

# Activation of Natural Killer T Cells by $\alpha$ -Galactosylceramide in the Presence of CD1d Provides Protection Against Colitis in Mice

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**Background & Aims:** CD1d is a major histocompatibility complex class I-like molecule that presents glycolipid antigens to a subset of natural killer (NK)1.1<sup>+</sup> T cells. These NK T cells exhibit important immunoregulatory functions in several autoimmune disease models.

**Methods:** To investigate whether CD1d and NK T cells have a similar role in intestinal inflammation, the effects of the glycolipid,  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer), on dextran sodium sulfate (DSS)-induced colitis were examined. Wild-type (WT), CD1d<sup>-/-</sup>, and RAG<sup>-/-</sup> mice were examined for their response to either  $\alpha$ -GalCer or the control analogue,  $\alpha$ -mannosylceramide ( $\alpha$ -ManCer). **Results:** WT mice, but not CD1d<sup>-/-</sup> and RAG<sup>-/-</sup> mice, receiving  $\alpha$ -GalCer had a significant improvement in DSS-induced colitis based on body weight, bleeding, diarrhea, and survival when compared with those receiving  $\alpha$ -ManCer. Elimination of NK T cells through antibody-mediated depletion resulted in a reduction of the effect of  $\alpha$ -GalCer. Furthermore, adoptive transfer of NK T cells preactivated by  $\alpha$ -GalCer, but not  $\alpha$ -ManCer, resulted in diminished colitis. Using a fluorescent-labeled analogue of  $\alpha$ -GalCer, confocal microscopy localized  $\alpha$ -GalCer to the colonic surface epithelium of WT but not CD1d<sup>-/-</sup> mice, indicating  $\alpha$ -GalCer binds CD1d in the intestinal epithelium and may be functionally active at this site.

**Conclusions:** These results show an important functional role for NK T cells, activated by  $\alpha$ -GalCer in a CD1d-restricted manner, in regulating intestinal inflammation.

present lipid-related antigens to CD4<sup>-</sup>/CD8<sup>+</sup> or CD4<sup>-</sup>/CD8<sup>-</sup> (double negative, or DN) T-cell receptor (TCR) $\alpha\beta$ <sup>+</sup> T cells.<sup>1-3</sup> The second group consists of the CD1d-like molecules that are expressed by humans, rodents (mice and rats), and rabbits and share close homology across species.<sup>2,4-6</sup> Mice express only 2 highly related CD1d-like molecules, CD1.1 and CD1.2.<sup>6</sup>

Recent studies in mice and humans have shown that the CD1d members function as essential restriction elements in the activation of a subset of CD4<sup>+</sup> or DN T cells. These CD1d-restricted lymphocytes are known to coexpress the natural killer marker, NK1.1 (CD161), and an invariant TCR $\alpha$  chain (TCRAV24AJQ in humans and TCRAV14AJ281 in mice) paired with polymorphic TCR $\beta$  chains from a restricted variety of TCRBV families.<sup>7-9</sup> NK T cells exist in low numbers in the peripheral blood (<1%-2% of peripheral blood T cells) and most other tissues, except for the liver, in which they are more highly represented.<sup>10</sup> However, in mice lacking expression of CD1d, these NK T cells do not develop in the thymus, resulting in their absence from peripheral tissues.<sup>11,12</sup> Upon stimulation, NK T cells produce significant quantities of interleukin (IL)-4 and interferon gamma, and exhibit enhanced cytolytic activity.<sup>11-16</sup> Such a phenotype implies that these cells play an important role in immunoregulation and immunosurveil-

**T**he CD1 gene family consists of 5 major histocompatibility complex (MHC) class I-like molecules, 4 of which are known to be functionally active.<sup>1</sup> These 4 gene products are subdivided into 2 groups based on sequence homology.<sup>2</sup> CD1a, CD1b, and CD1c form 1 group of antigen-presenting molecules, which have been shown to

*Abbreviations used in this paper:*  $\alpha$ -GalCer,  $\alpha$ -galactosylceramide;  $\alpha$ -ManCer,  $\alpha$ -mannosylceramide; DN, double negative; DSS, dextran sodium sulfate; IEC, intestinal epithelial cell; IL, interleukin; MHC, major histocompatibility complex; NK, natural killer (cell); RAG, recombinase-activating gene; TCR, T-cell receptor; Th, T helper; WT, wild-type.

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lance, which is consistent with studies in autoimmune diseases and antitumor responses.<sup>16–18</sup>

Human inflammatory bowel disease (IBD) is a chronic, relapsing, and remitting condition of unknown origin that exhibits a variety of autoimmune features.<sup>19</sup> Studies in animal models and humans with IBD have suggested abnormal intestinal epithelial cell (IEC) barrier function, excessive production of either T helper (Th)1 or Th2 cytokines, and the unrestrained activation of CD4<sup>+</sup> TCR $\alpha\beta$ <sup>+</sup> T cells by components of the normal luminal bacteria as contributing to the pathogenesis of these disorders.<sup>19</sup> Given the importance of NK T cells in the control of several autoimmune diseases and the observations that CD1d is expressed by normal human intestinal epithelium,<sup>20</sup> we sought to determine whether CD1d-related immune pathways may be functionally important to IBD. Therefore, we examined the effects of  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer), a lipid ligand known to bind CD1d and induce activation of NK T cells in a CD1d-restricted fashion,<sup>21</sup> in the dextran sodium sulfate (DSS) mouse model of colitis. These studies show that CD1d-mediated activation of NK T cells plays an important role in modulating the colitis associated with DSS administration.

## Materials and Methods

### Mice

Wild-type (WT) and recombinase-activating gene 2 knockout (RAG<sup>-/-</sup>) C57BL/6x129/Sv mice were purchased from Jackson Laboratories (Bar Harbor, ME). CD1d<sup>-/-</sup> mice have been generated as previously described<sup>22</sup> and were also used as a mixed background of C57BL/6x129/Sv. All animals were maintained under specific pathogen-free conditions on standard laboratory chow and water ad libitum until the desired age (8–10 weeks) and/or weight (20–30 g) were achieved. All mice were treated humanely according to the National Institutes of Health and SERI-BBRI Animal Care and Use Committee guidelines.

### Lipids

$\alpha$ -GalCer (AGL-582; KRN7000) and the control analogue lipid,  $\alpha$ -mannosylceramide ( $\alpha$ -ManCer; AGL-595), were developed and manufactured by the Pharmaceutical Research Laboratory of Kirin Brewery Co. (Gunma, Japan).<sup>23</sup> The glycolipids were administered via intraperitoneal injection into mice in a vehicle of sterile phosphate-buffered saline solution containing 0.5% polysorbate 20 (Nikko Chemical, Tokyo, Japan). For use in confocal microscopy, an NBD (7-nitrobenz-2-oxa-1,3-diazol)-labeled form of  $\alpha$ -GalCer was also generated by the Pharmaceutical Research Laboratory of Kirin Brewery Co.<sup>24</sup>

### DSS Colitis Model

DSS (ICN, lot no. 3073B; Aurora, OH) was added to the animals' water supply at a concentration of 2.5% (wt/vol) at day 0 of each experiment. Total body weight (grams) was measured at the same time each day and before injection of lipid compounds. Feces were collected from each mouse daily and stored for rehydration and fecal occult bleeding measurements. For analysis of fecal occult bleeding, daily stools were resuspended in 400  $\mu$ L of sterile water through vortexing for 5 minutes and microcentrifuged at 13,000 rpm for 2 minutes using an Eppendorf 5417C table-top centrifuge (Hamburg, Germany), and then 40  $\mu$ L of supernatant was added to paper from the Hemocult SENSE (SmithKline Diagnostics, Inc., San Jose, CA) kit. Scoring for diarrhea (0–3) and fecal blood (0–4) were performed as previously reported.<sup>25</sup> Intraperitoneal injections of  $\alpha$ -GalCer and  $\alpha$ -ManCer were initiated on day 1 and then performed every other day thereafter. All experiments were repeated at least twice with 5–10 mice per group. The data displayed provide representative results for 1 experiment.

### Histology

WT mice treated with 2.5% DSS and either  $\alpha$ -GalCer lipid or the control lipid,  $\alpha$ -ManCer, were randomly selected at day 6 for tissue harvesting. Colonic involvement was noted to be diffuse in the  $\alpha$ -ManCer group by both gross examination and analysis of multiple sections from different colonic regions in 4 mice from each group. Freshly isolated colonic tissue from the descending portion of the large intestine was frozen in liquid nitrogen using O.C.T. compound (Sakura, Torrance, CA). Sections were stained with H&E and then photographed with a Nikon TE300 inverted microscope (Melville, NY).

### Antibodies

The anti-mouse Ly-49C antibody (clone 5E6), anti-mouse NK-1.1 antibody (NKR-P1C, Ly-55; clone PK136), mouse isotype control immunoglobulin (Ig) G2a (clone G155-178), Cy-Chrome labeled anti-mouse TCR $\beta$  antibody (clone H57-597), and *FcBlock* were all obtained from Pharmingen (San Diego, CA).

### NK T-Cell Depletion

Depletion of NK T cells was achieved by coadministration of anti-mouse Ly-49C (50  $\mu$ g/mouse) and anti-mouse Ly-55 (50  $\mu$ g/mouse), as previously described.<sup>22</sup> Antibodies (100  $\mu$ L/mouse total volume) were given intravenously through tail vein injections on the first and third days of the experiment in WT C57BL/6x129/Sv mice. Similar quantities of control isotype IgG antibodies were used in all experiments. NK T-cell depletion (>90%) was confirmed by flow cytometry of isolated splenocytes as assessed by staining with anti-mouse NK-1.1 and TCR $\beta$  antibodies after initial blocking of nonspecific Fc receptors.

### NK T-Cell Adoptive Transfer

Intraperitoneal injections of either  $\alpha$ -GalCer or  $\alpha$ -ManCer were administered to WT C57BL/6x129/Sv mice

every other day for a period of 1 week. Subsequently, splenic lymphocytes were isolated from WT donor mice of equal number to RAG<sup>-/-</sup> recipient mice, stained with the Ly-55 antibody, and sorted by flow cytometry to yield a purified population of NK1.1<sup>+</sup> cells, which included NK1.1<sup>+</sup>/CD3<sup>+</sup> cells (data not shown). These cells were then introduced intraperitoneally in a sterile fashion into RAG<sup>-/-</sup> recipients at concentrations of  $1 \times 10^5$  cells per mouse. Donor and recipient mice were of the same sex and genetic background. DSS treatment, as described above, was initiated on the same day as the cell transfer. Lipid injections similar to those that were used to stimulate the adoptively transferred populations of cells were given on the following day and every other day thereafter to recipient animals for the duration of the experiment.

### Confocal Microscopy

The descending portion of the large intestine from 2 WT and 2 CD1d<sup>-/-</sup> mice was excised 4 hours after administration of NBD-labeled  $\alpha$ -GalCer, rinsed in phosphate-buffered saline, and frozen in isopentane cooled with liquid nitrogen. Frozen sections (5  $\mu$ m) were cut on a Leica CM 1850 cryostat (Leica Inc., Deerfield, IL) in longitudinal sections and fixed for 1 minute in 80% acetone at  $-20^\circ\text{C}$ . Sections were incubated for 30 minutes in frozen section buffer containing 150 mmol/L NaCl, 2 mg/mL gelatin, and 10 mmol/L NaPO<sub>4</sub> (pH 7.5) followed by staining for 30 minutes with rhodamine-labeled phalloidin (Molecular Probes, Eugene, OR). After staining, sections were washed in frozen section buffer, mounted in Vectashield mount (Vector Laboratories, Inc., Burlingame, CA), and examined in a Bio-Rad MRC 1024 confocal laser scanning system using the Imaging Core Facility of the Harvard Digestive Diseases Center.

### Statistics

All data are presented as SEM. Parametric data (body weight) were analyzed using a paired 2-tailed *t* test for the paired data or a 1-way analysis of variance (ANOVA) followed by a Dunnett Multiple Comparisons post-test. Nonparametric data (diarrhea and bleeding scoring) were analyzed using a Mann-Whitney *U* test for paired data or a Kruskal-Wallis test (nonparametric ANOVA) followed by a Dunn's Multiple Comparisons post-test. Survival fractions were calculated using the product limit or Kaplan-Meier method. All of the above comparisons were performed using either InStat or Prism Graph Pad programs (GraphPad Software Inc., San Diego CA). An associated probability (*P* value) of  $<0.05$  was considered significant.

## Results

### $\alpha$ -GalCer Protects WT Mice From Colitis Associated With DSS Administration

To evaluate  $\alpha$ -GalCer in the well-characterized DSS model of colitis,<sup>26</sup> mice were administered DSS (2.5%) in the drinking water and the glycolipid effects

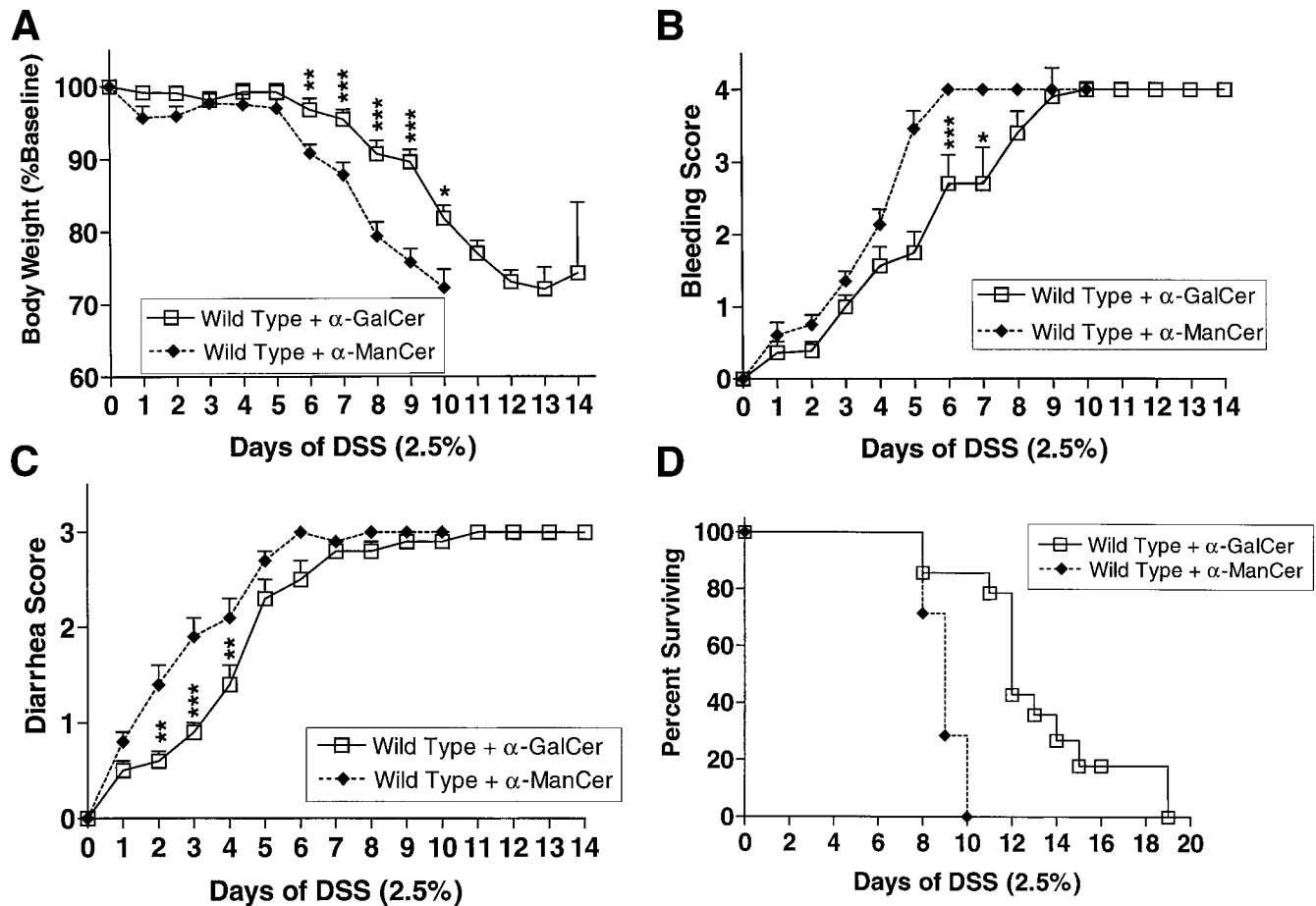
were analyzed after administration of the lipid on day 1 and every other day thereafter for the duration of the study. Preliminary investigations established an optimal dose of  $\alpha$ -GalCer at 100  $\mu$ g/kg body wt (data not shown). At this dose of  $\alpha$ -GalCer, there was a significant amelioration in the severity of colitis as observed in WT mice (Figure 1).

The control lipid analogue,  $\alpha$ -ManCer, which can bind CD1d but not activate NK T cells, had no effect on the DSS-induced colitis. To verify that  $\alpha$ -ManCer did not accelerate DSS-induced colitis and provide incorrect interpretation of  $\alpha$ -GalCer's effect, concomitant experiments were performed with WT mice receiving only DSS, which showed no difference in comparison to the  $\alpha$ -ManCer-treated group (data not shown).  $\alpha$ -GalCer-induced protection from colitis was evident as assessed by several parameters including body weights, diarrhea, fecal bleeding, and duration of survival (Figure 1A-D). The improvement in colitis was confirmed histologically with a reduction in neutrophilic and lymphocytic infiltrates, as well as the preservation of crypt architecture and surface epithelium in the  $\alpha$ -GalCer-treated group (Figure 2).

### Dependence of the Protective Effect of $\alpha$ -GalCer on the Presence of CD1d

Although  $\alpha$ -GalCer is a highly specific ligand for CD1d, it was important to confirm that the effects observed after  $\alpha$ -GalCer administration were dependent on CD1d. The DSS colitis model was therefore examined in mice that were deficient in CD1d in comparison to WT animals. As depicted in Figure 3,  $\alpha$ -GalCer was unable to confer any protective effect in the CD1d<sup>-/-</sup> mice in comparison with WT mice, which were protected by  $\alpha$ -GalCer. In addition, no differences were observed between either the  $\alpha$ -GalCer-treated or the  $\alpha$ -ManCer-treated CD1d<sup>-/-</sup> mice and the WT animals treated with  $\alpha$ -ManCer, indicating that the absence of CD1d per se did not lead to a worse colitis (Figure 3).

Because these results could be interpreted as either caused by the absence of CD1d on an antigen-presenting cell, such as the IEC, and/or caused by the lack of a T-cell population such as NK T cells, which do not develop in CD1d<sup>-/-</sup> mice,<sup>11,12</sup> the functional activity of  $\alpha$ -GalCer in the DSS colitis model was also assessed in RAG<sup>-/-</sup> mice. Despite the presence of CD1d and NK cells in RAG<sup>-/-</sup> mice,  $\alpha$ -GalCer exhibited no protective effect in the RAG<sup>-/-</sup> mice because the severity of colitis was similar to that observed in either RAG<sup>-/-</sup> or WT mice treated with  $\alpha$ -ManCer (Figure 3). These results imply



**Figure 1.** Effects of  $\alpha$ -GalCer (100  $\mu$ g/kg) and its control analogue  $\alpha$ -ManCer (100  $\mu$ g/kg) on WT mice continuously receiving DSS (2.5%, wt/vol). Colitis improvement by  $\alpha$ -GalCer in comparison with the  $\alpha$ -ManCer is noted in terms of (A) body weight, (B) fecal occult bleeding, (C) diarrhea, and (D) survival. The  $\alpha$ -ManCer-treated group exhibited no significant differences in comparison to WT mice receiving DSS alone (data not shown). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ;  $\alpha$ -GalCer vs.  $\alpha$ -ManCer. Data represent  $\pm$  SEM.

that the protective effect of  $\alpha$ -GalCer requires CD1d and either T or B cells, but not NK cells, and does not occur through a direct effect on the IEC or some other CD1d-bearing cell type alone.

#### Mediation of the Protective Effect of $\alpha$ -GalCer by NK T Cells

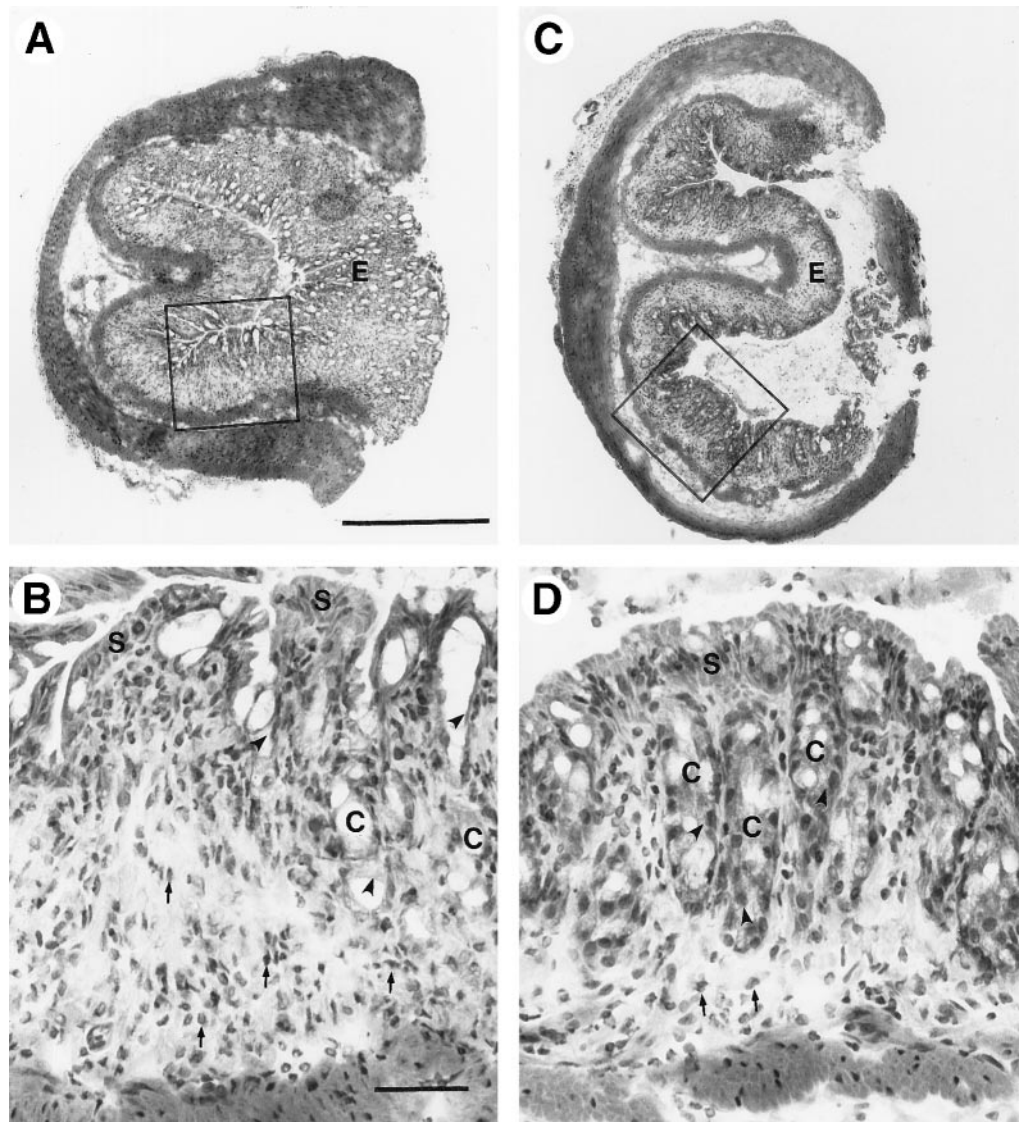
Because the beneficial effects of  $\alpha$ -GalCer require CD1d and lymphoid cells, but not CD1d alone, the results described above could be interpreted to mean that the major effector mechanism stimulated by  $\alpha$ -GalCer was through a CD1d-restricted T cell. An obvious candidate is the NK T cell, whose development and activation are dependent on CD1d in the thymus and periphery, respectively.<sup>11,12</sup> Therefore, the role for NK T cells in the effects observed with  $\alpha$ -GalCer were examined in the following series of experiments.

In the first approach, antibody-mediated depletion of NK1.1<sup>+</sup> cells, which include the NK T-cell subset, was used as a means to examine the role of NK T cells on the

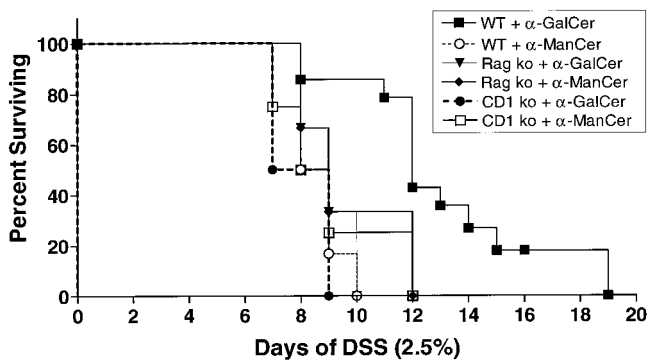
beneficial properties of  $\alpha$ -GalCer observed in the DSS colitis models. As shown in Figure 4, when NK1.1<sup>+</sup> cells were depleted, the protective effect of  $\alpha$ -GalCer was abrogated, whereas the control isotype Ig-treated group responded to  $\alpha$ -GalCer with evidence of a protective effect, as previously described for WT-treated mice.

Verification of depletion of NK T cells was determined by fluorescence-activated cell sorter analysis using antibodies specific for NK1.1 and the TCR $\beta$  chain (data not shown). Importantly, depletion of NK1.1<sup>+</sup> cells did not induce colitis by itself, making it unlikely that the antibody depletion had an effect independent of the DSS (data not shown).

In a second approach, a role for NK T cells was directly addressed by determining whether adoptively transferred NK T cells could confer protection to RAG<sup>-/-</sup> mice exposed to DSS. WT mice were treated with either  $\alpha$ -GalCer or  $\alpha$ -ManCer for 1 week. At this time, splenic lymphocytes were examined by flow cytometry and sorted for expression of the NK1.1 marker. These NK1.1<sup>+</sup>

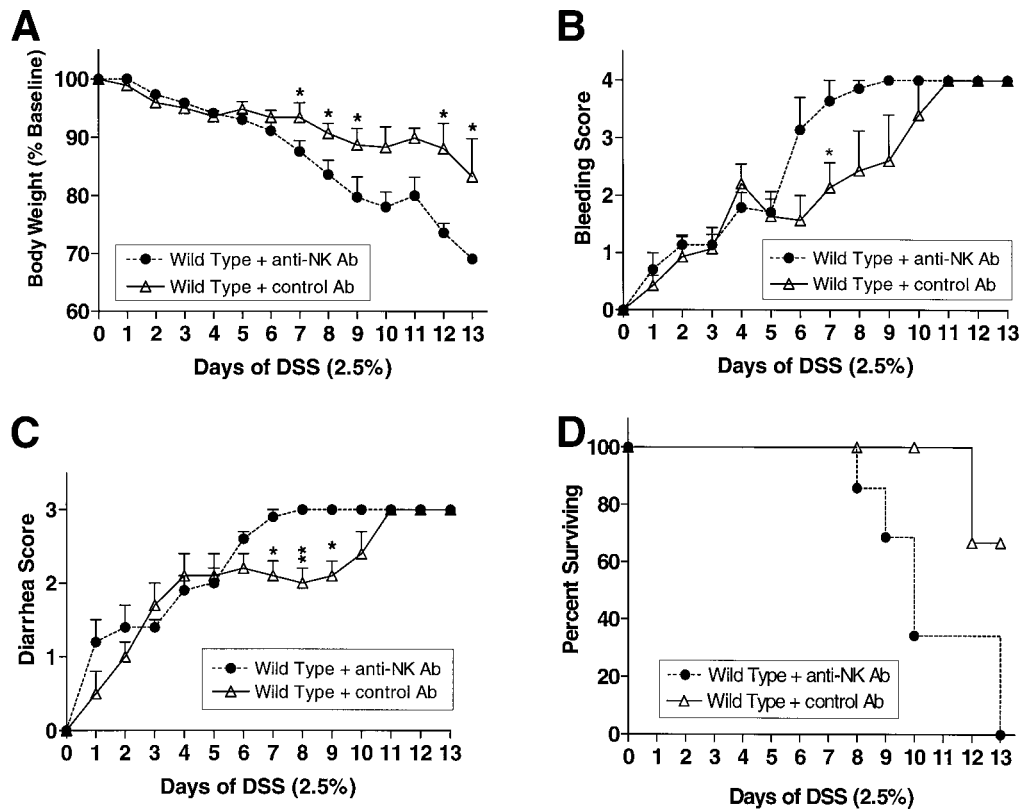


**Figure 2.** Frozen sections of descending colonic tissue depicting the histology from WT mice treated with either (A and B) the control analogue  $\alpha$ -ManCer or (C and D) the NK T-cell agonist  $\alpha$ -GalCer, while exposed to 2.5% DSS for 6 days. At low magnification, there was circumferential loss of the epithelial surface in (A) the  $\alpha$ -ManCer-treated group (E), compared with (C) the  $\alpha$ -GalCer-treated group. At higher magnification, (B) the  $\alpha$ -ManCer-treated group showed loss of normal-appearing surface epithelium (s) and had increased inflammatory infiltrates in the lamina propria (arrows) and flattening of the crypt epithelium (arrowheads) compared with (D) the  $\alpha$ -GalCer-treated group. c, colonic crypts in the high power fields. The boxes in the low-power magnification outline the regions displayed at higher magnification. The tissues are stained with H&E and are representative of 4 animals from each experimental group.



**Figure 3.** Effects on survival by  $\alpha$ -GalCer and its control analogue  $\alpha$ -ManCer in  $CD1d^{-/-}$  (CD1 ko) and  $RAG^{-/-}$  (Rag 2 ko) mice. Colitis was established and treatment initiated as described in Figure 1. There was no significant difference in survival by the  $CD1d^{-/-}$  and  $RAG^{-/-}$  mice administered  $\alpha$ -GalCer, compared with the significant improvement in survival seen in  $\alpha$ -GalCer-treated WT mice ( $P = 0.003$  vs.  $RAG^{-/-}$ ;  $P = 0.0005$  vs.  $CD1d^{-/-}$ ). The  $\alpha$ -ManCer-treated groups were overlapping in their survival curves.

cells, which contained NK T cells and were observed to be expanded in numbers by flow cytometry after treatment with  $\alpha$ -GalCer, but not  $\alpha$ -ManCer (data not shown), were subsequently transferred into  $RAG^{-/-}$  recipients of the same genetic background. These  $RAG^{-/-}$  mice were then treated with DSS to induce colitis. In the first group of experiments,  $RAG^{-/-}$  mice which received  $NK1.1^+$  cells from  $\alpha$ -GalCer treated WT mice were protected. To verify that activation with  $\alpha$ -GalCer was necessary,  $RAG^{-/-}$  mice that received  $NK1.1^+$  cells from  $\alpha$ -GalCer-treated WT mice were compared with  $RAG^{-/-}$  mice that received  $NK1.1^+$  cells from  $\alpha$ -ManCer-treated WT mice.  $RAG^{-/-}$  mice that received  $NK1.1^+$  cells preacti-



**Figure 4.** Effects of antibody-mediated NK1.1<sup>+</sup> cell depletion, including the NK T-cell subset, on DSS-induced colitis in WT mice. Colitis in the control-treated group was compared with the group depleted of NK1.1<sup>+</sup> cells (anti-NK Ab) in terms of (A) body weight, (B) fecal occult bleeding, (C) diarrhea, and (D) survival. Antibodies (50  $\mu$ g/mouse each) specific for Ly-49C and Ly-55 (NK1.1) cell markers were introduced intravenously before DSS (2.5%) administration and treatment with  $\alpha$ -GalCer, as described in Figure 1. \* $P$  < 0.05, \*\* $P$  < 0.01; anti-NK Ab vs. control IgG Ab. Data represent  $\pm$  SEM.

vated with  $\alpha$ -GalCer, but not the NK1.1<sup>+</sup> cells preactivated with  $\alpha$ -ManCer, were protected from colitis induced by DSS (Figure 5B).

These studies show that NK T cells, activated by a model glycolipid antigen, can down-regulate the colitis associated with administration of DSS, but only after activation.

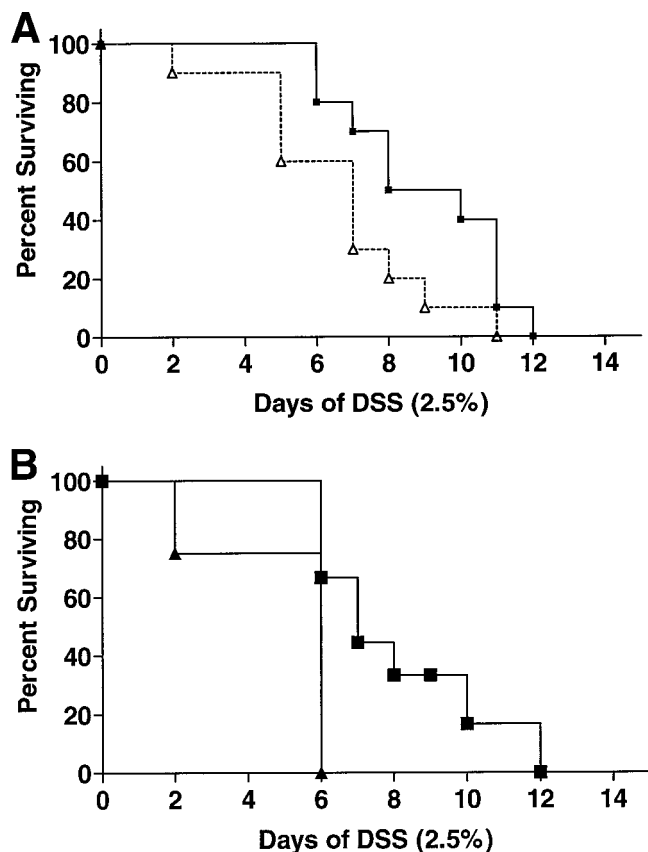
#### $\alpha$ -GalCer Localizes to the Intestinal Epithelium

CD1d has been shown to be expressed by IECs of the colon in humans, rats, and mice.<sup>20,27,28</sup> However, to determine whether CD1d expressed by IECs is capable of binding  $\alpha$ -GalCer as a potential mechanism to explain the effects observed, the localization of  $\alpha$ -GalCer was defined in vivo. Therefore, NBD-labeled  $\alpha$ -GalCer was injected into WT and CD1d<sup>-/-</sup> mice and, 4 hours after injection, colonic tissues were isolated and examined by confocal microscopy. As indicated in Figure 6A by the strong fluorescence signal, the NBD-labeled lipid was clearly detected on the surface, but poorly in the crypt, epithelium. In WT animals, expression of NBD-labeled  $\alpha$ -GalCer was greatest in the subapical and basolateral regions of the IEC, consistent with previously published studies using CD1d-specific antibodies.<sup>20</sup> In contrast, colonic tissue from CD1d<sup>-/-</sup> mice exhibited diffuse background staining but did not show specific labeling of the colonic surface epithelium (Figure 6B).

These studies indicate that CD1d, expressed by IECs of the mouse colon, is capable of binding  $\alpha$ -GalCer and raises the possibility that CD1d bound  $\alpha$ -GalCer in the intestinal epithelium may play a role in down-regulating DSS-induced colitis through an as yet to be defined mechanism.

#### Discussion

In this report, we have used a well-established model of colitis associated with administration of DSS to show that NK T cells activated by  $\alpha$ -GalCer exhibit an important ameliorative effect on the severity of colitis associated with DSS. DSS is a sulfated polymer that causes a self-limited colitis with acute administration or colitis with chronic features on continuous exposure.<sup>26</sup> This model allows for direct testing of cause and effect relationships between specific interventions and colitis. DSS is believed to cause colitis by interfering with IEC barrier function and/or stimulating regional inflammation within the colon through the up-regulation of both Th1 and Th2 cytokines and inflammatory mediators.<sup>29</sup> Susceptibility to colitis associated with DSS is genetically determined, although the nature of these genes remains unknown.<sup>30,31</sup> Although DSS was originally viewed as a T-cell independent model because it is observed in T cell-deficient animals such as SCID mice,<sup>32,33</sup> such observations deal only with disease initiation and not mecha-



**Figure 5.** Effects on survival by adoptive transfer of NK1.1<sup>+</sup> cells into RAG<sup>-/-</sup> mice that were exposed to DSS (2.5%). (A)  $\alpha$ -GalCer-primed NK1.1<sup>+</sup> cells (■) were adoptively transferred into RAG<sup>-/-</sup> recipient mice and compared with RAG<sup>-/-</sup> recipient mice ( $P = 0.0492$ ) that did not undergo cell transfer ( $\Delta$ ). (B) Survival comparisons were also made between RAG<sup>-/-</sup> recipient mice adoptively receiving  $\alpha$ -GalCer primed NK1.1<sup>+</sup> cells (■) and mice receiving  $\alpha$ -ManCer primed NK1.1<sup>+</sup> cells ( $\blacktriangle$ ) ( $P = 0.0159$ ).

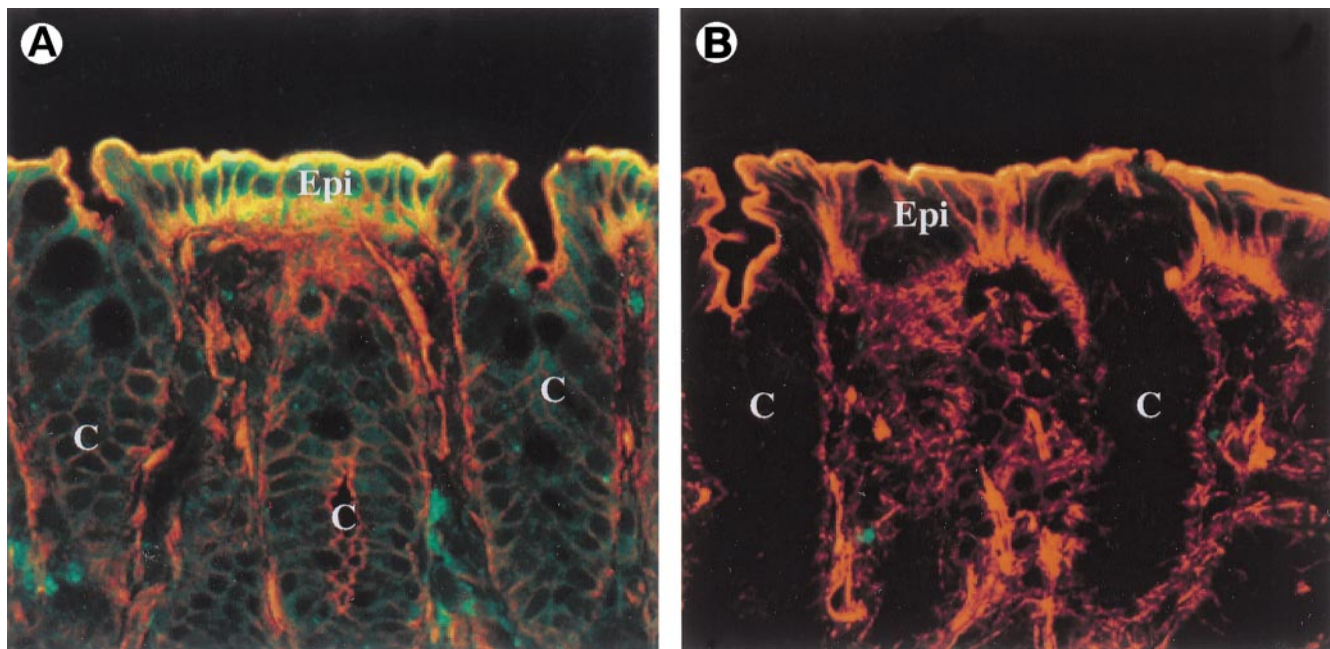
nisms of disease regulation. It has recently been appreciated that DSS-associated colitis is more severe in a T and B cell-deficient environment, raising the possibility that regulatory T cells may be operative in later phases of the colitis associated with DSS administration.<sup>34</sup> This is consistent with recent studies that show that specific subpopulations of T cells may be capable of regulating inflammation and/or promoting healing. Potential examples include IL-10 producing T-regulatory 1 cells that may down-regulate proinflammatory networks and keratinocyte growth factor-producing  $\gamma\delta$  T cells, which encourage restitution of the IEC barrier.<sup>35,36</sup>

In this context, we believe that our results are consistent with a similar role for NK T cells in colitis pathogenesis. We have shown that  $\alpha$ -GalCer, but not its nonfunctional analogue,  $\alpha$ -ManCer, significantly down-regulates the severity of colitis in WT but not in either CD1d- or RAG-deficient animals, or in animals that have been depleted of NK and NK T cells. This benefit of  $\alpha$ -GalCer is notably restored to RAG<sup>-/-</sup> animals through

the transfer of NK1.1<sup>+</sup> cells. However, this protection by NK1.1<sup>+</sup> cells only occurs after activation by  $\alpha$ -GalCer. Moreover, the absence of CD1d and consequently NK T cells does not lead to a worse course of colitis, but rather to a loss of the protection induced by CD1d and NK T-cell activation. Taken together, these studies indicate that activation of NK T cells stimulates powerful down-regulatory and/or repair mechanisms that lead to significant improvement in colitis. Although the details of this ameliorative effect are unknown, the present results do raise several possibilities.

Our results clearly indicate that the effectiveness of  $\alpha$ -GalCer requires the presence of CD1d, which is somewhat predictable given the role of CD1d as a prerequisite ligand for  $\alpha$ -GalCer presentation and/or function. The mechanism of the dependence of  $\alpha$ -GalCer on CD1d expression can either be through the role of CD1d in NK T-cell activation and/or the direct activation of CD1d-bearing cells by  $\alpha$ -GalCer. As noted, our results strongly support the first possibility because NK T cells must be present and exposed to  $\alpha$ -GalCer and thus activated to reveal the beneficial effect of  $\alpha$ -GalCer. This is especially supported by the NK T-cell transfer experiments, which directly show that activation is required for NK T cells to effect a diminution in the severity of colitis. Given the fact that transfer of preactivated splenic NK T cells via intraperitoneal injection into recipient animals is capable of down-regulating colitis, as shown here, it is possible that activation in the periphery with or without trafficking to intestinal tissues in response to  $\alpha$ -GalCer administration is sufficient for the observed effect. In fact, in response to  $\alpha$ -GalCer we observed a significant expansion of NK T cells in the spleen.

These activated NK T cells are likely the source of potent anti-inflammatory cytokines. NK T cells produce high concentrations of both Th1 (interferon gamma) and Th2 (IL-4 and IL-10) cytokines.<sup>14,37,38</sup> Recent investigations, however, have shown that the Th1 response may be short lived with continued production of Th2 cytokines, and it may be this response that is occurring in the in vivo state.<sup>37</sup> The role of IL-10 in down-regulating the immune response has been shown in several colitis models and is particularly appealing in the current model given the ability of NK T cells to produce IL-10.<sup>37</sup> The exact effector mechanism involved in NK T-cell protection from colitis is an area of active investigation and remains to be defined. However, it is instructive that NK T cells exhibit their beneficial effect in the absence of T and B cells as shown by the adoptive transfer experiments in RAG<sup>-/-</sup> mice, suggesting a novel mode of regulation. These data also support the argument that RAG<sup>-/-</sup> mice have functional CD1d present, such as on the surface



**Figure 6.** Detection of  $\alpha$ -GalCer binding to intestinal epithelial cells of the colon by confocal microscopy. NBD-labeled  $\alpha$ -GalCer (50  $\mu$ g/kg) was injected intravenously into (A) WT and (B)  $CD1d^{-/-}$  mice and tissue from the descending colon isolated 4 hours after injection. The surface epithelial cells (Epi) in the WT mice show uptake (green fluorescence), as well as some basolateral uptake in the crypt epithelium (C) and scattered uptake within the lamina propria, whereas similar green fluorescent cells are not detected in the  $CD1d^{-/-}$  mice (B). The red represents counterstaining with phalloidin.

epithelium of their colon. Overall, this strongly suggests that NK T cells function either directly by themselves or indirectly through an effect on some other cell type such as the IEC, macrophage, or NK cell, which has been shown to play a critical role in another colitis model.<sup>39</sup>

These observations and interpretations raise the possibility that NK T cells within the intestine may be activated by  $\alpha$ -GalCer bound to CD1d in this location and contribute to the ameliorative effects on colitis. NK T cells likely normally represent a minor subset of cells within intestinal tissues, as has been described in peripheral blood.<sup>40</sup> However, this does not rule out the possibility that the number of cells present may be sufficiently large, given the size of the gut-associated lymphoid tissue, to have an important regulatory role in mucosal inflammation.

If NK T cells were to function within the intestine, the question then becomes which cell type(s) would be capable of activating local NK T cells through presentation of  $\alpha$ -GalCer if this process were occurring locally. One obvious candidate for a CD1d-bearing mucosal antigen-presenting cell is the IEC, which has been shown to express CD1d in humans, rats, and mice.<sup>20,27,28,41</sup> These observations were confirmed in these studies and indicate that labeled  $\alpha$ -GalCer localizes dominantly to the colonic IECs in a CD1d-dependent fashion. This raises the possibility that the effects of  $\alpha$ -GalCer may be mediated in part by the IEC. Because we have observed

that ligation of CD1d on the IEC under conditions of cross-linking leads to IL-10 production<sup>42</sup> and functionally inhibits the disruptive effects of interferon gamma on IEC barrier function in an autocrine pathway, a similar pathway may be engaged by  $\alpha$ -GalCer. This could either be by a direct effect of  $\alpha$ -GalCer on the IEC or indirectly through CD1d-restricted,  $\alpha$ -GalCer-specific antigen presentation to NK T cells. In either case, IL-10 production by the IEC, perhaps in conjunction with other NK T cell-derived cytokines, would lead to enhancement of barrier function and improvement in DSS-induced colitis. The fact that the protective effect of  $\alpha$ -GalCer was lost in the  $RAG^{-/-}$  animals, as observed in this report, suggests that the putative IEC barrier enhancing effects by  $\alpha$ -GalCer requires both a CD1d-bearing antigen-presenting cell and the NK T cell.

In summary, we have provided direct evidence for a role of NK T cells in the immunobiology of colitis. Through the use of  $\alpha$ -GalCer, a specific lipid agonist of CD1d-restricted NK T cells, we have shown that NK T cells play a beneficial role in colitis associated with DSS administration. Although the mechanism of this effect is unknown, we hypothesize that it is caused by a suppressive effect of NK T cells on mucosal inflammation and/or an important effect of NK T cells in positively regulating IEC barrier function. Thus, NK T cells must be considered a novel class of T cells involved in down-regulating the severity of colitis.

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