a microtome HM 505 cryostat (Microcom Laboratory), fixed with PBS/paraformaldehyde (4%), permeabilized by PBS/Triton (0.1%), contrastained with rhodamine phallodin (Molecular Probes), and mounted with Vectashield (Vector Laboratories). Preparations were analyzed with fluorescent microscope Axioplan 2 imaging coupled with an ApoTome system (Zeiss). GFP-NKT cell trafficking was also assessed by FACS analysis performed on day 7 after infection with cell suspensions obtained from lamina propria and livers.

Bone marrow chimeric mice

Recipient mice were lethally irradiated (900 rad) with a ^{137}Ce source. Then they received i.v. bone marrow cells (1×10^7) recovered from femurs and tibias of donor mice. To generate mice with only NKT cells devoid of the IL-10 gene, a mix (50/50%) of bone marrow cells from Jα281$^{-/-}$ mice and IL-$10^{-/-}$ mice was prepared. Control mice received cells from B6, Jα281$^{-/-}$ or IL-$10^{-/-}$ mice alone. Six weeks after reconstitution, mice were bled, and the presence of CD4$^+$, CD19$^+$ (1D3), and CD11c$^+$ (HL3) cells was monitored by flow cytometric analysis. Reconstitution with NKT was assessed (two mice per group) by staining the CD1d/α-GalCer-allophycocyanin tetramer cell suspensions obtained from the liver and lamina propria of the chimera. Chimeric mice were then infected. At different times after infection, LPLs and MLN cell suspensions were phenotyped by FACS analysis. Morbidity was evaluated daily by recording the weight loss, and mortality was also recorded.

RNA extraction, cDNA preparation, and real-time RT-PCR

Tissue samples from intestines and purified cells were kept frozen (~ 70°C) until mRNA extraction. Specimens were disrupted in a Polytron (Brinkmann Instruments) and homogenized in 350 ml of RLT buffer (Qiagen).

RNA extraction and cDNA preparation were conducted following standard procedures using oligo(dT)$_{17}$ primers, and 10 U of avian myeloblastosis virus reverse transcriptase. Quantitative PCR was performed with the GeneAmp 7000 (Applied Biosystems) as indicated by the supplier. Primers and probes for the quantitative PCR assay of cytokines and actin were designed as previously described (31). Foxp3 mRNA were analyzed with applied assay on demand n°Mm00475156_m1 (Applied Biosystems).

Parasite burden

DNA was extracted from the different organ samples using a DNAeasy kit (Qiagen). The *Toxoplasma* B1 gene was amplified by quantitative real-time PCR (32). Parasite titration by real-time PCR was performed with the GeneAmp 7000 (Applied Biosystems). The standard curve established from the serial 10-fold dilutions of *T. gondii* DNA of parasite concentrations ranging from 1×10^6 to 10, showed linearity over a 6-log concentration range and was included in each amplification run. At different time points after infection, tissue samples were recovered, and their DNA were extracted with the DNeasy Tissue Kit (Qiagen). For each sample, parasite count was calculated by interpolation from the standard curve. The parasite burden was expressed as the number of parasites per milligram of samples. Cerebral parasite burden was evaluated by enumeration of the cysts on day 30 after infection.

Statistical analysis

Results are expressed as the mean ± SD. Statistical differences between groups were analyzed using Student's *t* test. A value of $p < 0.05$ was considered significant.

Results

Presence of NKT cell in the lamina propria

The presence of the NKT lymphocyte subpopulation within the gut was demonstrated by FACS analysis using the CD1d/α-GalCer tetramers. In the lamina propria of naive B6 mice, 2% of the mononuclear cells (LPLs) were detected (Fig. 1*A*). NKT cells were not detected in cell suspensions from the IEL compartment (Fig. 1*A*). Seventy to 80% of the tetramer-positive cells were CD4$^+$: the remainder were CD4$^-$CD8$^-$ double negative. During the days following infection, a decrease in the number of tetramer-positive cells was observed (Fig. 1*B*) that could be due to TCR down-regulation. Serial time point phenotyping after infection demonstrated that all NKT cells were CD25$^-$. To assess NKT cell trafficking into the intestine after infection, Jα281$^{-/-}$ mice were transferred with NKT-GFP$^+$ cells (1×10^6) highly purified from the livers of GFP transgenic mice on the basis of CD5 and NK1.1 expression (Fig. 1*D*, *a*). At 7 days after infection, GFP$^+$ cells were found in cell suspension obtained from the liver (Fig. 1*D*, *b*) and lamina propria (Fig. 1*D*, *c*) of the transferred mice. Histological examination by confocal microscopy revealed that within the liver, NKT-GFP$^+$ cells were distributed among hepatocytes near the sinusoids (Fig. 1*E*). Within the gut, NKT-GFP$^+$ cells were always

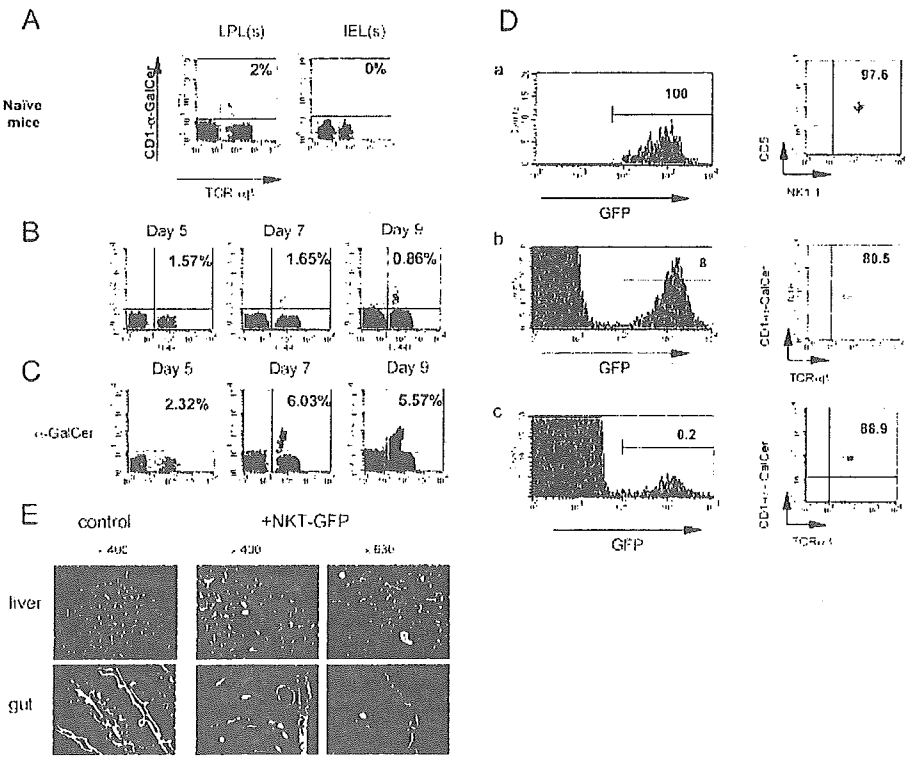

FIGURE 1. NKT cells are present in the lamina propria compartment. *A*, Representative FACS profiles showing Vα14 CD1d/α-GalCer$^+$ TCRαβ$^+$ cells obtained from LPL and IEL suspension from naive mice. The numbers indicate the proportion of tetramer-positive T cells in the lymphocyte gate. This analysis was performed with five mice and was repeated twice. *B*, Representative FACS profiles showing Vα14 CD1d/α-GalCer$^+$ TCRαβ$^+$ cells obtained from LPL suspensions of mice at different times after infection. This analysis was performed with five mice individually and was repeated twice. *C*, Representative FACS profiles showing Vα14 CD1d/α-GalCer$^+$ TCRαβ$^+$ cells obtained from LPL suspensions of α-GalCer-treated mice at different times after infection. This analysis was performed with five mice and was repeated twice. *D*, NKT cell populations from actin-GFP mice were purified on the basis of GFP, CD5, and NK1.1 (PE) expression and were positively selected with magnetic beads directed against PE. *D*, *a*, Purity of the selected NKT cell population. One million purified NKT cells were injected i.v. into Jα281$^{-/-}$ mice. The following day, these mice were infected with *T. gondii*. On day 7 after the adoptive transfer, Jα281$^{-/-}$ recipients showed a significant presence of GFP$^+$ cells in the liver (*b*) and lamina propria (*c*) cell suspension, which are almost all NKT cells as revealed by CD1d/α-GalCer$^+$ staining after gating on GFP$^+$ cells. Four mice were adoptively transferred with NKT-GFP cells, requiring 24 GFP transgenic donor mice. *E*, GFP$^+$ NKT cells were detected in paraformaldehyde-fixed cryosections of liver and intestine from Jα281$^{-/-}$ recipient mice (the control was sections from naive Jα281$^{-/-}$ mice). Actin filaments were stained in red with rhodamine phallodin to visualize the organ structure. Original magnifications: ×400 and ×630. The pictures shown are representative of observations made with the four NKT-GFP cell recipient mice.

localized in the lamina propria and were never associated with the IEL compartment (Fig. 1*E*). These data indicated that NKT cells traffic to the intestine, where they localize within the lamina propria.

Importance of NKT cells in the development of acute inflammatory ileitis in B6 mice

The involvement of NKT cells in the initiation of the intestinal inflammation after oral infection with *T. gondii* was investigated by comparing the outcome of the infection in wild-type B6 mice and mice genetically deficient in NKT cells ($J\alpha281^{-/-}$ mice). As expected, all control B6 mice died within 7–10 days of severe ileitis after oral challenge with 35 cysts (Fig. 2*A*). The intestinal inflammation and subsequent morphological changes were characterized by cellular infiltration within the lamina propria; short, thickened villi; and patchy transluminal necrosis. In contrast, $J\alpha281^{-/-}$ mice developed a less severe disease (Fig. 2*B*) associated with 1) a decrease in the length/thickness ratio of the villi compared with B6 infected mice (Fig. 2*C*), 2) a significantly delayed time of death, and 3) a decrease in the mortality rate compared with B6 mice (Fig. 2*A*). This outcome was not parasite dose dependent, as determined using a lower infectious dose of cysts (10 cysts/mouse) in which all the $J\alpha281^{-/-}$ mice survived, whereas 25% of the B6 died (Fig. 2*D*). These results indicate that the absence of NKT cells correlates with a more resistant phenotype. However, $CD1d^{-/-}$ mice were even more susceptible than B6 mice (Fig. 2*A*). In addition to NKT depletion, regulatory cells, such as IEL and B cells, are also reduced in $CD1d^{-/-}$ mice (33, 34).

To further explore the potential role of NKT cells in the inflammatory process, mice that overexpressed NKT cells (Vα14Tg mice) were infected. Both B6 and Vα14Tg mice died within 7–10 days when infected with 35 cysts (Fig. 2*A*). However, in the experiment using a lower dose of cysts (10 cysts/mouse), all the Vα14Tg mice died, whereas only 25% of the B6 mice died (Fig. 2*D*). These data confirm that NKT cells are important in the innate host response to oral parasite infection and are involved in disease susceptibility.

NKT cell activation correlates with intestinal IFN-γ production after T. gondii *infection*

IFN-γ is an important cytokine in mediating host defense against *T. gondii* infection. It limits parasite replication, but, at the same time, if overproduced, it leads to the development of overwhelming intestinal inflammation. Therefore, because NKT cell-deficient

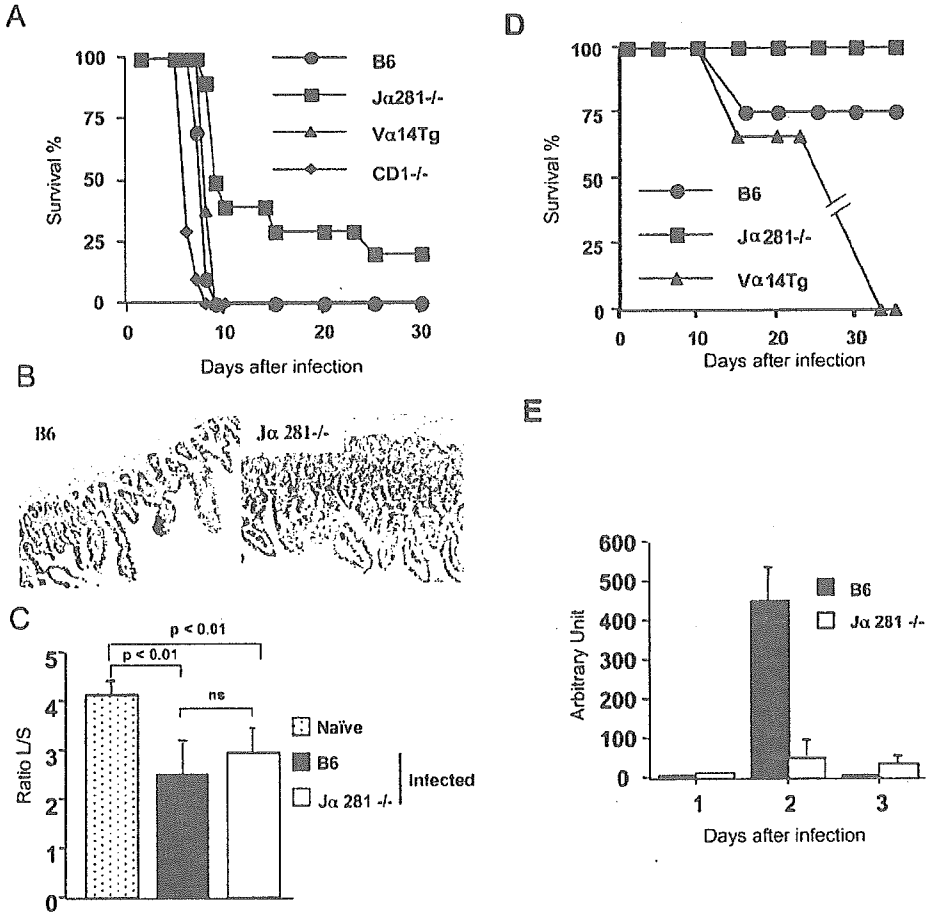

FIGURE 2. NKT cells are involved in the development of acute inflammatory ileitis in B6 mice. *A*, Survival rate of B6, $J\alpha281^{-/-}$, Vα14Tg, and $CD1d^{-/-}$ mice after challenge with 35 cysts of *T. gondii* (n = 10/group). Data are representative of three independent experiments with similar results. *B*, Intestinal H&E histology from B6 and $J\alpha281^{-/-}$ mice on day 7 after challenge (magnification, ×200). Results are representative of two independent experiments performed with four mice each time. *C*, Intestinal lesions from B6 and $J\alpha281^{-/-}$ on day 7 after challenge were scored as the ratio of the villi length to its thickness. These data were the mean of 20 measures obtained with four different fields and repeated with two mice per group. *D*, Survival rate of B6, $J\alpha281^{-/-}$, Vα14Tg after challenge with 10 cysts of *T. gondii* (n = 5/group). Data are representative of two independent experiments with similar results. *E*, Early IFN-γ mRNA expression in the intestine after infection is dependant upon the presence of NKT cells. Samples from the ileum of B6 and $J\alpha281^{-/-}$ mice were analyzed for mRNA expression of IFN-γ by real-time RT-PCR. Results are expressed as the fold increase relative to noninfected control mice after normalization with the housekeeping gene. The mean ± SD were calculated from two samples from two mice. Results are representative of three independent experiments.

mice (Jα281$^{-/-}$) were more resistant to the development of lethal ileitis after *T. gondii* infection, the expression of IFN-γ in their intestines was measured at different times after oral challenge with the cysts. Between days 2 and 3 after infection, IFN-γ mRNA expression peaked in the intestine of B6 mice, and there was a significant difference in IFN-γ mRNA expression between B6 mice and Jα281$^{-/-}$ mice. By quantitative RT-PCR, the level of mRNA expression in B6 mice was 9–10 times higher than that in Jα281$^{-/-}$ mice (Fig. 2*E*). Over time, inflammatory cytokine production in Jα281$^{-/-}$ mice may increase, contributing in the delayed time to death due to lethal intestinal inflammation. The lack of early production of IFN-γ might also explain the 2-fold increase in parasite burden in Jα281$^{-/-}$ mice on day 8 after infection. These findings strongly suggest that NKT cell activation after oral infection with *T. gondii* is associated with early initiation of the Th1 process observed in the intestines of B6 mice.

Treatment with α-GalCer protects against the development of lethal ileitis

Because α-GalCer can influence the nature of the cytokines produced by NKT cells and consequently the orientation of the adaptative Th response, mice were treated with α-GalCer the day before infection. Up to 30 days after infection, this treatment prevented death in both B6 (100%) and V14αTg mice overexpressing NKT cells (80%; Fig. 3*A*). Histological examination performed on day 7 after infection revealed that treatment with α-GalCer interfered with the development of ileitis (Fig. 3, *B* and *C*). In addition, B6 mice treated with α-GalCer exhibited less weight loss compared with untreated infected controls (Fig. 3*D*). To assess the cell population targeted by α-GalCer treatment, NKT-deficient mice (Jα281$^{-/-}$) were treated with α-GalCer the day before infection. This treatment had no effect on the infection outcome in Jα281$^{-/-}$ mice (Fig. 3*A*), as attested by the early time of death and the histological damages observed in treated mice (Fig. 3*B*). These observations strongly suggest that α-GalCer modulates the functional abilities of NKT cells. Treatment with α-GalCer was not directly toxic to the parasite, because there was no difference in parasite burden in Jα281$^{-/-}$ mice treated or not treated on day 30 after infection (data not shown). Treatment with α-GalCer 2 days after infection failed to impact the development of the hyperinflammatory response in small intestine.

Treatment with α-GalCer induces preferential production of IL-4 and IL-10 in T. gondii*-infected mice*

One of the consequences of α-GalCer treatment was the increase in the number of NKT cells in the lamina propria of infected mice (Fig. 1*C*). The production of selected cytokines in the whole intestine of α-GalCer-treated mice was monitored by quantitative RT-PCR. A significant increase in IL-10 (180-fold) and IL-4 (80-fold) mRNA expression was observed in the intestines of α-GalCer-treated mice on days 3 and 5, respectively, after infection. In contrast, no increase in IL-13 mRNA expression in the whole intestine of α-GalCer-treated mice was measured at serial time points after infection. mRNA for IFN-γ was also significantly decreased (10-fold) in α-GalCer-treated mice (data not shown). This result demonstrated a shift in cytokine production toward a Th2-like profile after treatment with α-GalCer and infection and a decline in the Th1-like immune response. To better assess the contribution of intestinal NKT cells in this shift, α-GalCer-treated mice and untreated control mice were killed on day 8 after infection, and NKT cells were purified from the lamina propria (Fig. 4*A*). As shown in Fig. 4*A*, the purity of the sorted population was

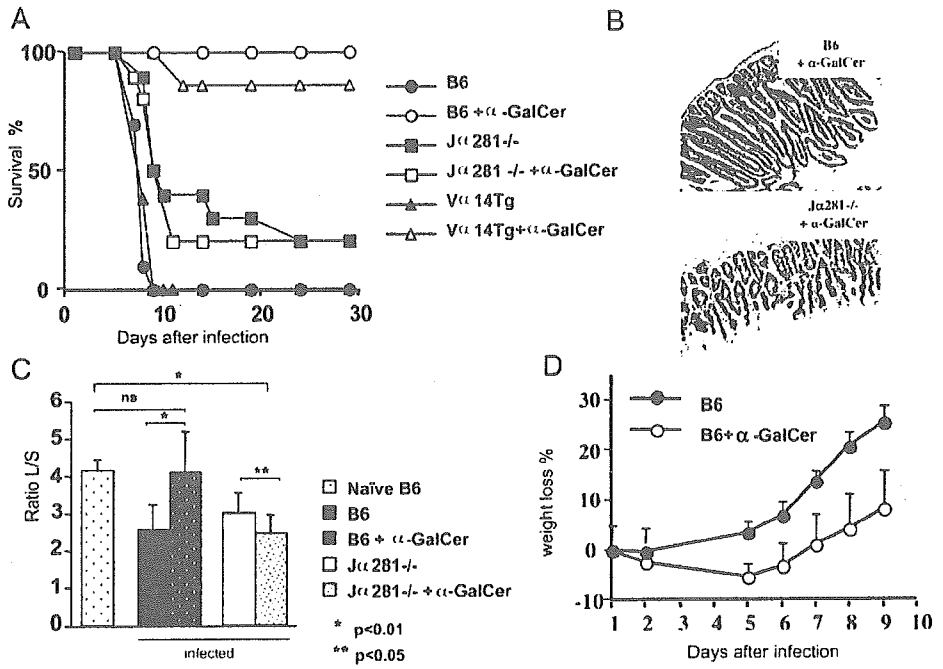

FIGURE 3. α-GalCer treatment protects the infected mice against the development of lethal ileitis. *A*, Survival rates of B6, Jα281$^{-/-}$, and Vα14Tg mice after i.p. administration of 5 μg of α-GalCer the day before challenge with *T. gondii* (n = 10/group). Results are representative of two independent experiments. *B*, Intestinal H&E histology of α-GalCer-treated or untreated mice on day 7 after infection (magnification, ×200). *C*, Intestinal lesions in α-GalCer-treated or untreated mice on day 7 after infection were scored as the ratio of the villi length to its thickness. These data were the mean of 20 measures obtained with four different fields and repeated with two mice per group. *D*, B6 mice treated with α-GalCer exhibited only mild weight loss compared with untreated infected controls. Infected B6 mice treated, or not, with α-GalCer were weighed daily. Weight loss is expressed as a percentage of the animal's initial weight.

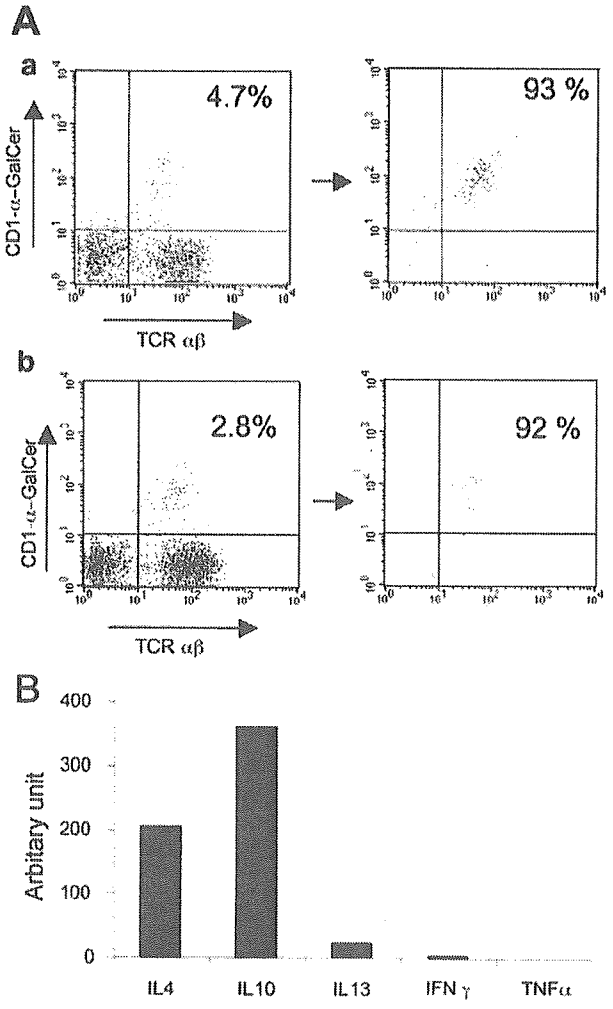

FIGURE 4. NKT cells produce Th2 cytokine after α-GalCer administration and *T. gondii* infection. *A*, NKT cell populations were isolated from the lamina propria of mice treated (*a*) or not treated (*b*; two mice per group) with α-GalCer on day 8 after infection. The cells were then purified on the basis of CD1d/α-GalCer tetramer staining using anti-allophycocyanin magnetic beads. The purity of sorted cells was confirmed by FACS analysis. This experiment was repeated twice. *B*, Purified NKT cells were analyzed for mRNA expression of Th1 and Th2 cytokines by real-time RT-PCR. Results are expressed as the relative increase in the cytokines in NKT cells from treated mice compared with the untreated NKT cells after normalization with the housekeeping gene. Results are representative of two independent experiments.

>90% in both α-GalCer-treated (Fig. 4*A*, *a*) and untreated (Fig. 4*A*, *b*) animals. The mRNA production of different cytokines by the purified NKT cell population was measured by RT-PCR. The results are expressed as the relative increase or decrease in mRNA expression for different cytokines in NKT cells isolated from α-GalCer-treated mice compared with control infected, but untreated, mice. Compared with controls, IL-10, IL-4, and IL-13 mRNA expressions were increased in the NKT cell population isolated on day 8 from mice treated with α-GalCer and infected (Fig. 4*B*). These data indicate that treatment with α-GalCer shifts the NKT cell cytokine pattern to a Th2-like profile.

The production of IL-10 and IL-4 by NKT cells stimulated with α-GalCer was increased in the intestines of treated mice. In contrast, IL-13 production by NKT cells after treatment with α-GalCer did not lead to an increase in this cytokine in the whole intestine throughout the serial time points after infection.

Role of IL-4 in protection against T. gondii*-induced death*

The contribution of IL-4 production associated with α-GalCer treatment to interference with the induction of *T. gondii*-induced death was evaluated by a series of experiments using blocking Ab. Blocking of IL-4 the day before α-GalCer treatment partially reversed its beneficial effect, as shown by a 50% survival rate compared with 100% survival of mice in the α-GalCer alone-treated group (Fig. 5*A*). These observations suggest a partial role for IL-4 in the protection induced by α-GalCer in this model.

Critical role of IL-10 in protection against T. gondii*-induced ileitis*

The contribution of IL-10 production associated with α-GalCer treatment in interfering with the induction of *T. gondii*-induced death was evaluated using genetically deficient and chimeric mice. Strikingly α-GalCer treatment had no beneficial effect on protection in IL-$10^{-/-}$ mice (Fig. 5*B*). These observations suggest a pivotal role for IL-10.

To determine whether IL-10 produced by NKT cells was sufficient to suppress lethal intestinal inflammatory lesions, double-chimeric mice were generated. B6 mice were irradiated and reconstituted by a 50/50% mix of bone marrow cells from Jα281$^{-/-}$ (NKT cell-deficient) and IL-$10^{-/-}$ mice. After reconstitution, the double-chimeric mice expressed a normal immunological phenotype, except for the NKT cells that were IL-$10^{-/-}$ (NKT IL-$10^{-/-}$). These NKT IL-$10^{-/-}$ chimeric mice and their appropriate controls (B6 mice, Jα281$^{-/-}$ and IL-$10^{-/-}$ mice) were treated with α-GalCer the day before infection. NKT IL-$10^{-/-}$ chimeric mice treated with α-GalCer rapidly lost more weight than α-GalCer-treated B6 mice (Fig. 5*C*), indicating that the lack of IL-10 production by the NKT cells alone conferred greater susceptibility to the infection.

However, in contrast to what was expected, the decreased protective effect of α-GalCer treatment in NKT IL-$10^{-/-}$ chimeric mice did not lead to a significant increase in the mortality rate (80% survival; Fig. 5*D*). These results, demonstrating the complete lack of effect of α-GalCer treatment in IL-$10^{-/-}$ mice (Fig. 5*B*), and a reduced effect of this treatment in NKT IL-$10^{-/-}$ chimeric mice (Fig. 5, *C* and *D*), suggested that other cell types might be the source of the IL-10 that is critical for protection. T regulatory cells ($CD4^+CD25^+$) that express the transcription factor FoxP3 and are known as important IL-10 producers were assessed after treatment with α-GalCer and infection. Interestingly, the number of $CD4^+CD25^+$ cells from intestines and MLNs were increased on days 6 and 9, respectively (data not shown), after infection, and this correlates with an increased expression of FoxP3 in the intestine on day 6 and in MLNs on day 9 from B6 mice, but not from Jα281$^{-/-}$ mice (Fig. 6*A*). The sorted $CD4^+CD25^+$ cell subpopulation exhibited IL-10 mRNA expression (data not shown). Whatever the time after infection and the treatment with or without α-GalCer, the sorted NKT cell population failed to express either FoxP3 or CD25. To better characterize the implication of these T regulatory cell subpopulations to the protective process induced by α-GalCer, the effect of this treatment in mice also treated with blocking anti-CD25 Abs was studied. Treatment with anti-CD25 abrogated the protection (Fig. 6*B*), indicating the crucial role of these cells in the anti-inflammatory process induced by treatment with α-GalCer.

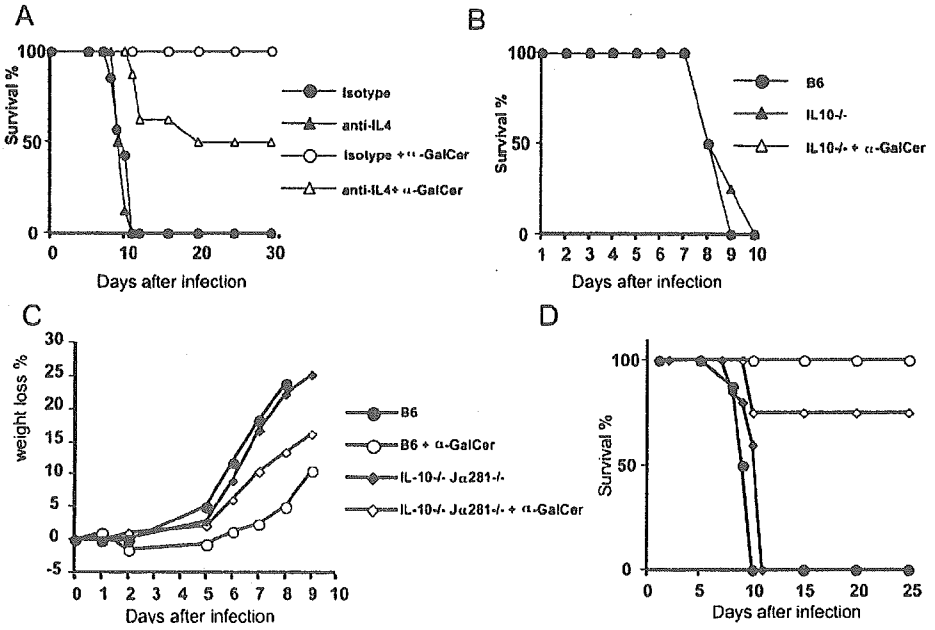

FIGURE 5. Roles of IL-4 and IL-10 in the protective process. *A*, Neutralization of IL-4 the day before α-GalCer treatment partially altered its protective effect. B6 mice were treated with 1 mg of anti-IL-4 Abs 24 h before α-GalCer treatment and 48 h before infection with *T. gondii*. Similarly infected α-GalCer-treated B6 mice and B6 mice treated with rat IgG were used as controls (eight mice per group). The survival rate of these mice was determined. Similar results were obtained in another separate experiment. *B*, α-GalCer treatment fails to protect IL-10$^{-/-}$ mice. IL-10$^{-/-}$ mice were treated (eight mice per group) with 5 μg of α-GalCer i.p. 24 h before infection. As a control, IL-10$^{-/-}$ mice and B6 mice sham-treated with DMSO alone were infected, and the survival rates of all mice were determined. *C* and *D*, IL-10 produced by NKT is partially responsible for the protective effect of α-GalCer. To assess the role of IL-10 produced by NKT cells, chimeric mice were generated. B6 mice were irradiated and then received i.v. 1×10^7 bone marrow cells recovered from femurs and tibias of donor mice. To generate mice in which only NKT cells were devoid of the IL-10 gene, a mixture (50/50%) of bone marrow cells from Jα281$^{-/-}$ mice and IL-10$^{-/-}$ mice was used for reconstitution (12 mice/group). Control mice were reconstituted with bone marrow from B6, Jα281$^{-/-}$, or IL-10$^{-/-}$ mice (six mice per group). Six weeks later, the efficiency of the reconstitution was determined. Chimeric mice were then infected, and weight loss (*C*) and survival rate (*D*) were recorded. This experiment has been performed twice with similar results.

Discussion

In contrast to B6 mice that develop acute lethal ileitis after oral infection with *T. gondii*, mice deficient in NKT cells, although permissive to parasite replication, are more resistant to this severe immunopathological manifestation, suggesting a critical role of these cells in the intestinal inflammation. NKT cells, present in the intestine at early stages after infection, can secrete IFN-γ that will initiate a Th1-like immune response mediating the lethal ileitis. The critical role of IFN-γ was confirmed by studies showing that mice deficient in IFN-γ production do not develop ileitis (27).

Results from this study show that the harmful effect of NKT cells can be neutralized by treatment with a single injection of α-GalCer. When intestinal NKT cells were stimulated by α-GalCer the day before infection, minor intestinal lesions developed, and the mice survived the infection. The beneficial effect of α-GalCer was accompanied by a shift in cytokine production by the intestinal NKT cells toward a Th2 profile (IL-4 and IL-10) and a dramatic increase in CD4$^+$CD25$^+$Foxp3$^+$ cells in MLNs. Depletion of regulatory T cells abrogated the protective effect of treatment with α-GalCer before the infection. This observation indicates that activation of NKT cells by α-GalCer triggers a regulatory T cell response that helps control the inflammatory intestinal disease observed after *T. gondii* infection.

We showed for the first time that conventional CD1d-restricted NKT cells are present in the small intestine of *T. gondii*-infected mice; more precisely, they are located within the lamina propria compartment. They are not associated with IELs in this model, contrary to what was described in previous studies that have identified NK-like T cells within the intraepithelial compartment of the mouse small intestine (35). The presence of unconventional NKT cells, non-CD1d-restricted cells, was also described in the large intestine (36). In this study it was observed that the purified NKT cells were mainly of the CD4$^+$ phenotype, with double-negative CD4$^-$8$^-$ cells making up the difference.

Upon polyclonal or Ag-specific stimulation through the TCR, CD1d-restricted NKT cells have the capacity to produce IL-4 and IFN-γ (11). In this model of pathogen-driven ileitis, we observed, that intestinal CD1d-restricted NKT cells promote an IFN-γ response, as reflected by the marked reduction of IFN-γ mRNA expression at serial time points after infection in Jα281$^{-/-}$ mice devoid of NKT cells compared with wild-type control mice. This early IFN-γ production by intestinal NKT cells may influence the Th1/Th2 balance and thus favor the switch toward a local inflammatory Th1 immune response. Secretion of IFN-γ by intestinal NKT cells may induce DC to secrete IL-12, resulting in an increased production of IFN-γ and TNF-α by lamina propria CD4$^+$ T cells that are important effector cells in the hyperinflammatory process associated with oral *T. gondii* infection. IFN-γ produced by NKT may activate other cell types, such as macrophages and neutrophils (37), that will act on NK cells and CD8 T cells to enhance their IFN-γ production. Our data confirmed the findings of previous studies in which NK1.1$^+$ cells were identified as a source of IFN-γ that is essential to limit parasite replication (32, 46) and also point out their role in triggering an exacerbated IFN-γ response leading to immunopathology.

NKT cells are certainly not the only source of IFN-γ. In Jα281$^{-/-}$ mice, characterized by the absence of NKT cells, a limited amount of IFN-γ was secreted after infection, followed by a

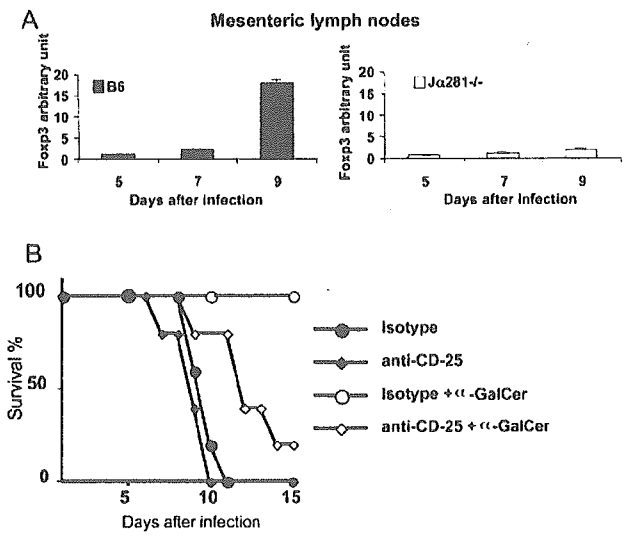

FIGURE 6. Implication of regulatory T cells after α-GalCer treatment. *A*, An increased number of Foxp3 regulatory T cells in MLNs from α-GalCer-treated mice was observed. cDNAs obtained from total MLNs of infected B6 and Jα281$^{-/-}$ mice treated, or not, with α-GalCer were submitted to quantitative real-time PCR with specific primers and probed for Foxp3 and actin (five mice per group). After normalization to actin expression, results were expressed as an increase in Foxp3 expression in α-GalCer-infected B6 or Jα281$^{-/-}$mice compared with infected B6 or Jα281$^{-/-}$ mice. This experiment was repeated twice with similar results. *B*, B6 mice were depleted of CD25^{+} cells with anti-CD25 mAb i.p. 3 days before α-GalCer treatment. Twenty-four hours later, all mice were infected. Similarly infected α-GalCer-treated B6 mice, anti-CD25-treated B6 mice, and B6 mice treated with isotype Abs were used as controls (five mice per group). Results are representative of two independent experiments.

significant increase in cytokine production with time (day 8). This late IFN-γ production indicates that other cells within the responding immune population (e.g., CD4^{+} T cells from the lamina propria) are specifically activated and probably are responsible for the death of 75% of the Jα281$^{-/-}$ mice and the mild inflammation observed in the intestines of surviving mice.

NKT cells can be activated through different pathways. Activation through TCR ligation by CD1d-associated glycolipid is one possibility. Alternatively, IL-12 might activate NKT cells directly, in the absence of TCR engagement (38, 39), or might synergize its effect to that of TCR engagement (40). The activation pathway responsible for NKT cells activation after *T. gondii* infection remains unclear. It is indeed unknown whether TCR engagement by *Toxoplasma* Ag or through recognition of self Ag is required. Recently, Brigl et al. (40) have described a model in which NKT cells in the presence of IL-12 were activated after recognition of self Ags presented by CD1d. IL-12 was first made by DCs in response to microbial products, and this cytokine, in turn, activated NKT cells to up-regulate CD69 expression and IFN-γ production. One of the potential *Toxoplasma* Ag responsible directly or indirectly for NKT activation is the surface Ag-1 (SAG1) protein, the major surface protein of the parasite. The SAG1 molecule induces the dominant Ab response during infection (41) and a strong Th1 immune response characterized by high levels of IFN-γ production by CD4 T cell from the lamina propria and CD8 T lymphocytes (42, 43). SAG1 is a GPI-anchored protein and could be a potential ligand for CD1d molecule.

The hypothesis of the activation of NKT cells through TCR recognition of CD1d-presented Ag is attractive in our model. However, after oral infection with *T. gondii*, CD1$^{-/-}$ (B6 background) mice developed an acute and lethal ileitis within 7 days despite the absence of NKT cells. This suggests that CD1d may act via several alternative pathways. Besides its activity on NKT cell activation, CD1d is important for the activation of IELs (33) that down-regulate the intestinal inflammation after *T. gondii* infection. Indeed, upon Ag activation these IEL secrete copious amounts of TGF-β that participate in the maintenance of gut homeostasis (28). The lack of CD1 expression leads to the absence of protective IELs, and the absence of regulatory mechanisms overcome the absence of inflammatory NKT cells. In addition, the CD1d molecule is expressed on both the apical and the basolateral membranes of intestinal epithelial cells (44), and its ligation induces IL-10 secretion by these cells (45). Thus, the regulation of CD1 expression and its recognition by the TCR could play important roles in the regulation of intestinal inflammatory processes.

In this model of pathogen-driven inflammatory disease, NKT cells are important for the initiation of the robust Th1 inflammatory immune response in the intestine after oral parasite infection. Alternatively, α-GalCer and related glycolipids can modulate NKT cell responses toward a Th2-like profile (11, 12, 46). Our observations demonstrate that α-GalCer treatment has an impact on the intestinal immune response by shifting the cytokine profile production by NKT cells toward a Th2 phenotype, resulting in orientation of the lamina propria CD4 response. A single dose of α-GalCer prevented the development of lethal ileitis after infection with *T. gondii*. This treatment resulted in a Th2 immune response characterized by the production of IL-4, IL-10, and IL-13 by intestinal NKT cells. The major cytokine implicated in this protection is IL-10, because the beneficial effect of α-GalCer treatment was completely abrogated in IL-10-deficient mice.

Our data are in full agreement with previous work reporting the high susceptibility of IL-10-deficient mice to the development of lethal ileitis after oral *T. gondii* infection (47). This susceptibility is associated with the defect of T cells to produce IL-10, because mice with an inactivation of the IL-10 gene restricted to T cells generated by Cre/loxP-mediated targeting of the IL-10 gene succumb to severe immunopathology upon infection with *T. gondii* (48).

IL-10 secreted by NKT cells also participated in the protective effect of α-GalCer treatment, because double-chimeric mice in which NKT cells alone were impaired in IL-10 secretion were more susceptible to the development of ileitis than controls after α-GalCer injection. However, other IL-10-producing cells are also implicated, because treatment with α-GalCer reduced the mortality of these double-chimeric mice. Regulatory CD25^{+} T cells are the likely candidates, because they are present in the intestine, and the anti-CD25 treatment blocked the protective effect of α-GalCer injection.

IL-10 produced by NKT cells has been shown to exert an important regulatory function in experimental models of different pathologies, such as diabetes (49) and allergic encephalomyelitis (50). The link between the shift in the cytokine profile produced by NKT cells toward a Th2 profile and the activation of regulatory CD4^{+} T cells is as yet unknown. IL-10-producing CD4^{+} NKT cells are involved in the generation of regulatory CD8^{+} T cells after Ag exposure in the anterior chamber of the eye (51). Several reports indicate that NKT cells may contribute to immunoregulation via DC maturation (52). DC maturation in the presence of IL-10 may equally induce T regulatory 1 or Th3 regulatory T cells (53). Secretion of IL-4 and IL-10 by intestinal NKT cells after α-GalCer treatment may act directly on local DCs during induction of the polarization of the immune response and promote a Th2 profile. There is evidence that DCs that mature in the presence of NKT cells produce greater amounts of IL-10

and lose the ability to secrete IL-12, a phenotype consistent with a tolerogenic function (17).

The participation of IL-4 in this process cannot be ruled out. The role of IL-4 seems to be complex in toxoplasmosis. Our data indicate that neutralization of IL-4 cannot render α-GalCer-treated mice as susceptible as wild-type, infected, untreated mice, indicating the participation of other cytokine, such as IL-10. In addition, these experiments might indicate, as suggested by Nickdel et al. (54), that IL4-deficient mice are more resistant than wild-type mice to the development of ileitis. However, our data for IL-4 corroborate previous findings reporting that treatment with α-GalCer or OCH (a synthetic glycolipid that has shorter hydrophobic chain) improves mucosal Th1/Th2 cytokine balance by increasing IL-10 and IL-4 production and prevents experimental colitis in mice (55).

The important role played by NKT cells in the regulation of the intestinal immune response has also been previously suggested in a colitis model induce by chemical agents such as dextran sodium sulfate (56) or oxazolone (57). The pathogenic pathway leading to tissue injury in dextran sodium sulfate-induced colitis and, by extension, in Crohn's disease was attributed to production of Th1 cytokines such as IFN-γ and to the presence of NK1.1$^+$ T cells (56). However, the pathogenic pathway leading to tissue injury in oxazolone colitis was also associated with NKT cells secreting IL-13 (57).

The presence of IL-10-secreting T regulatory lymphocytes has been associated with regulation of intestinal inflammation (33), and in our model these cells may be ultimately responsible for the protective effect seen after treatment with α-GalCer. These data illustrate the dual potential of NKT cells in orienting distinct (i.e., Th1 or Th2) immune responses depending on the stimuli used.

After activatión with *T. gondii*, NKT cells are important mediators of the immune response via a robust IFN-γ-mediated effect that limits parasite replication and allows for parasite clearance. However, this early and influential response is not without drawbacks and can be detrimental to the host. This response, when uncontrolled, leads to the development of an acute inflammatory process and death within 7 days of infection in this experimental model of pathogen-driven ileitis. Our data highlight the crucial role of NKT cells derived from the gut in the modulation of intestinal homeostasis.

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Disclosures

The authors have no financial conflict of interest.

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