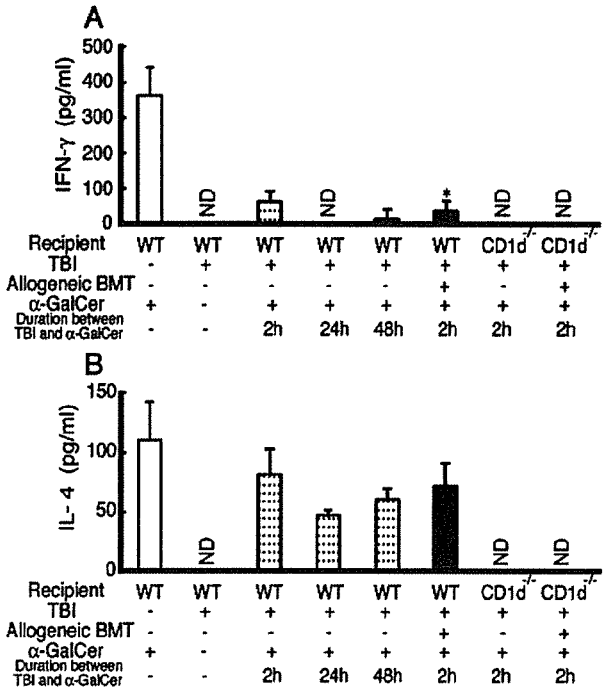


FIGURE 1. Cytokine responses to α-GalCer in lethally irradiated mice with or without BMT. WT and CD1d$^{-/-}$ B6 mice received 13 Gy TBI. Two, 24, or 48 h later, mice were injected i.p. with α-GalCer (100 μg/kg) or diluent. A cohort of animals were transplanted with allogeneic BM cells ($5 \times 10^6$) and spleen cells ($5 \times 10^6$) 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.

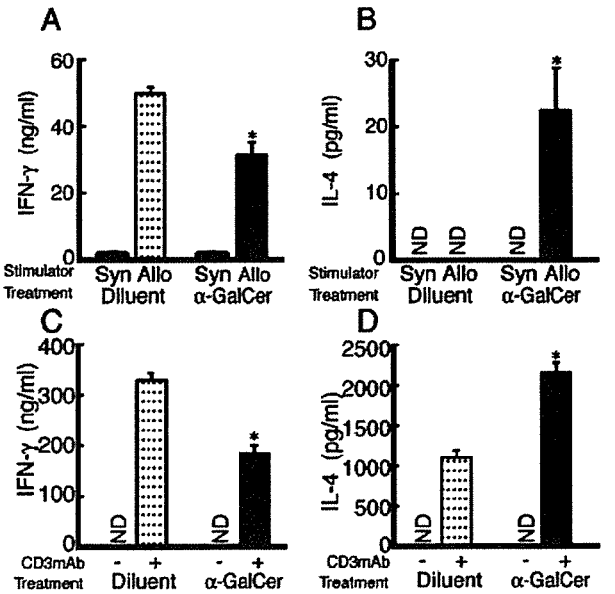


FIGURE 2. Administration of α-GalCer to recipients of allogeneic BMT polarizes donor T cells toward Th2 cytokine secretion. Lethally irradiated (13 Gy) B6 mice were transplanted with BM cells ($5 \times 10^6$) and spleen cells ($5 \times 10^6$) isolated from BALB/c mice, followed by injection of either 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 $5 \times 10^4$/well and were stimulated with $1 \times 10^5$/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

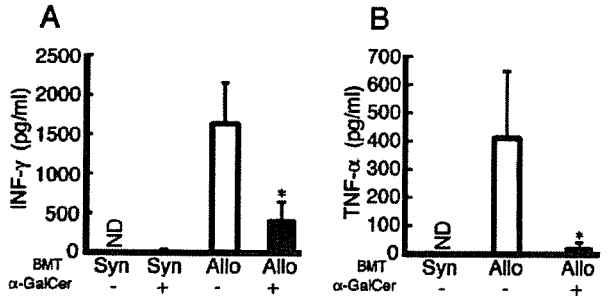


**FIGURE 3.** A single injection of α-GalCer to recipients of allogeneic BMT markedly reduces serum levels of IFN-γ and TNF-α. WT B6 mice were transplanted as in Fig. 2. Sera ($n$ = 3–10/group) were obtained from diluent-treated (□) and α-GalCer-treated (■) recipients on day 6 after BMT, and serum levels 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

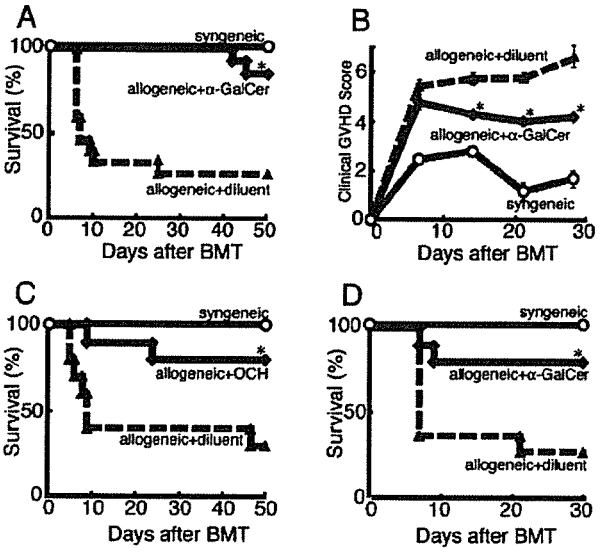


**FIGURE 4.** A single injection of NKT ligands to BMT recipients modulates acute GVHD. BMT was performed as in Fig. 2. *A*, Survival curves of syngeneic control group (○, solid line; $n$ = 9); allogeneic, diluent-treated recipients (▲, dotted line; $n$ = 15); and allogeneic, α-GalCer-treated recipients (♦, 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.

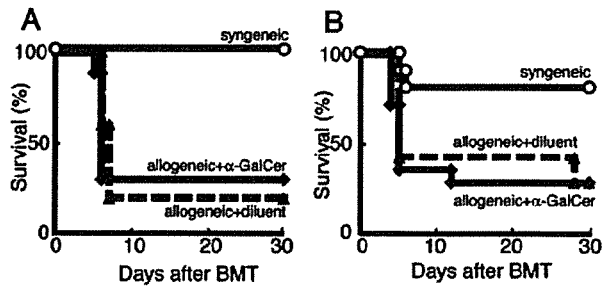


**FIGURE 5.** Host NKT cells and host IL-4 production are required for suppression of GVHD by α-GalCer. *A*, Lethally irradiated CD1d$^{-/-}$ B6 mice were transplanted as in Fig. 2. Survival curves of syngeneic control group (○, solid line; $n$ = 6); allogeneic, diluent-treated recipients (▲, dotted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

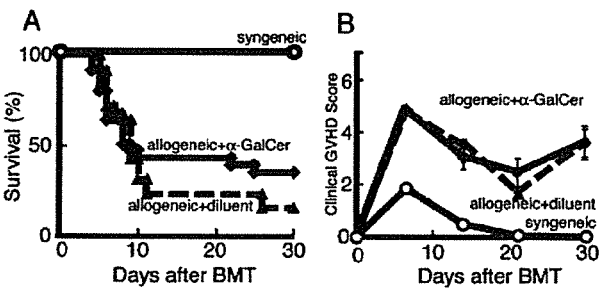


**FIGURE 6.** The protective effects of α-GalCer against GVHD are dependent upon the STAT6 pathway of donor T cells. Lethally irradiated B6 mice were transplanted with TCD-BM cells (4 × 10$^6$) from WT BALB/c mice and spleen cells (5 × 10$^6$) from STAT6$^{-/-}$ BALB/c mice. *A*, Survival curves of the 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-$\gamma$ in the lamina propria. The implication of NKT cells was confirmed by the observation that NKT cell-deficient mice (J$\alpha$281$^{-/-}$) are more resistant than C57BL/6 mice to the development of lethal ileitis. J$\alpha$281$^{-/-}$ mice failed to overexpress IFN-$\gamma$ in the intestine early after infection. This detrimental effect of NKT cells is blocked by treatment with $\alpha$-galactosylceramide, which prevents death in C57BL/6, but not in J$\alpha$281$^{-/-}$, 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 $\alpha$-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 V$\alpha$14-J$\alpha$281 TCR $\alpha$-chain paired with V$\beta$8, -7, or -2 TCR $\beta$-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 $\alpha$-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 $\alpha$-galactosylceramide ($\alpha$-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-$\gamma$ 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-$\gamma$) 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 $\alpha$-GalCer, NKT cells exhibit the ability to proliferate and to produce both Th1 and Th2 cytokines (9, 19). However administration of $\alpha$-GalCer at the time of priming of mice with Ag results in the generation of only Ag-specific Th2 cells. Thus, $\alpha$-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-$\gamma$ 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-$\gamma$ 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: $\alpha$-GalCer, $\alpha$-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 J$\alpha$$281^{-/-}$ mice by Dr. M. Taniguchi (Riken Research Center for Allergy and Immunology, Yokohama, Japan) (9), V$\alpha$14Tg 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 ($1 \times 10^6$) were injected i.v. into J$\alpha$$281^{-/-}$ 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 ($1 \times 10^7$) 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 J$\alpha$$281^{-/-}$ mice and IL-$10^{-/-}$ mice was prepared. Control mice received cells from B6, J$\alpha$$281^{-/-}$ 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 $1 \times 10^6$ 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 ($1 \times 10^6$) 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

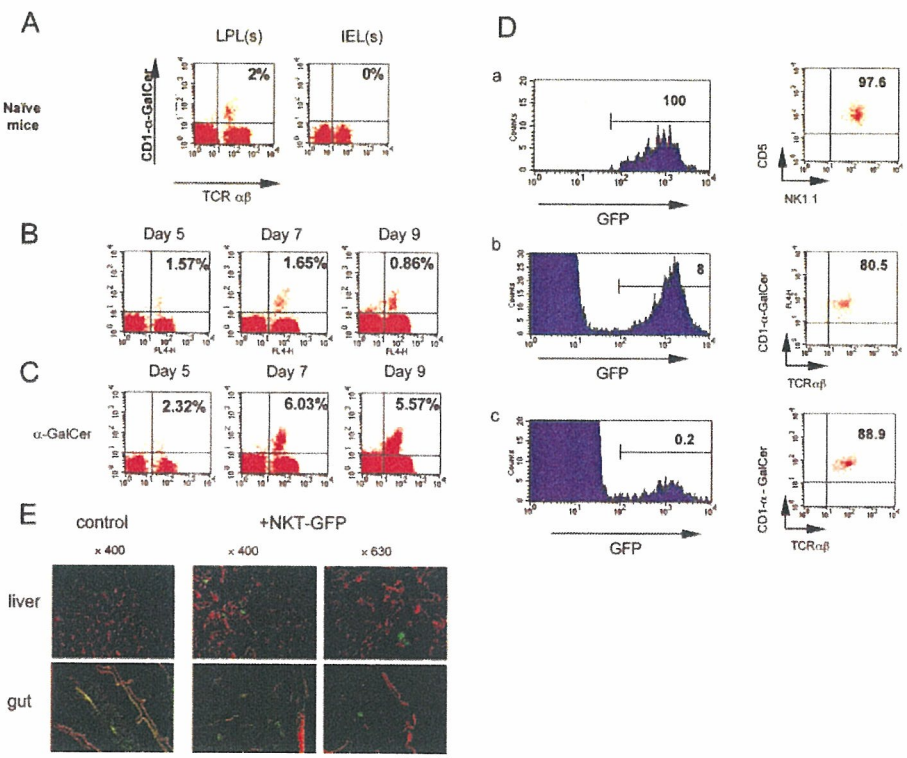


**FIGURE 1.** NKT cells are present in the lamina propria compartment. *A*, Representative FACS profiles showing Vα14 CD1d/α-GalCer$^+$TCRαβ$^+$ cells obtained from LPL and IEL suspension from naive mice. The numbers indicate the proportion of tetramer-positive T cells in the lymphocyte gate. This analysis 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$\alpha$281$^{-/-}$ 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$\alpha$281$^{-/-}$ 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$\alpha$281$^{-/-}$ 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 (V$\alpha$14Tg mice) were infected. Both B6 and V$\alpha$14Tg mice died within 7–10 days when infected with 35 cysts (Fig. 2*A*). However, in the experiment using a lower dose of cysts (10 cysts/mouse), all the V$\alpha$14Tg 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-$\gamma$ production after* T. gondii *infection*

IFN-$\gamma$ 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

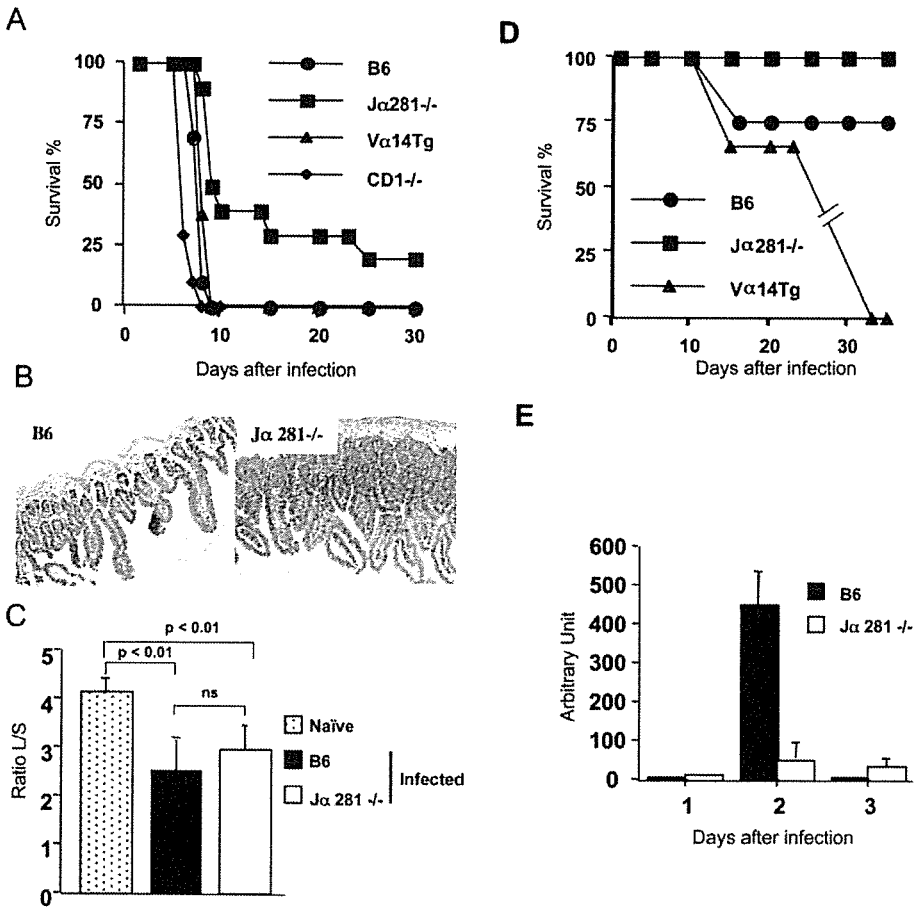


**FIGURE 2.** NKT cells are involved in the development of acute inflammatory ileitis in B6 mice. *A*, Survival rate of B6, J$\alpha$281$^{-/-}$, V$\alpha$14Tg, and CD1d $^{-/-}$ mice after challenge with 35 cysts of *T. gondii* ($n$ = 10/group). Data are representative of three independent experiments with similar results. *B*, Intestinal H&E histology from B6 and J$\alpha$281$^{-/-}$ 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$\alpha$281$^{-/-}$ 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$\alpha$281$^{-/-}$, V$\alpha$14Tg after challenge with 10 cysts of *T. gondii* ($n$ = 5/group). Data are representative of two independent experiments with similar results. *E*, Early IFN-$\gamma$ mRNA expression in the intestine after infection is dependant upon the presence of NKT cells. Samples from the ileum of B6 and J$\alpha$281$^{-/-}$ mice were analyzed for mRNA expression of IFN-$\gamma$ 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 (J$\alpha$281$^{-/-}$) were more resistant to the development of lethal ileitis after *T. gondii* infection, the expression of IFN-$\gamma$ in their intestines was measured at different times after oral challenge with the cysts. Between days 2 and 3 after infection, IFN-$\gamma$ mRNA expression peaked in the intestine of B6 mice, and there was a significant difference in IFN-$\gamma$ mRNA expression between B6 mice and J$\alpha$281$^{-/-}$ mice. By quantitative RT-PCR, the level of mRNA expression in B6 mice was 9–10 times higher than that in J$\alpha$281$^{-/-}$ mice (Fig. 2*E*). Over time, inflammatory cytokine production in J$\alpha$281$^{-/-}$ mice may increase, contributing in the delayed time to death due to lethal intestinal inflammation. The lack of early production of IFN-$\gamma$ might also explain the 2-fold increase in parasite burden in J$\alpha$281$^{-/-}$ 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 $\alpha$-GalCer protects against the development of lethal ileitis*

Because $\alpha$-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 $\alpha$-GalCer the day before infection. Up to 30 days after infection, this treatment prevented death in both B6 (100%) and V14$\alpha$Tg mice overexpressing NKT cells (80%; Fig. 3*A*). Histological examination performed on day 7 after infection revealed that treatment with $\alpha$-GalCer interfered with the development of ileitis (Fig. 3, *B* and *C*). In addition, B6 mice treated with $\alpha$-GalCer exhibited less weight loss compared with untreated infected controls (Fig. 3*D*). To assess the cell population targeted by $\alpha$-GalCer treatment, NKT-deficient mice (J$\alpha$281$^{-/-}$) were treated with $\alpha$-GalCer the day before infection. This treatment had no effect on the infection outcome in J$\alpha$281$^{-/-}$ 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 $\alpha$-GalCer modulates the functional abilities of NKT cells. Treatment with $\alpha$-GalCer was not directly toxic to the parasite, because there was no difference in parasite burden in J$\alpha$281$^{-/-}$ mice treated or not treated on day 30 after infection (data not shown). Treatment with $\alpha$-GalCer 2 days after infection failed to impact the development of the hyperinflammatory response in small intestine.

### *Treatment with $\alpha$-GalCer induces preferential production of IL-4 and IL-10 in* T. gondii*-infected mice*

One of the consequences of $\alpha$-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 $\alpha$-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 $\alpha$-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 $\alpha$-GalCer-treated mice was measured at serial time points after infection. mRNA for IFN-$\gamma$ was also significantly decreased (10-fold) in $\alpha$-GalCer-treated mice (data not shown). This result demonstrated a shift in cytokine production toward a Th2-like profile after treatment with $\alpha$-GalCer and infection and a decline in the Th1-like immune response. To better assess the contribution of intestinal NKT cells in this shift, $\alpha$-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

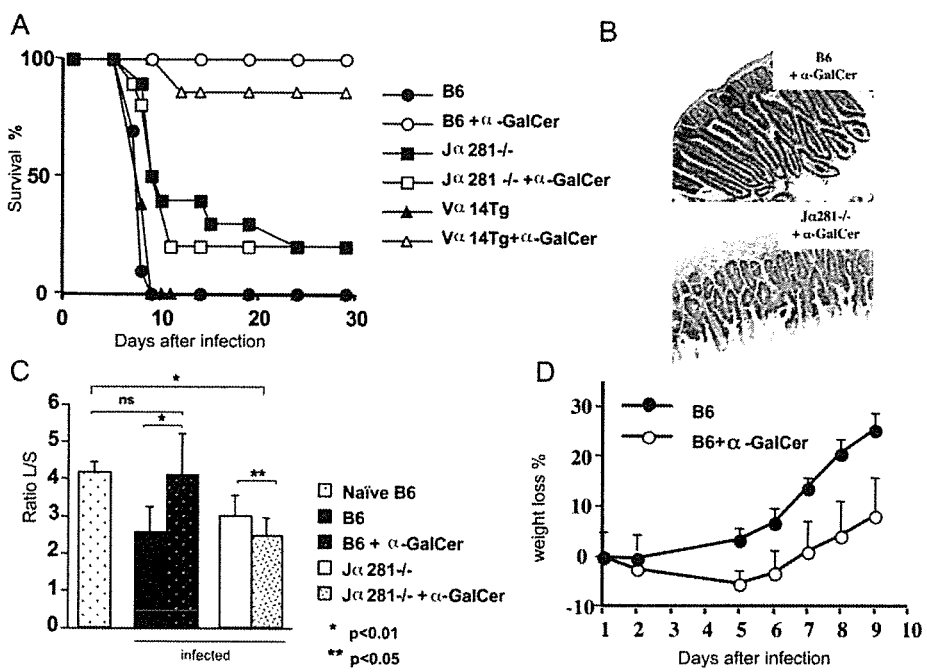


**FIGURE 3.** $\alpha$-GalCer treatment protects the infected mice against the development of lethal ileitis. *A*, Survival rates of B6, J$\alpha$281$^{-/-}$, and V$\alpha$14Tg mice after i.p. administration of 5 $\mu$g of $\alpha$-GalCer the day before challenge with *T. gondii* ($n$ = 10/group). Results are representative of two independent experiments. *B*, Intestinal H&E histology of $\alpha$-GalCer-treated or untreated mice on day 7 after infection (magnification, ×200). *C*, Intestinal lesions in $\alpha$-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 $\alpha$-GalCer exhibited only mild weight loss compared with untreated infected controls. Infected B6 mice treated, or not, with $\alpha$-GalCer were weighed daily. Weight loss is expressed as a percentage of the animal's initial weight.

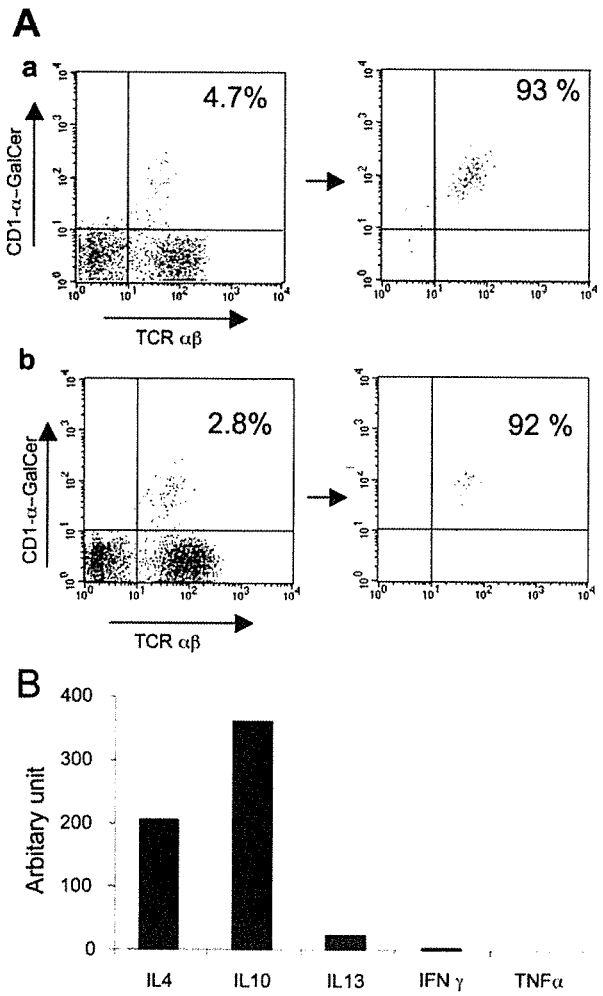


**FIGURE 4.** NKT cells produce Th2 cytokine after α-GalCer administration and *T. gondii* infection. *A*, NKT cell populations were isolated from the lamina propria of mice treated (*a*) or not treated (*b*; two mice per group) with α-GalCer on day 8 after infection. The cells were then purified on 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 J$\alpha$281$^{-/-}$ (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, J$\alpha$281$^{-/-}$ 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 J$\alpha$281$^{-/-}$ 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.

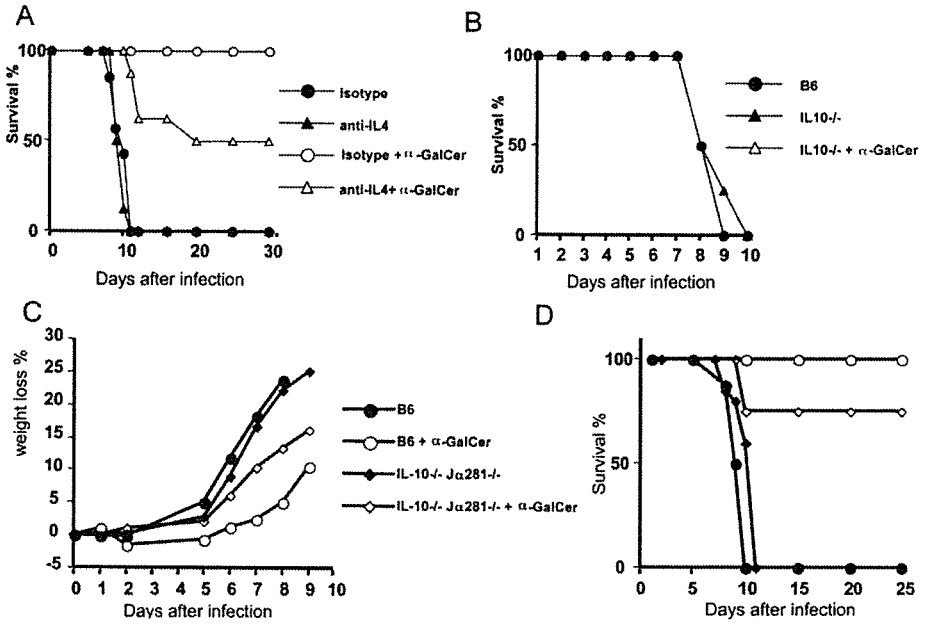


**FIGURE 5.** Roles of IL-4 and IL-10 in the protective process. *A*, Neutralization of IL-4 the day before α-GalCer treatment partially altered its protective effect. B6 mice were treated with 1 mg of anti-IL-4 Abs 24 h before α-GalCer treatment and 48 h before infection with *T. gondii*. Similarly 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. $1 \times 10^7$ 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 Jα$281^{-/-}$ mice and IL-$10^{-/-}$ mice was used for reconstitution (12 mice/group). Control mice were reconstituted with bone marrow from B6, Jα$281^{-/-}$, 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 Jα$281^{-/-}$ 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 Jα$281^{-/-}$ mice, characterized by the absence of NKT cells, a limited amount of IFN-γ was secreted after infection, followed by a

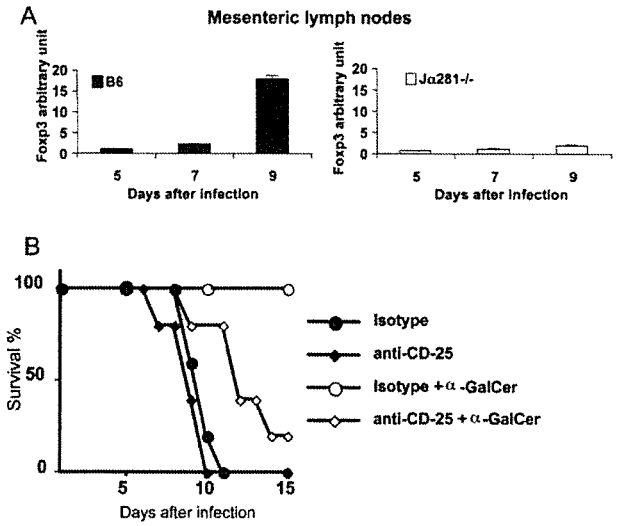


**FIGURE 6.** Implication of regulatory T cells after α-GalCer treatment. *A*, An increased number of Foxp3 regulatory T cells in MLNs from α-GalCer-treated mice was observed. cDNAs obtained from total MLNs of infected B6 and $J\alpha281^{-/-}$ mice treated, or not, with α-GalCer were submitted to quantitative 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 $J\alpha281^{-/-}$mice compared with infected B6 or $J\alpha281^{-/-}$ 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 $J\alpha281^{-/-}$ 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 activation 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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# IFN-γ–mediated negative feedback regulation of NKT-cell function by CD94/NKG2

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**Activation of invariant natural killer T (iNKT) cells with CD1d-restricted T-cell receptor (TCR) ligands is a powerful means to modulate various immune responses. However, the iNKT-cell response is of limited duration and iNKT cells appear refractory to secondary stimulation. Here we show that the CD94/NKG2A inhibitory receptor plays a critical role in down-regulating iNKT-cell responses. Both TCR and NK-cell receptors expressed by iNKT cells were rapidly down-modulated by priming with α-galactosylceramide (α-GalCer) or its analog OCH [(2*S*,3*S*,4*R*)-1-*O*-(α-D-galactopyranosyl)-*N*-tetracosanoyl-2-amino-1,3,4-nonanetriol)]. TCR and CD28 were re-expressed more rapidly than the inhibitory NK-cell receptors CD94/NKG2A and Ly49, temporally rendering the primed iNKT cells hyperreactive to ligand restimulation. Of interest, α-GalCer was inferior to OCH in priming iNKT cells for subsequent restimulation because α-GalCer–induced interferon γ (IFN-γ) up-regulated Qa-1$^b$ expression and Qa-1$^b$ in turn inhibited iNKT-cell activity via its interaction with the inhibitory CD94/NKG2A receptor. Blockade of the CD94/NKG2–Qa-1$^b$ interaction markedly augmented recall and primary responses of iNKT cells. This is the first report to show the critical role for NK-cell receptors in controlling iNKT-cell responses and provides a novel strategy to augment the therapeutic effect of iNKT cells by priming with OCH or blocking of the CD94/NKG2A inhibitory pathway in clinical applications. (Blood. 2005;106:184-192)**



## Introduction

Natural killer T (NKT) cells are a special T-cell population coexpressing the T-cell receptor (TCR) and NK-cell receptors such as NK1.1.[1-4] Invariant NKT (iNKT) cells express a Vα14Jα18 chain (Vα24Jα15 in humans) and a semivariant TCR β-chain that is largely biased toward Vβ8.2 (Vβ11 in humans), Vβ2, and Vβ7.[1-4] This TCR recognizes glycolipid antigens, such as α-galactosylceramide (α-GalCer), its analogs including OCH (a sphingosine-truncated analog of α-GalCer; (2*S*,3*S*,4*R*)-1-*O*-(α-D-galactopyranosyl)-*N*-tetracosanoyl-2-amino-1,3,4-nonanetriol), and isoglobotrihexosylceramide (iGb3), presented on the major histocompatibility complex (MHC) class I–like molecule CD1d.[1-10] In addition, costimulatory signals, mediated through antigen-presenting cells that express CD80/86 interacting with CD28 expressed by iNKT cells, critically regulate iNKT-cell activation in a similar manner to conventional T cells.[11,12] One of most striking characteristics of iNKT cells is their ability to promptly secrete various cytokines, including both interferon γ (IFN-γ) and interleukin 4 (IL-4), after their encounter with antigens (Ag's),[1-4] which is reminiscent of effector/memory T cells. Accordingly, iNKT cells are thought to be potent immunoregulatory cells and their activation by ligands, such as α-GalCer and OCH, has been shown to be a powerful means to modulate various immune responses, including protective immunity, autoimmunity, and antitumor immunity.[1-4,6,9,11,13,14]

Despite an accumulation of studies concerning iNKT-cell activation of bystander immune cells, relatively little is known about the fate of Ag-primed iNKT cells themselves. Previous studies have shown that iNKT cells disappear quickly after their activation following TCR ligation or IL-12 stimulation.[15-17] This phenomenon was initially attributed to increased activation-induced cell death (AICD) of iNKT cells, consistent with repopulation and homeostatic proliferation of peripheral iNKT cells after their rapid recruitment from the bone marrow.[15] More recently, however, several studies have reported that down-regulation of TCR and NK1.1 cell surface expression on iNKT cells is the primary reason for the apparent disappearance of iNKT cells following α-GalCer treatment.[18-20] Indeed a substantial iNKT-cell proliferation was observed in peripheral lymphoid organs following

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α-GalCer treatment.[18-20] These findings suggested an interesting possibility that Ag-primed iNKT cells may develop an effector/memory subpopulation, which exerts even more potent function than unprimed iNKT cells. Here, we now demonstrate that priming of iNKT cells with their TCR ligands induces a dynamic modulation of TCR, NK-cell receptors (NK1.1, CD94/NKG2, NKG2D, and Ly49), and a costimulatory receptor (CD28). Differential kinetics of re-expression of these molecules on the cell surface temporally renders the primed iNKT cells hyperresponsive to secondary Ag stimulation. Of importance, the recall response of α-GalCer–primed iNKT cells is strictly regulated by an IFN-γ–dependent negative feedback mechanism where IFN-γ upregulates Qa-$1^b$ expression and subsequent ligation of CD94/NKG2 inhibits iNKT-cell activity. Although Qa-$1^b$ can interact with NKG2A, NKG2C, and NKG2E, it has been previously reported that the majority of CD94/NKG2 expressed in mice is the inhibitory CD94/NKG2A receptor and that the Qa-$1^b$–CD94/NKG2A interaction plays a critical role in negative regulation of NK-cell responses to self.[21-23] We confirm that iNKT cells preferentially express CD94/NKG2A and hence blockade of the Qa-$1^b$–NKG2A interaction markedly augmented recall responses of primed iNKT cells and primary responses of naive iNKT cells to α-GalCer. These findings are an important step to improve the efficacy of iNKT-cell–targeting therapeutics against tumor, infection, and autoimmune diseases since they demonstrate a means to modulate the adjuvant nature of iNKT cells by combined treatment with iNKT-cell glycolipid ligands and antagonistic monoclonal antibodies (mAbs).

## Materials and methods

### Mice

Wild-type (WT) C57BL/6 (B6) mice were obtained from Charles River Japan (Yokohama, Japan). IFN-γ–deficient (IFN-$\gamma^{-/-}$) B6 mice were kindly provided by Y. Iwakura (University of Tokyo).[24] All mice were maintained under specific pathogen-free conditions and used in accordance with the institutional guidelines of Juntendo University.

### Reagents

A synthetic form of α-GalCer was obtained from Kirin Brewery (Gunma, Japan) and OCH was derived as described previously.[6] In most experiments, mice were intraperitoneally injected with 2 μg of α-GalCer or OCH in 200 μL of phosphate-buffered saline (PBS) for priming and boosting. Dimethyl sulfoxide (DMSO; 0.1%) was used as the vehicle control. Phycoerythrin (PE)–conjugated tetrameric CD1d molecules loaded with α-GalCer (α-GalCer/CD1d) were prepared as described.[17] The anti-NKG2A/C/E (NKG2) mAb, 20d5, and the anti-NKG2D mAb (CX5) were generated as described previously.[21,25] Fab fragments of 20d5 and anti-Qa-$1^b$ mAb (BD Pharmingen, San Diego, CA) were prepared using the Fab preparation kit (Pierce, Rockford, IL) as described.[26]

### Flow cytometric analysis

Mononuclear cells (MNCs) were prepared from spleen and liver as described.[11] Cells were first preincubated with antimouse CD16/32 (2.4G2) mAb to avoid nonspecific binding of mAbs to FcγR. Surface and intracellular expression of molecules by iNKT cells were analyzed on electronically gated α-GalCer/CD1d tetramer$^+$ cells on 4-color flow cytometry using a FACSCaliber (BD Bioscience, San Jose, CA). Intracellular staining was performed with a BD Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer's instructions. Intracellular TCR in NKT cells was detected with a mixture of PE-conjugated antimouse Vβ2 TCR mAb (B20.6), anti-Vβ7 TCR mAb (TR310), and anti-Vβ8 TCR mAb (F23.1), or α-GalCer–loaded recombinant soluble dimeric mouse CD1d:immunoglobulin (CD1d:Ig; BD Pharmingen) and PE-conjugated anti-mouse IgG1 mAb (A85-1; BD Pharmingen). Surface and intracellular molecules were analyzed on electronically gated intracellular Vβ2/7/8$^+$ cells 1 day after α-GalCer or OCH injection. Surface and intracellular molecules were stained with fluorescein isothiocyanate (FITC)– or allophycocyanin (APC)–conjugated NK1.1 mAb (PK136); FITC- or biotin-conjugated antimouse CD94 mAb (18d3); FITC- or biotin-conjugated antimouse NKG2A/C/E (NKG2) mAb (20d5); biotin-conjugated antimouse NKG2A$^{B6}$ (16a11); FITC-conjugated antimouse Ly49 mAbs (anti-Ly49D mAb [4E5] or a mixture of anti-Ly49A$^{B6}$ [A1], anti-Ly49C/I mAb [5E6], anti-Ly49G2 mAb [4D11], and antimouse Ly49I mAb [YL1-90]); biotin-conjugated anti-NKG2D mAb (CX5); APC- or biotin-conjugated anti-mouse CD28 mAb (37.51); biotin-conjugated antimouse Qa-$1^b$ mAb (6A8.6F10.1A6); FITC-, PE-, PE-cyanin 5 (PE-Cy5)–, APC-, or biotin-conjugated isotype-matched control mAbs (G155-178, MOPC-31C, R35-95, A95-1, R3-34, and Ha4/8); and Cy5-conjugated streptavidin. All of these reagents were purchased from BD Pharmingen, except for antimouse CD94 mAb, anti-NKG2A$^{B6}$ mAb, anti-NKG2D mAb, and anti-CD28 mAb from eBioscience (San Diego, CA).

### Cell preparation and in vitro stimulation

Freshly isolated splenic MNCs from vehicle-, α-GalCer–, or OCH-primed mice ($5 \times 10^5$) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, and 25 mM $NaHCO_3$ in humidified 5% $CO_2$ at 37°C in 96-well U-bottom plates (Corning, Corning, NY) as previously described.[11] Cells were stimulated with 100 ng/mL α-GalCer, OCH, or vehicle (0.1% DMSO) in the presence or absence of 10-μg/mL Fab fragments of isotype-matched control mAbs, anti-NKG2 mAb, or antimouse Qa-$1^b$ mAb, or intact antimouse CD80 (16-10A1) and antimouse CD86 (PO 3.1) mAbs (eBioscience). After 24 to 48 hours, cell-free culture supernatants were harvested to determine IFN-γ and IL-4 levels by enzyme-linked immunosorbent assay (ELISA).

### Coculture of iNKT cells and DCs

Freshly isolated hepatic MNCs were stained with PE-conjugated α-GalCer/CD1d tetramer, and positive cells were enriched by autoMACS using anti-PE microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Enriched iNKT cells were then sorted on a FACS Vantage (BD Bioscience) to obtain highly purified (98%-99%) iNKT cells. Splenic dendritic cells (DCs) were prepared according to the reported method.[27,28] Purified iNKT cells ($10^5$) and DCs ($5 \times 10^4$) were cocultured as previously described[11,29,30] with 100 ng/mL α-GalCer or vehicle (0.1% DMSO) in the presence or absence of 10-μg/mL Fab fragments of isotype-matched control mAbs, anti-NKG2A mAb, or anti-Qa-$1^b$ mAb. After 24 to 72 hours, cell-free culture supernatants were harvested to determine IFN-γ and IL-4 levels by ELISA.

### ELISA

IFN-γ and IL-4 levels in the culture supernatants or the sera were determined by using mouse IFN-γ– or IL-4–specific ELISA kits (OptEIA; BD Bioscience Pharmingen) according to the manufacturer's instructions.

### Cytotoxicity assay

Cytotoxic activity was tested against NK-cell–sensitive YAC-1 cells and NK-cell–resistant P815 cells by a standard 4-hour $^{51}Cr$-release assay as previously described.[11] Effector cells (hepatic and splenic MNCs) were prepared from mice 24 hours after the last intraperitoneal injection of α-GalCer, OCH, or vehicle. Some mice were administered with 300 μg of isotype-matched control Ig or anti-NKG2 mAb intraperitoneally 2 days before the last α-GalCer injection. Specific cytotoxicity was calculated as previously described.[11]

### Experimental lung metastases

B6 mice were intraperitoneally injected with OCH, α-GalCer, or vehicle, and then intravenously inoculated with B16 melanoma cells ($1 \times 10^5$,

$3 \times 10^5$, or $5 \times 10^5$/200 μL) 2 hours later on day 0. B16 melanoma cells were prepared as previously described.[11,16] Some mice were primed 2.5 days earlier with OCH or α-GalCer. Some mice were administered with 300 μg of isotype-matched control Ig or anti-NKG2 mAb intraperitoneally 2 days before the last α-GalCer injection. On day 14, the number of tumor colonies on the lungs was counted under a dissecting microscope (Olympus, Tokyo, Japan).

### Statistical analysis

Data were analyzed by a 2-tailed Student *t* test. *P* values less than .05 were considered significant.

## Results

### Modulation of costimulatory and NK-cell receptors on iNKT cells upon priming with TCR ligands

We analyzed the modulation of TCR, inhibitory NK-cell receptors (CD94/NKG2 and Ly49A/C/I/G2), activating NK-cell receptors (NK1.1, NKG2D, and Ly49D), and a costimulatory receptor (CD28) on iNKT cells after in vivo priming with synthetic TCR ligands, α-GalCer or OCH. Amongst liver iNKT cells from B6 WT mice, approximately 50% expressed CD94/NKG2 and NKG2D, less than 10% expressed Ly49A/C/I/G2, less than 2% expressed Ly49D, and all constitutively expressed CD28 (Figure 1A). Staining with an NKG2A-specific mAb showed that CD94/NKG2 expressed on iNKT cells was mainly composed of NKG2A (data not shown) but not NKG2C or NKG2E, as previously reported.[22] Upon priming with α-GalCer or OCH, α-GalCer/CD1d tetramer$^+$ iNKT cells seemingly began to disappear within 6 hours (data not shown) and almost completely disappeared at 16 to 24 hours, as previously reported.[15-20] Consistent with recent reports,[18-20] intracellular staining with anti-Vβ2/7/8 mAbs, detecting the predominant TCR β-chains expressed by iNKT cells, clearly showed the presence of liver iNKT cells expressing intracellular TCR at 24 hours after α-GalCer or OCH priming. NK-cell receptors and CD28 were also internalized, although some retention of cell surface CD28 was still detected (Figure 1A). Although staining intensity was relatively weak, intracellular staining with α-GalCer–loaded recombinant soluble dimeric mouse CD1d:Ig also demonstrated the internalized α-GalCer/CD1d–specific TCR coexpressed with intracellular NK1.1 one day after α-GalCer (Figure 1B) or OCH injection (data not shown). Similar results were obtained with spleen MNCs after in vivo priming and with liver MNCs after in vitro priming (data not shown).

After 2 to 3 days, the primed iNKT cells re-expressed TCR and CD28 on their surface. By contrast, a reduced level of surface NK-cell receptors (NK1.1, CD94/NKG2, Ly49, and NKG2D) was maintained for at least 3 to 4 days. After 5 to 7 days, some iNKT cells still expressed a low level of surface NK1.1, but another iNKT-cell population expressed relatively higher levels of NK1.1 compared with naive iNKT cells. After activation, the proportions of CD94/NKG2-, Ly49-, or NKG2D-expressing iNKT cells increased, and the expression levels of CD94/NKG2 and Ly49 were relatively higher than those found on naive iNKT cells. Again, CD94/NKG2 on these iNKT cells was mainly composed of NKG2A as estimated by staining with NKG2A-specific mAb. Consistent with previous reports,[7,9,18-20] TCR ligand priming induced iNKT-cell expansion, although the expansion level was reduced following OCH priming (1.5-3 fold) compared with α-GalCer priming (5-8 fold) (data not shown). Similar results were obtained with spleen MNCs after in vivo priming and with liver MNCs after in vitro priming (data not shown).

Consistent with a previous report that OCH selectively induced T-helper 2 (Th2) cytokine production by iNKT cells,[6] a minor but significant serum IL-4 elevation was observed 3 to 5 hours after priming with OCH, but serum IFN-γ was not detected (Figure 2A). Moreover, we observed a similar modulation of iNKT-cell surface

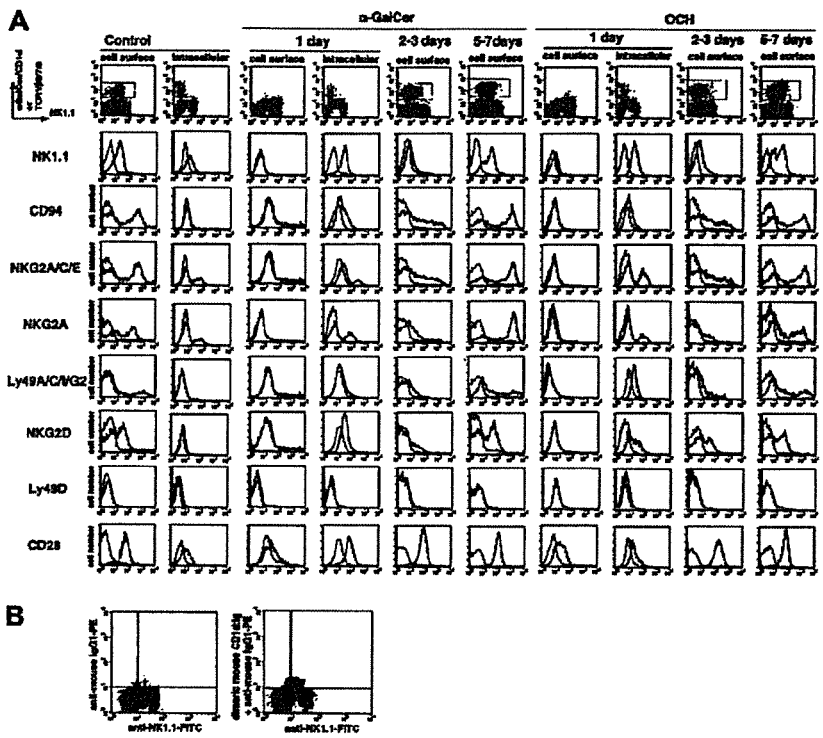


**Figure 1. Modulation of NK1.1, CD94/NKG2, Ly49, NKG2D, and CD28 on α-GalCer– or OCH-activated liver iNKT cells.** (A) Cell surface expression of the indicated molecules was analyzed on electronically gated α-GalCer/CD1d$^+$ iNKT cells on the indicated days after intraperitoneal injection of α-GalCer or OCH. One day after α-GalCer or OCH injection, both cell surface and intracellular expression of the indicated molecules were analyzed in electronically gated intracellular Vβ2/7/8$^+$ iNKT cells. The analysis gates are indicated by the gray line in dot plot panels. Bold lines indicate the staining with the respective mAb, and the thin lines indicate the staining with isotype-matched control Ig. Similar results were obtained from 3 independent experiments. (B) Existence of a cell population expressing intracellular α-GalCer/CD1d–specific TCR 1 day after α-GalCer injection. Liver MNCs were intracellularly stained with α-GalCer–loaded recombinant soluble dimeric mouse CD1d:Ig and PE-conjugated anti–mouse IgG1 mAb, or PE-conjugated anti–mouse IgG1 mAb together with FITC-conjugated anti-NK1.1 mAb, 1 day after α-GalCer injection. Quadrant gates were set by staining with FITC-conjugated isotype-matched control and PE-conjugated anti–mouse IgG1 mAb.

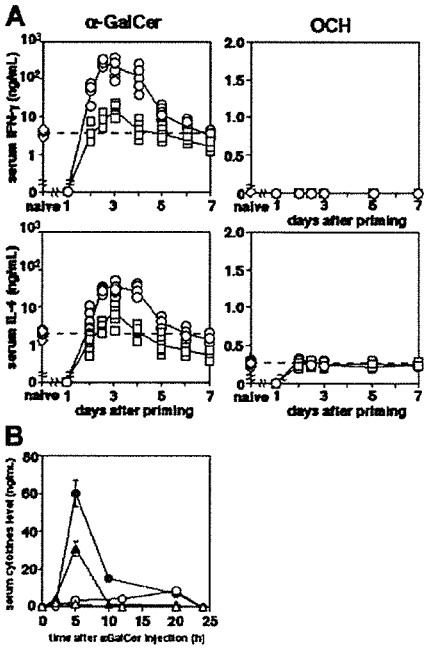


**Figure 2. Augmented serum IFN-γ and IL-4 following α-GalCer treatment in OCH-primed mice.** (A) Mice were primed with intraperitoneal injection of α-GalCer (□) or OCH (○) and then boosted with α-GalCer or OCH on the indicated day. Serum samples were obtained from 3 to 10 mice in each group 5 hours after the boost or the priming. Serum IFN-γ and IL-4 levels of primed naive mice were indicated on the y-axis (◇), and the dotted horizontal line in each panel shows the mean level of the primary response. Serum IFN-γ or IL-4 in the vehicle-injected mice were not detectable (data not shown). (B) Kinetics of serum IFN-γ (circles) and IL-4 (triangles) levels following α-GalCer boost of vehicle-primed (open symbols) or OCH-primed (closed symbols) mice on day 2.5. Data are represented as the mean ± SD of 5 mice in each group. Similar results were obtained from 3 independent experiments.

receptors by α-GalCer or OCH priming in IFN-γ$^{-/-}$ mice or in anti–IFN-γ mAb– and/or anti–IL-4 mAb–treated WT mice (data not shown). Taken together, these results indicated that priming of iNKT cells with TCR ligands resulted in a dramatic modulation of not only TCR and NK1.1 but also CD28 and inhibitory or activating NK-cell receptors on their surface, and this modulation was independent of IFN-γ or IL-4 production.

### Augmented recall responses of OCH-primed iNKT cells to α-GalCer in vivo

Since the modulation of surface receptors on iNKT cells by Ag priming might modify iNKT-cell responses to subsequent Ag challenge, we next examined the responses of α-GalCer– or OCH-primed mice to secondary α-GalCer or OCH administration. We measured serum IFN-γ and IL-4 5 hours after α-GalCer or OCH injection to avoid the contribution of NK cells, since it has been previously reported that NK cells are activated after iNKT-cell activation and contribute significantly to IFN-γ production within 12 hours after α-GalCer injection.[16,31] Of interest, serum IFN-γ and IL-4 levels were dramatically increased (10-30 fold) by secondary α-GalCer injection 2 to 4 days after OCH priming compared with primary α-GalCer injection (Figure 2A). By contrast, serum IFN-γ and IL-4 levels were only slightly increased by secondary α-GalCer injection in α-GalCer–primed mice compared with primary α-GalCer injection (Figure 2A). Very little serum IFN-γ and IL-4 were detected in mice injected with α-GalCer 1 day after α-GalCer or OCH priming, possibly due to the initial internalization of TCR in iNKT cells. OCH administration after α-GalCer or OCH priming did not augment serum IL-4 levels compared with primary administration of OCH, and serum IFN-γ was never detected (Figure 2A). We also examined the kinetics of serum IFN-γ and IL-4 induction by α-GalCer injection 2.5 days after OCH priming. Both IFN-γ and IL-4 levels were dramatically increased by the OCH priming and peaked at 5 hours after secondary α-GalCer injection (Figure 2B).

We next examined the α-GalCer–induced cytotoxic activity and antitumor effect in α-GalCer– or OCH-primed mice. OCH priming markedly augmented the α-GalCer–induced cytotoxic activities of liver and spleen MNCs against either NK-cell–sensitive YAC-1 or NK-cell–resistant P815 target cells compared with priming with the vehicle (Figure 3A). However, the iNKT-cell proportions in MNCs were similar among the groups when the mice were boosted by secondary α-GalCer injection (Table 1). In contrast, α-GalCer priming did not significantly augment the secondary α-GalCer–induced cytotoxicity (Figure 3A). Moreover, α-GalCer administration 2.5 days after OCH priming markedly augmented the antimetastatic effect against B16 melanoma compared with other prime/boost regimens (eg, α-GalCer/α-GalCer; Figure 3B). These results indicated that iNKT cells were hyperresponsive to secondary α-GalCer stimulation 2 to 3 days after OCH priming in vivo, resulting in a dramatic augmentation of effector functions, including IFN-γ and IL-4 production, cytotoxicity, and antitumor effect.

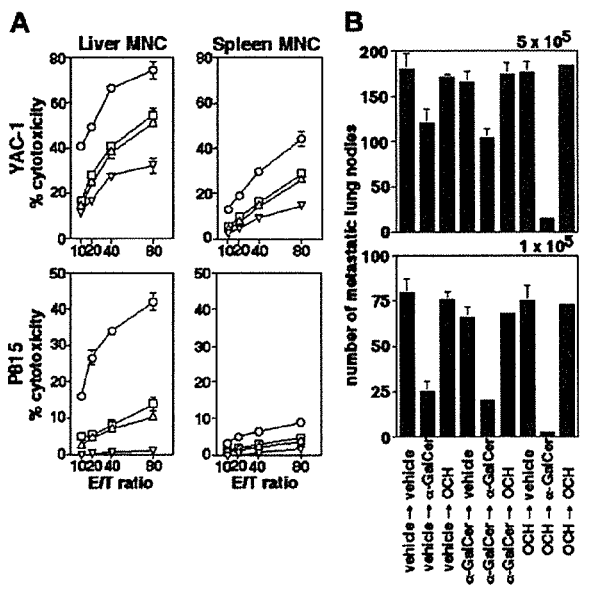


**Figure 3. Induction of cytotoxic activity and antimetastatic effect by priming and boosting with α-GalCer and OCH.** (A) Cytotoxic activity of liver and spleen MNCs was tested against NK-cell–sensitive YAC-1 cells or NK-cell–resistant P815 cells 24 hours after α-GalCer injection into mice primed 2.5 days earlier with vehicle (△), αGalCer (□), or OCH (○). Control mice were primed and boosted with vehicle (▽). Proportion of iNKT cells (%) in respective MNC populations at the time of boosting injection is indicated in Table 1. Data are represented as the mean ± SD of triplicate samples. Similar results were obtained from 3 independent experiments. E/T indicates effector-to-target ratio. (B) Antimetastatic effect. Mice were primed and boosted with α-GalCer, OCH, or vehicle on days −3 and 0 as indicated. Then, the indicated number of B16 melanoma cells were intravenously inoculated into the mice 2 hours after the boost. On day 14, the number of tumor metastatic colonies in the lungs was counted. Data are represented as the mean ± SD of 5 mice in each group. Similar results were obtained from 3 independent experiments.

**Table 1. Proportion of iNKT cells in respective MNC populations at the time of boosting injection**

| | Liver MNCs, % | Spleen MNCs, % |
|---|---|---|
| Vehicle primed | 22.7 ± 4.4 | 1.7 ± 0.8 |
| α-GalCer primed | 24.5 ± 3.4 | 3.3 ± 1.8 |
| OCH primed | 23.6 ± 5.1 | 2.5 ± 1.5 |

### Suppression of α-GalCer–induced iNKT-cell activation by Qa-1$^b$ and CD94/NKG2A interaction

To evaluate the priming effects more precisely, spleen MNCs were periodically isolated from naive, α-GalCer–primed, or OCH-primed mice and then stimulated with α-GalCer or OCH in vitro (Figure 4A). At 24 hours after the in vitro stimulation with α-GalCer or OCH, spleen MNCs from naive mice did not produce either IFN-γ or IL-4 at detectable levels, but by contrast spleen MNCs from α-GalCer– or OCH-primed mice produced substantial amounts of IFN-γ and IL-4 (Figure 4A). Maximal cytokine secretion was obtained from spleen MNCs isolated 2.5 days after priming. Consistent with the in vivo data (Figure 2), OCH-primed spleen MNCs secreted greater amounts of cytokines compared with α-GalCer–primed spleen MNCs. At 48 to 72 hours after the in vitro stimulation, naive splenic MNCs produced substantial amounts of IFN-γ and IL-4 in response to α-GalCer, but the OCH-primed MNCs still produced increased levels of both IFN-γ and IL-4 in response to α-GalCer restimulation compared with naive or α-GalCer–primed MNCs (data not shown). These results indicated that OCH priming sensitized iNKT cells to secondary α-GalCer stimulation more effectively than α-GalCer priming.

We next explored the mechanism by which OCH or α-GalCer priming sensitized iNKT cells to α-GalCer restimulation. We first examined whether the CD94/NKG2 inhibitory receptor might regulate the hyperresponsiveness of iNKT cells, since CD94/NKG2 was down-modulated on the sensitized iNKT cells 2 to 3 days after α-GalCer or OCH priming (Figure 1). Blockade of the CD94/NKG2 and Qa-1$^b$ interaction by Fab fragments of anti-NKG2 mAb or anti–Qa-1$^b$ mAb markedly enhanced IFN-γ and IL-4 production by α-GalCer–primed MNCs in response to restimulation in vitro with α-GalCer (Figure 4B). Albeit to a lesser extent, IFN-γ and IL-4 production by OCH-primed or naive MNCs was also significantly enhanced by the blockade of Qa-1$^b$ or CD94/NKG2. Notably, while α-GalCer–primed MNCs produced lower levels of IFN-γ and IL-4 than OCH-primed MNCs in response to α-GalCer restimulation in vivo, this difference was abrogated by the blockade of Qa-1$^b$ or CD94/NKG2. The contribution of activating CD94/NKG2C/E NK-cell receptors may be negligible, since blockade of the Qa-1$^b$–CD94/NKG2 interaction did not inhibit cytokine production by α-GalCer–activated iNKT cells in all cocultures, even if anti-NKG2 mAb or anti–Qa-1$^b$ mAb inhibited the function of activating CD94/NKG2C/E. Blockade of the CD80/CD86 interaction with CD28 abolished the cytokine production by naive or primed MNCs, irrespective of Qa-1$^b$ or CD94/NKG2 blockade. These results indicated that the Qa-1$^b$ and CD94/NKG2 interaction suppressed the TCR- and CD28-mediated activation of iNKT cells by α-GalCer, especially when the iNKT cells had first been primed with α-GalCer.

We also examined the impact of OCH or α-GalCer priming on antigen-presenting cells (APCs) by coculturing of purified iNKT cells and purified splenic DCs separately isolated 2.5 days after priming with α-GalCer, OCH, or vehicle (Figure 4C). Neither IFN-γ nor IL-4 was detected when iNKT cells were cocultured with any DCs in the absence of α-GalCer (data not shown). Notably, α-GalCer–primed DCs induced significantly lower levels of IFN-γ and IL-4 production by vehicle-primed iNKT cells compared with vehicle- or OCH-primed DCs. Of importance, this difference was abrogated by the blockade of CD94/NKG2. Moreover, OCH-primed iNKT cells produced significantly higher levels of IFN-γ and IL-4 compared with vehicle- or α-GalCer–primed iNKT cells. Again, the difference of cytokine production between OCH-primed and α-GalCer–primed iNKT cells was abrogated by CD94/NKG2 blockade. Significantly higher levels of IFN-γ and IL-4 were attained by both types of primed iNKT cells compared with vehicle-primed iNKT cells. A similar level (approximately 200 pg/mL) of IL-12 p40 was detected in the supernatants of all

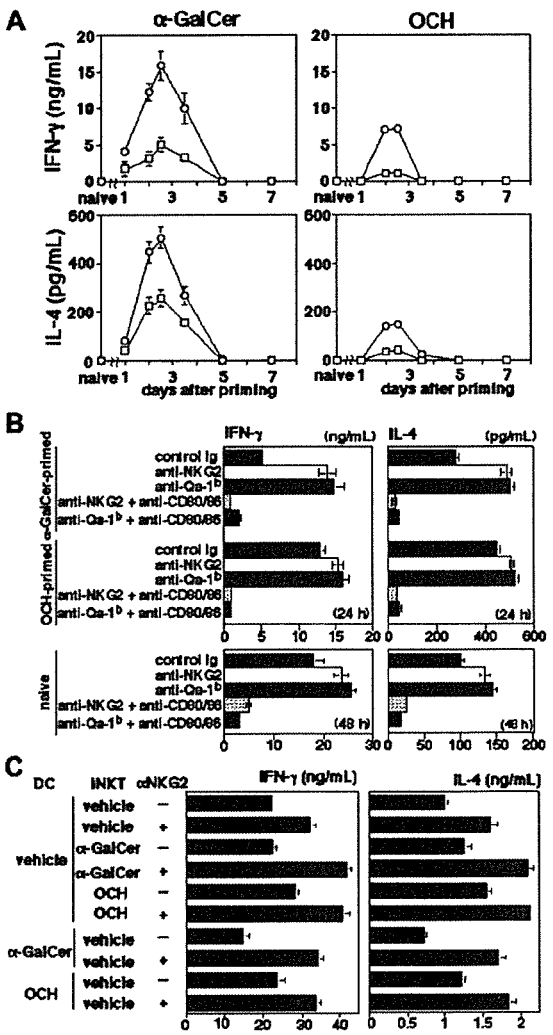


**Figure 4. NKG2 and CD28 regulate activation of naive or primed iNKT cells by α-GalCer.** (A) Mice were primed with intraperitoneal injection of α-GalCer (□) or OCH (○). Splenic MNCs were prepared on the indicated days after priming and stimulated with α-GalCer or OCH in vitro for 24 hours. Data are represented as the mean ± SD of triplicate wells. Similar results were obtained from 3 independent experiments. (B) Mice were primed with intraperitoneal injection of α-GalCer, OCH, or vehicle on day −2.5. Then, splenic MNCs were prepared on day 0 and stimulated with α-GalCer in vitro for 24 or 48 hours in the presence or absence of the indicated mAbs. Data are represented as the mean ± SD of triplicate wells. Similar results were obtained from 3 independent experiments. (C) Liver iNKT cells and splenic DCs were isolated from naive mice or primed with α-GalCer or OCH 2.5 days before and then cocultured with α-GalCer for 48 hours in the presence or absence of anti-NKG2 mAb. Data are represented as the mean ± SD of triplicate wells. Similar results were obtained from 2 independent experiments.

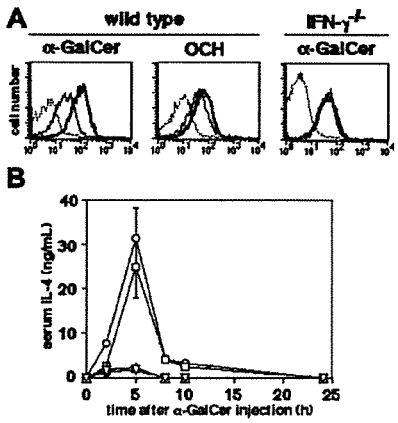


**Figure 5. IFN-γ–induced Qa-1$^b$ inhibits reactivation of α-GalCer–primed iNKT cells by α-GalCer.** (A) Qa-1$^b$ expression on splenic MNCs isolated from WT and IFN-$\gamma^{-/-}$ mice was analyzed 2.5 days after intraperitoneal administration of α-GalCer or OCH. Thin lines indicate the staining of MNCs from vehicle-treated mice with anti–Qa-1$^b$ mAb; bold lines, the staining of MNCs from α-GalCer– or OCH-treated mice with anti–Qa-1$^b$ mAb; and dotted lines, the staining with isotype-matched control Ig. Similar results were obtained from 3 independent experiments. (B) Kinetics of serum IL-4 induction after α-GalCer injection into vehicle-primed wild-type mice (▽), vehicle-primed IFN-$\gamma^{-/-}$ mice (△), α-GalCer–primed IFN-$\gamma^{-/-}$ mice (□), or OCH-primed IFN-$\gamma^{-/-}$ mice (○). Priming was performed 2.5 days before. Serum IL-4 was not detectable in the vehicle-injected mice (data not shown). Data are represented as the mean ± SD of 5 mice in each group. Similar results were obtained from 3 independent experiments.

cocultures (data not shown), which suggested that DC function was not impaired by α-GalCer or OCH priming. These results suggested that the recall responses of iNKT cells in α-GalCer–primed mice were more strictly regulated by Qa-1$^b$ and CD94/NKG2-mediated suppression than in naive or OCH-primed mice. Consistent with this notion, Qa-1$^b$ expression on splenic MNCs was markedly up-regulated in α-GalCer–primed mice compared with naive or OCH-primed mice (Figure 5A).

### IFN-γ–induced Qa-1$^b$ expression negatively regulates recall NKT-cell responses in vivo

Although iNKT cells primed with α-GalCer strongly up-regulated the Qa-1$^b$ expression on splenic MNCs, those primed with OCH did so only weakly (Figure 5A). Notably, the α-GalCer–induced Qa-1$^b$ up-regulation was not observed in IFN-$\gamma^{-/-}$ mice (Figure 5A). Moreover, α-GalCer priming increased the secondary α-GalCer–induced serum IL-4 to a level comparable with that induced by OCH priming in IFN-$\gamma^{-/-}$ mice (Figure 5B). These results indicated that IFN-γ induced by α-GalCer priming was responsible for Qa-1$^b$ up-regulation, which in turn resulted in CD94/NKG2-mediated suppression of recall iNKT-cell response in α-GalCer–primed mice.

We finally evaluated whether the blockade of CD94/NKG2 could augment the α-GalCer–induced iNKT-cell function in α-GalCer–primed mice. The in vivo treatment with anti-NKG2 mAb alone did not induce cytokine production or cytotoxicity and did not deplete NK cells or iNKT cells (data not shown). The CD94/NKG2 blockade dramatically increased α-GalCer–induced serum IFN-γ and IL-4 levels, particularly in α-GalCer–primed mice (Figure 6A). It was notable that high levels of serum IFN-γ and IL-4 were inducible 10 days after α-GalCer priming if CD94/NKG2 was blocked at the time of boosting. Moreover, the CD94/NKG2 blockade also significantly augmented the α-GalCer–induced cytotoxicity of liver and spleen MNCs and the antimetastatic effect of treatment, particularly in α-GalCer–primed mice (Figure 6B-C). Even if anti-NKG2 mAb possibly inhibited the function of activating CD94/NKG2C/E, the contribution of activating CD94/NKG2C/E NK-cell receptors may be negligible, since blockade of the Qa-1$^b$–CD94/NKG2 interaction did not inhibit

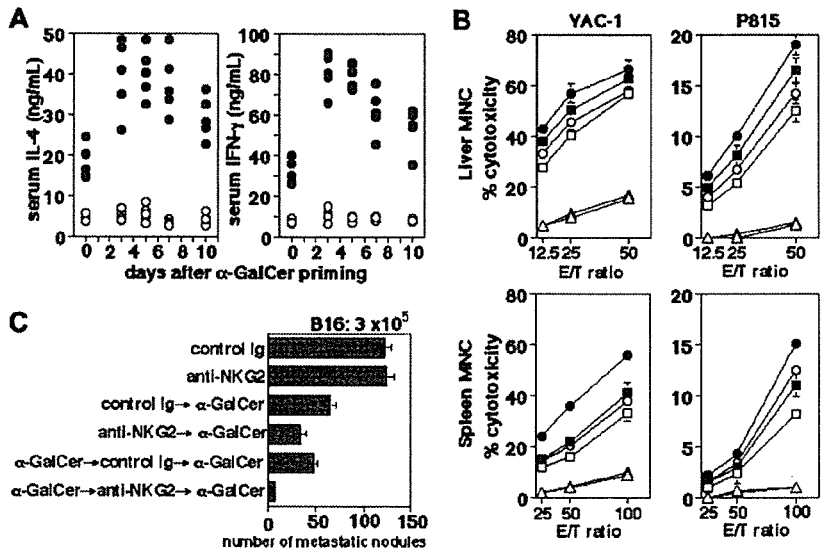


**Figure 6. Blockade of NKG2 enhances activation of naive and α-GalCer–primed iNKT cells by α-GalCer in vivo.** (A) Mice were primed with α-GalCer on day 0 and then boosted with α-GalCer on the indicated day. Anti-NKG2 mAb (●) or control Ig (○) was administered 2 days before the boost. Serum samples were obtained 5 hours after the boost. The mice indicated on day 0 were treated once with α-GalCer injection on day 0. (B) Cytotoxic activity of liver and spleen MNCs was tested against NK-sensitive YAC-1 cells and NK-resistant P815 cells 24 hours after the last α-GalCer injection. Mice were intraperitoneally injected with α-GalCer on day 0 (squares) or days −3 and 0 (circles), or injected with vehicle on days −3 and 0 (triangles), and intraperitoneally administered with anti-NKG2 mAb (closed symbols) or control Ig (open symbols) on day −2. Data are represented as the mean ± SD of triplicate samples. Similar results were obtained from 3 independent experiments. (C) Antimetastatic effect. Mice were intraperitoneally injected with α-GalCer on day 0 or days −3 and 0, and then intravenously inoculated with $3 \times 10^5$ B16 melanoma cells 2 hours later. Anti-NKG2 mAb or control Ig was intraperitoneally administered on day −2. On day 14, the number of tumor colonies in the lungs was counted under a dissecting microscope. Data are represented as the mean ± SD of 5 to 8 mice in each group. Similar results were obtained from 3 independent experiments.