

# CD1-restricted NK T Cells Protect Nonobese Diabetic Mice from Developing Diabetes

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## Abstract

NK T cells are a unique subset of T cells that recognize lipid antigens presented by CD1d. After activation, NK T cells promptly produce large amounts of cytokines, which may modulate the upcoming immune responses. Previous studies have documented an association between decreased numbers of NK T cells and the progression of some autoimmune diseases, suggesting that NK T cells may control the development of autoimmune diseases. To investigate the role of NK T cells in autoimmune diabetes, we crossed CD1 knockout (CD1KO) mutation onto the nonobese diabetic (NOD) genetic background. We found that male CD1KO NOD mice exhibited significantly higher incidence and earlier onset of diabetes compared with the heterozygous controls. The diabetic frequencies in female mice showed a similar pattern; however, the differences were less profound between female CD1KO and control mice. Early treatment of NOD mice with  $\alpha$ -galactosylceramide, a potent NK T cell activator, reduced the severity of autoimmune diabetes in a CD1-dependent manner. Our results not only suggest a protective role of CD1-restricted NK T cells in autoimmune diabetes but also reveal a causative link between the deficiency of NK T cells and the induction of insulin-dependent diabetes mellitus.

Key words: CD1 • NK T cells • autoimmunity • diabetes • cytokines

## Introduction

Nonobese diabetic (NOD)\* mice, which develop autoimmune (type I) diabetes spontaneously, have long been used as animal models to study the mechanisms involved in insulin-dependent diabetes mellitus (IDDM) in humans (1). In female mice, inflammation of the pancreatic islets (insulinitis) usually begins at  $\sim$ 3–5 wk of age, eventually leading to  $\beta$  cell destruction and overt diabetes by 4–6 mo of age. Both CD4<sup>+</sup> T cells, especially the Th1 subset, which preferentially secretes IFN- $\gamma$  and TNF- $\alpha$ , and CD8<sup>+</sup> T cells have been implicated in the pathogenesis of IDDM (2–5). In parallel with these effector cells, the presence of regulatory T cells in prediabetic NOD mice that can block the development of IDDM has been suggested by adoptive transfer studies. These include CD4<sup>+</sup>CD62L<sup>+</sup> thymocytes (6), CD4<sup>+</sup>CD25<sup>+</sup> splenocytes (7), and NK T cells (8–10). Although the mechanisms of action of the regulatory T cells are not fully understood, it is believed that an imbalance between autoreactive effector T cells and regulatory T

cells may trigger the development of destructive insulinitis and IDDM.

NK T cells are a unique subset of T cells that coexpress receptors of the NK lineage and  $\alpha/\beta$  TCR. Most NK T cells express an invariant TCR  $\alpha$  chain encoded by the V $\alpha$ 14J $\alpha$ 281 gene segment, paired preferentially to V $\beta$ 8, V $\beta$ 7, and V $\beta$ 2 chain (11). V $\alpha$ 14<sup>+</sup> NK T cells recognize lipid antigens presented by the MHC class I-like molecule CD1d (12). Similar subsets of T cells are also present in humans (13). CD1d-restricted NK T cells localize preferentially to thymus and liver and are mainly of CD4<sup>+</sup> or CD4<sup>-</sup>CD8<sup>-</sup> phenotype (14). Upon TCR stimulation, NK T cells promptly produce large amounts of cytokines, including IL-4 and IFN- $\gamma$  (11). In addition, NK T cells have cytotoxic effects to certain targets, such as tumor cells and immature thymocytes (15, 16). Although the physiological ligand for CD1-restricted NK T cells is unknown,  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer), a glycolipid isolated from marine sponges that specifically binds CD1d, has been shown to be a potent activator for V $\alpha$ 14<sup>+</sup> NK T cells (17). After injection of  $\alpha$ -GalCer into mice, the activation of NK T cells results in rapid production of cytokines, which leads to activation of cell types involved in both innate immunity (NK cells and dendritic cells) and adaptive immunity (T cells and B cells) (18–21). Recent studies indicated that NK T cells

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\*Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; KO, knockout; NOD, nonobese diabetic.

may be involved in various immune responses, including tumor rejection (22), early protection of infectious agents (23, 24), and regulation of autoimmune diseases (25, 26).

The potential role of NK T cells in autoimmune diabetes is suggested by the finding that the number and function of NK T cells is diminished in the thymus and peripheral lymphoid organs of young NOD mice (8, 25, 27). In particular, anti-CD3-induced IL-4 secretion is barely detectable in young NOD mice (27). Adoptive transfer of cell populations enriched for NK T cells prevents IDDM in NOD recipients (8–10). Thus, a relative lack of IL-4 production by NK T cells is associated with and might be causal to the onset of IDDM. This notion is further supported by the observation that the frequency of IL-4-producing NK T cells is decreased in the peripheral blood of patients with IDDM (28).

In this study, we directly addressed the question of whether functional deficiency in NK T cells has a causative effect on the onset of IDDM by comparing the cumulative incidence of diabetes and progression of insulinitis in CD1-deficient NOD mice with CD1-heterozygous controls. We also examined the effect of  $\alpha$ -GalCer treatment on the development of diabetes in NOD and CD1-deficient NOD mice. We found that lack of CD1-restricted NK T cells promotes the development of diabetes, whereas activation of V $\alpha$ 14<sup>+</sup> NK T cells by  $\alpha$ -GalCer suppresses disease in the NOD model. Our data suggests that NK T cells could be used as therapeutic targets to modulate autoimmune diabetes.

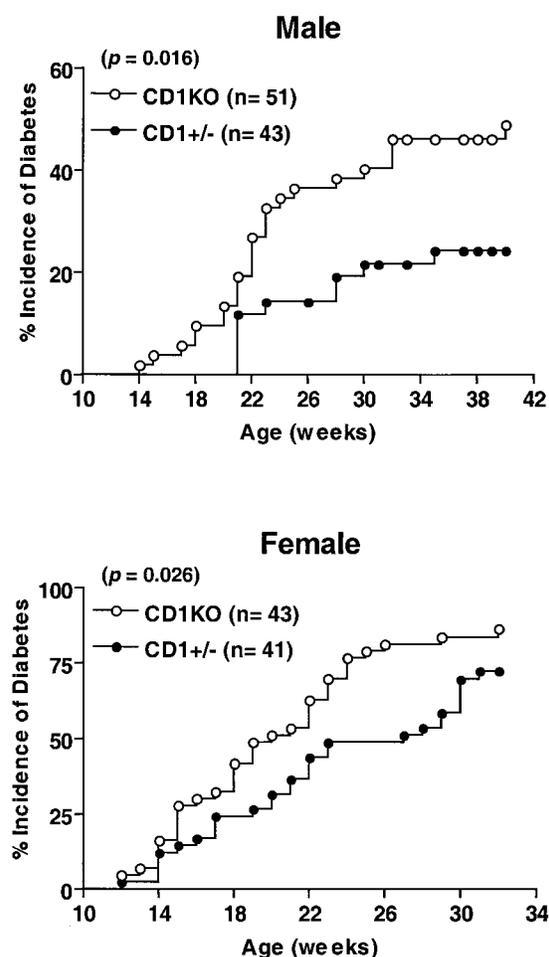
## Materials and Methods

**Generation and Genotyping of CD1-deficient NOD Mice.** Specific pathogen-free NOD/Lt and B6 mice were purchased from The Jackson Laboratory. In our NOD colony, the incidence of spontaneous diabetes at 24 wk is ~70% in females and 20% in males. CD1-deficient mice were generated by homologous recombination in our laboratory as described previously (29) and were backcrossed up to nine generations onto NOD/Lt background and then intercrossed to generate CD1<sup>+/-</sup> and CD1 knockout (CD1KO) NOD mice. The CD1 genotype was determined with PCR using the following three primers: CD1-for, 5'-ATGTTTGATGGCTGCCTCTGCTCACTG-3'; CD1-rev, 5'-TGAGTACAGAGAAGCCAGTGGCCTGGT-3'; and neo-, 5'-CCAAGTGCCAGCGGGGCTGCTAAAG-3', which yields a 150-bp fragment for the mutated allele and 330-bp fragment for the wild-type allele. At the third backcross generation, three diabetes susceptibility loci, *MHC I-A<sup>g7</sup>* (for *Idd1*), *D3Mit5* (for *Idd3*), and *D3Nds7* (for *Idd10*), were screened by PCR as described by Lenschow et al. (30). The specific primers used are as follows: for *I-A<sup>g7</sup>*, 5'-TGTCTTTTCTGTACCCCTAGAACA-3' and 5'-CTCTTCAGGCTGGGATGCTCCAC-3'; for *D3Mit5*, 5'-AGCCCTTCCAAGTGTCTCT-3' and 5'-GGTTTCGGAA-TGAGATGAGC-3'; and for *D3Nds7*, 5'-TGCTCCTACCA-GTCATGC-3' and 5'-TAGTGTTCATGGCTCTC-3'. The PCR products were resolved on a 4% agarose gel. The CD1<sup>+/-</sup> mice homozygous for the NOD allele in *I-A<sup>g7</sup>* and *D3Mit5* loci were selected for further backcross.

**Histopathology.** Pancreatic tissues were harvested at various time points, fixed with 10% buffered formalin, and embedded in

paraffin. Four serial 5- $\mu$ m sections were cut at intervals of 100  $\mu$ m and stained with hematoxylin and eosin. A minimum of 30 islets from each animal was examined. The severity of insulinitis was determined and graded using the following criteria according to the magnitude of mononuclear cell infiltration: grade 0, no mononuclear cell infiltration; grade 1, periinsulinitis, mononuclear cells surrounding islets and ducts but no intraislet infiltration; grade 2, mononuclear cell infiltration in and around the islet, but intraislet infiltration in <20% of the islet area; grade 3, intraislet mononuclear cell infiltration in 20–50% of the islet area; grade 4, extensive intraislet infiltration occupying >50% of the islet area.

**Assessment of Diabetes.** Starting at 10 wk of age, the urinary glucose analysis was performed every 2 wk with Diastix<sup>®</sup> reagent strips (Bayer Corporation). Hyperglycemia was confirmed by blood glucose determination with ONE-TOUCH<sup>®</sup> test strips (LifeScan, Inc.). Animals were considered diabetic after their urine glucose levels were >500 mg/dl for two consecutive days and their blood glucose levels were >250 mg/dl.



**Figure 1.** Increased onset and incidence of diabetes in CD1KO NOD mice. CD1KO mice were backcrossed six to nine generations onto NOD/Lt background and then intercrossed to generate CD1<sup>+/-</sup> and CD1KO NOD mice. Beginning at 10 wk of age, the urine glucose levels of NOD mice were monitored every 2 wk. Male (top) and female (bottom) mice were considered to have developed diabetes if two consecutive glucosuria levels were >500 mg/dl and blood glucose levels were >250 mg/dl. ○, homozygous CD1-deficient NOD mice; ●, CD1 heterozygous NOD littermate mice.

**In Vivo  $\alpha$ -GalCer Treatment.** A synthetic form of  $\alpha$ -GalCer, KRN7000, was used to treat NOD and CD1KO NOD mice. At 3 wk of age, the mice in the experiment group were injected twice per week intraperitoneally with 2  $\mu$ g per mouse of KRN7000 in 200  $\mu$ l of 0.025% polysorbate-20 in PBS, and the control mice were injected with 0.025% polysorbate-20 in PBS.

**In Vitro Activation of Lymphocytes and Analysis of Cytokine Production.** CD8<sup>+</sup> T cells and B cells were depleted from the splenocytes of CD1KO NOD or CD1<sup>+/-</sup> NOD mice by incubating cells with CD8 $\alpha$ -FITC and B220-FITC (PharMingen) and then incubated with avidin-magnetic beads and applied to magnetic separation (PerSeptive Biosystems). The CD4<sup>+</sup>-enriched cells ( $5 \times 10^5$  cells per well) were stimulated with either 100 ng/ml of  $\alpha$ -GalCer or with an anti-CD3 (2C11) coated 96-well plate in a final volume of 200  $\mu$ l of RPMI 1640 medium (supplemented with 10% FCS, 2 mM L-glutamine, 20  $\mu$ M 2-ME, and 100 U/ml penicillin/streptomycin). After 48 h, the culture supernatants were harvested, and the levels of IL-4 and IFN- $\gamma$  were quantitated by ELISA (PharMingen).

**Statistical Analysis.** The time course of spontaneous diabetes onset was analyzed using the Kaplan Meier curve. The curves were tested for significance by log-rank test. Mean values for cytokine levels and degree of insulinitis were compared using the unpaired Student's *t* test. Statistical significance was considered to be  $P < 0.05$ . All statistical analyses were performed with the Prism® program (GraphPad Software, Inc.).

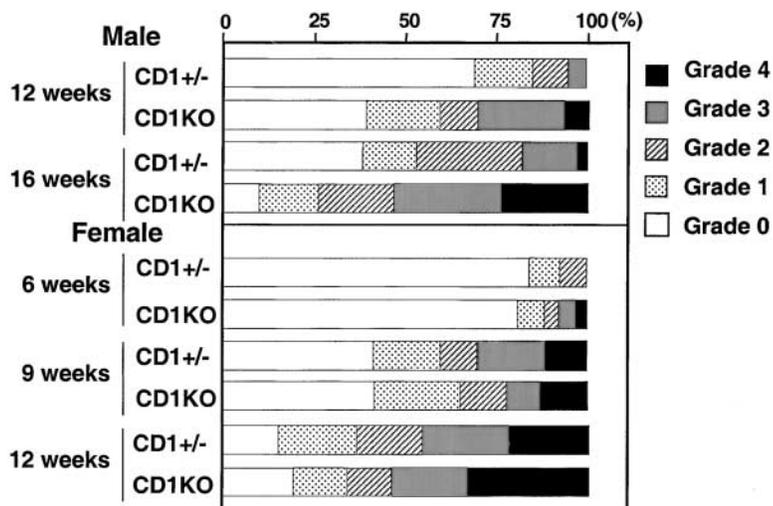
## Results

**Increased Incidence of Diabetes in CD1-deficient NOD Mice.** The CD1KO mutation replaced a 12-kb DNA fragment encompassing exon 4–6 from both *CD1d1* and *CD1d2* with a neomycin-resistant cassette, which resulted in a complete loss of CD1 expression and impaired NK T cell development in homozygous mutant animals (29). We crossed the CD1KO mutation onto the NOD/Lt background to investigate the role of CD1-restricted NK T cells in the regulation of autoimmune diabetes. The development of diabetes in CD1KO NOD and control mice (from six to nine generations backcrossed) was monitored at biweekly intervals beginning after 10 wk of age. As seen in Fig. 1,

>50% of the male CD1KO NOD mice were hyperglycemic by 40 wk of age, as compared with only 20% of the male CD1<sup>+/-</sup> NOD mice. Nearly 15% of the male CD1KO NOD mice developed diabetes by 20 wk of age, while all the CD1<sup>+/-</sup> controls were diabetes free within the same period. This data suggests that CD1 deficiency not only results in an increased incidence of diabetes but also accelerates the onset of diabetes in male NOD mice ( $P < 0.05$ ;  $n = 51$  and 43 for CD1KO and CD1<sup>+/-</sup>, respectively). Although the female CD1KO NOD mice also displayed a slightly increased incidence of diabetes over a period of 32 wk ( $P < 0.05$ ;  $n = 43$  and 41 for CD1KO and CD1<sup>+/-</sup>, respectively), we observed no significant differences in the onset of diabetes between the female CD1KO NOD and CD1<sup>+/-</sup> controls.

A hallmark of the NOD mouse model for IDDM is the progressive infiltration of mononuclear cells into the pancreatic islets. In our wild-type NOD colony, insulinitis becomes noticeable at 5–6 wk of age in females and 10–12 wk of age in males. To quantitate the development of insulinitis, 30–40 islets from individual CD1KO NOD and heterozygous control mice (nine generations backcrossed) were assessed for the severity of insulinitis (Fig. 2). In the male CD1<sup>+/-</sup> NOD mice, almost 70% of islets are free of infiltrating cells at 12 wk of age, at which time <40% of islets from CD1KO NOD mice are free of insulinitis ( $n = 5$ ,  $P < 0.05$ ). At 16 wk of age, 35–45% of islets from CD1<sup>+/-</sup> controls still have no detectable insulinitis, while only 10–15% of islets from CD1KO NOD male mice are free of infiltrating cells ( $n = 4$ ,  $P < 0.05$ ). In addition, the percentages of islets with heavy infiltrates (grades 3 and 4) were also significantly greater in CD1KO groups. In contrast, we found no significant difference in the degree of insulinitis between female CD1KO NOD mice and their heterozygous littermate controls at 6, 9, and 12 wk of age (Fig. 2).

**Reduced IL-4 and IFN- $\gamma$  Secretion in CD1-deficient NOD Mice.** Cytokine imbalance has been attributed to be one of the mechanisms to trigger the development of autoimmune diabetes (31). To investigate whether lack of CD1-restricted NK T cells affects the balance between Th1 and



**Figure 2.** Histopathologic examination of pancreatic insulinitis in CD1KO NOD and CD1<sup>+/-</sup> NOD littermates. Pancreatic tissues were taken at serial time points. After hematoxylin and eosin staining, a minimum of 30 islets from each mouse was examined. The severity of insulinitis was determined and graded semiquantitatively using the criteria as described in Materials and Methods. Data shown represent mean of four to five animals in each age group.

Th2 cells in NOD mice, we analyzed the cytokine profile of CD4<sup>+</sup> T cells from CD1KO and control littermates upon anti-CD3 stimulation *in vitro*. As shown in Fig. 3 A, splenic CD4<sup>+</sup> T cells from both male and female CD1KO NOD mice produced significantly lower quantities of IL-4 compared with heterozygous control mice in all of the age groups ( $n = 5$ ,  $P < 0.05$ ). We found considerable variability in the levels of IFN- $\gamma$  produced in individual mice with no statistical significance between CD1KO and heterozygous littermate controls (Fig. 3 A). Thus, lack of IL-4 secreted by NK T cells did not promote the Th1 response in CD1KO NOD mice. Nevertheless, the ratios of IFN- $\gamma$ /IL-4 are consistently greater in CD1KO NOD mice than those in the control mice (Fig. 3 B), which might be an important factor in controlling the IDDM.

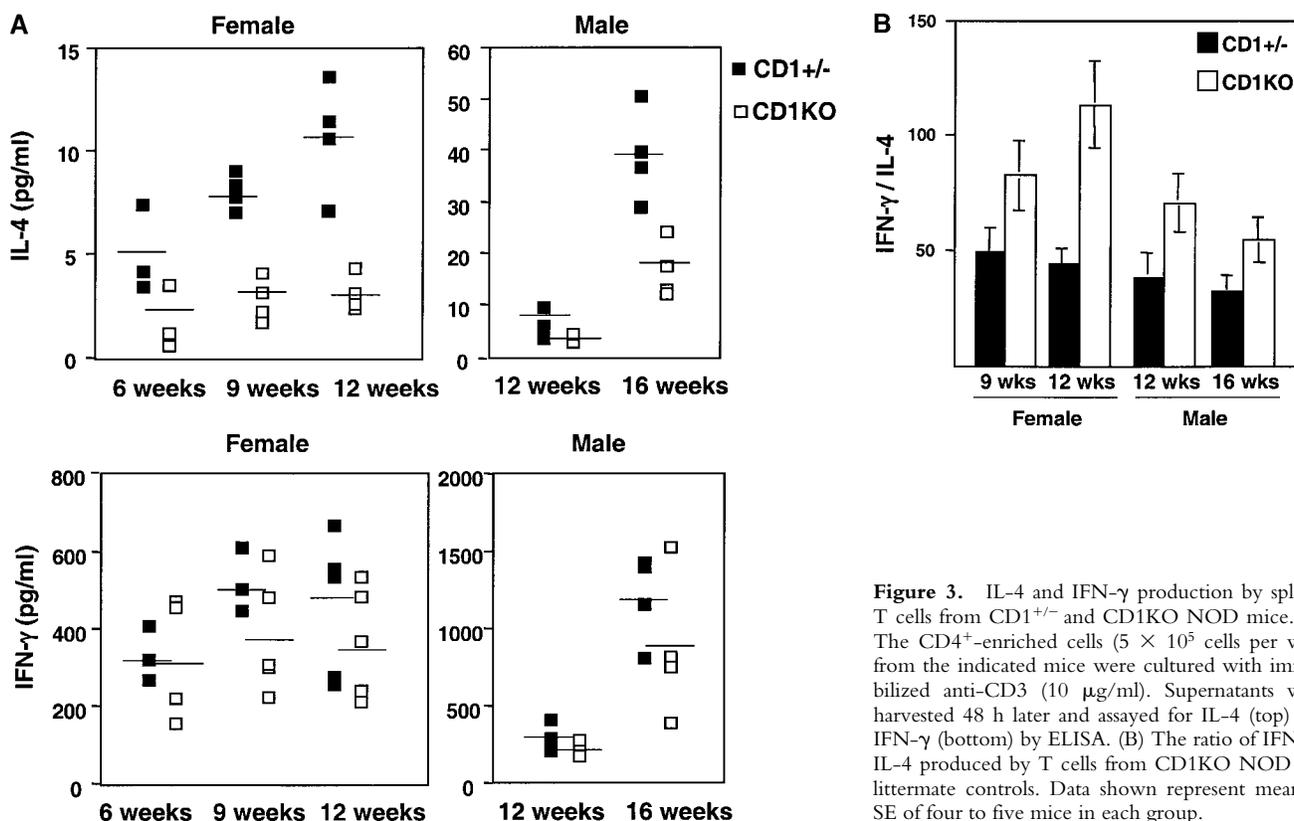
*$\alpha$ -GalCer Treatment Delays the Onset and Reduces the Incidence of Diabetes in NOD Mice.*  $\alpha$ -GalCer has been shown to be a potent immunostimulator for CD1-restricted NK T cells both *in vivo* and *in vitro* (17). As NOD mice have diminished NK T cell activity compared with other strains of mice, we examined whether  $\alpha$ -GalCer can activate NK T cells from NOD mice. Fig. 4 A shows that splenic T cells from NOD mice secrete both IL-4 and IFN- $\gamma$  upon stimulation with  $\alpha$ -GalCer, although the response is significantly weaker than that in B6 mice. To determine the effect of enhanced NK T cell activity on the development of spontaneous autoimmune diabetes, NOD mice were treated, starting at 3 wk of age, with  $\alpha$ -GalCer or vehicle control. Treating NOD mice with  $\alpha$ -GalCer significantly reduced

the cumulative incidence of diabetes at the age of 32 wk (from 80 to 40%,  $n = 15$ ,  $P < 0.05$ ; Fig. 4 B). There was also a delayed onset of diabetes in  $\alpha$ -GalCer-treated NOD mice. None of the  $\alpha$ -GalCer-treated mice developed diabetes before 16 wk of age, whereas nearly 30% of vehicle-treated NOD mice had already developed diabetes. In contrast, treating CD1KO NOD mice with  $\alpha$ -GalCer did not have any effect on the development of diabetes. Therefore, the effect of  $\alpha$ -GalCer on the development of diabetes is strictly CD1 dependent, presumably mediated through CD1-restricted NK T cells.

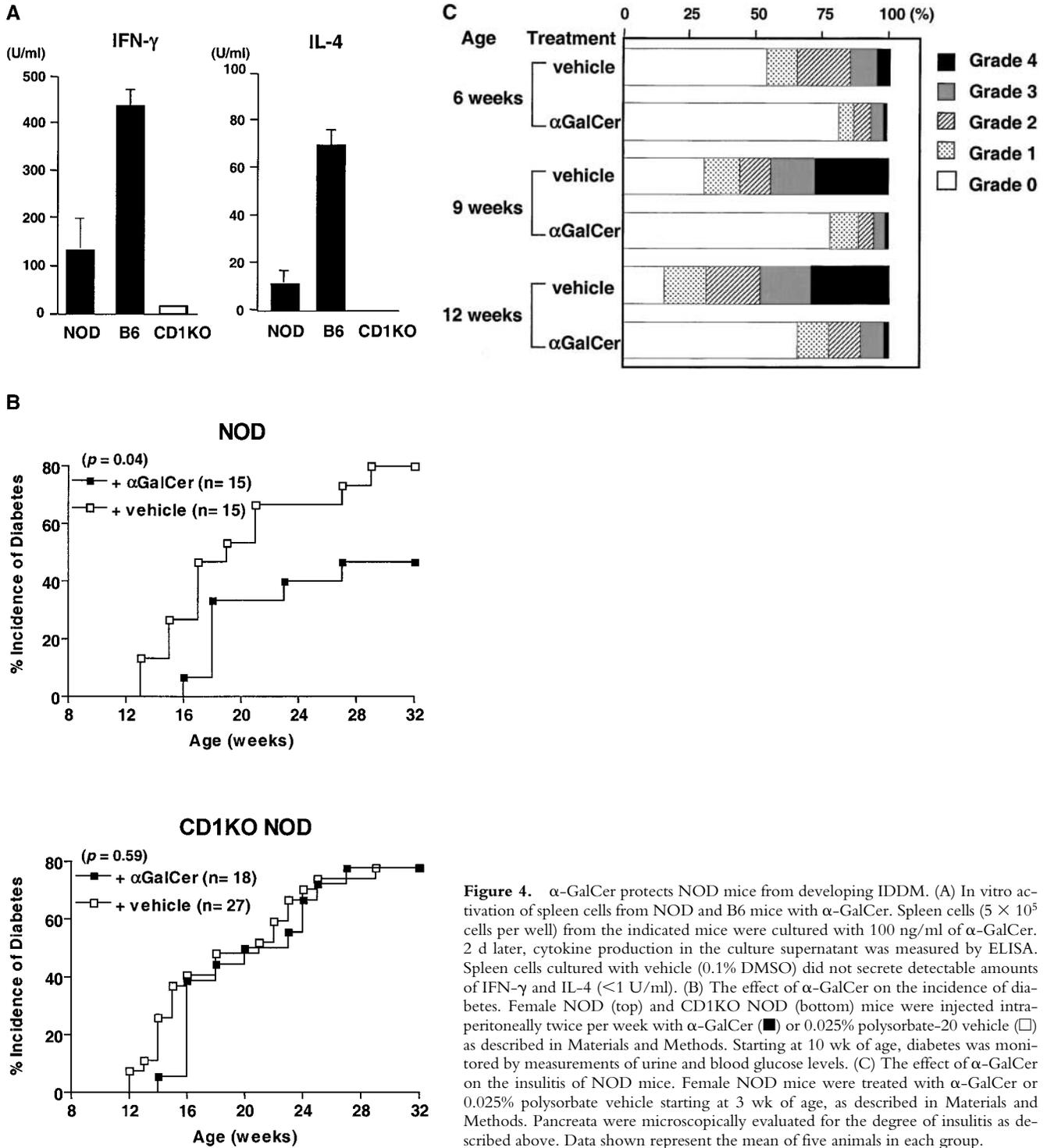
To examine the effect of  $\alpha$ -GalCer treatment on pre-diabetic islet infiltration, we analyzed hematoxylin and eosin-stained sections of pancreata from  $\alpha$ -GalCer- and vehicle-treated mice at weeks 3, 6, and 9 after the start of treatment. As shown in Fig. 4 C, significantly greater percentages of islets were free of insulitis from  $\alpha$ -GalCer-treated mice than those from the control mice at all three time points ( $n = 5$ ,  $P < 0.05$ ). Furthermore, the degree of insulitis in  $\alpha$ -GalCer-treated mice is less severe than that in the vehicle-treated mice. Thus, activation of NK T cells by  $\alpha$ -GalCer suppressed the inflammatory infiltration of the islets in NOD mice, which could subsequently delay the onset and reduce the incidence of diabetes.

## Discussion

As in human IDDM, diabetes development in NOD mice is controlled by a number of disease susceptibility or



**Figure 3.** IL-4 and IFN- $\gamma$  production by splenic T cells from CD1<sup>+/-</sup> and CD1KO NOD mice. (A) The CD4<sup>+</sup>-enriched cells ( $5 \times 10^5$  cells per well) from the indicated mice were cultured with immobilized anti-CD3 (10  $\mu$ g/ml). Supernatants were harvested 48 h later and assayed for IL-4 (top) and IFN- $\gamma$  (bottom) by ELISA. (B) The ratio of IFN- $\gamma$ /IL-4 produced by T cells from CD1KO NOD and littermate controls. Data shown represent mean  $\pm$  SE of four to five mice in each group.



**Figure 4.**  $\alpha$ -GalCer protects NOD mice from developing IDDM. (A) In vitro activation of spleen cells from NOD and B6 mice with  $\alpha$ -GalCer. Spleen cells ( $5 \times 10^5$  cells per well) from the indicated mice were cultured with 100 ng/ml of  $\alpha$ -GalCer. 2 d later, cytokine production in the culture supernatant was measured by ELISA. Spleen cells cultured with vehicle (0.1% DMSO) did not secrete detectable amounts of IFN- $\gamma$  and IL-4 ( $<1$  U/ml). (B) The effect of  $\alpha$ -GalCer on the incidence of diabetes. Female NOD (top) and CD1KO NOD (bottom) mice were injected intraperitoneally twice per week with  $\alpha$ -GalCer (■) or 0.025% polysorbate-20 vehicle (□) as described in Materials and Methods. Starting at 10 wk of age, diabetes was monitored by measurements of urine and blood glucose levels. (C) The effect of  $\alpha$ -GalCer on the insulinitis of NOD mice. Female NOD mice were treated with  $\alpha$ -GalCer or 0.025% polysorbate vehicle starting at 3 wk of age, as described in Materials and Methods. Pancreata were microscopically evaluated for the degree of insulinitis as described above. Data shown represent the mean of five animals in each group.

resistance genes (*Idds*). The CD1 gene is close to the *Idd3* and *Idd10* loci in murine chromosome 3 (32). Based on the PCR typing (data not shown), all of the mice (CD1 KO and CD1 $^{+/-}$ ) used in this study were homozygous for the NOD allele in *Idd3* locus. The CD1 gene is tightly linked with *Idd10*. The CD1 $^{+/-}$  mice used in this study are heterozygous for the *Idd10* locus, while CD1KO mice retain the 129 allele. As CD1KO NOD mice contain one less

*Idd*-susceptible allele than controls, it is unlikely that the increased susceptibility to diabetes is due to a linked gene carried along during the backcross. Our observation of the acceleration of autoimmune disease in CD1KO NOD mice suggested that CD1-restricted NK T cells play a role in resistance to diabetes in NOD mice even though NK T cell function is genetically diminished relative to that in other strains of mice (25). Activation of NK T cells with  $\alpha$ -Gal-

Cer inhibited IDDM development in NOD but not CD1KO NOD mice, which further supports the protective role of CD1-restricted NK T cells in autoimmune diabetes.

In the NOD model, there exists a remarkable gender disparity in the cumulative incidence of diabetes, indicating that female NOD mice might have a greater deficit in various immunoregulatory mechanisms. Therefore, lack of functional activity from NK T cells, one out of many immunoregulatory mechanisms, appears to have less profound effect on the progression of diabetes in female NOD mice than in male NOD mice. However, we cannot eliminate the possibility that NK T cells may play a more dominant role in regulating the IDDM in male NOD mice.

There are several distinct mechanisms that may explain why lack of NK T cells in CD1KO NOD mice would render them more susceptible to autoimmune disease. First, lower levels of IL-4 detected in CD1KO NOD mice might not be sufficient to stimulate mice to downregulate Th1 diabetogenic cells. However, as IL-4KO NOD mice do not develop accelerated disease (33), lack of IL-4 secreted by NK T cells could not be solely responsible for the increased susceptibility to diabetes in CD1KO NOD mice. A recent study showed that IFN- $\gamma$  secreted by NK T cells in the periphery might be critical for protection against diabetes (10). Although the majority of CD1KO NOD mice secreted lower levels of IFN- $\gamma$  compared with controls, some CD1KO NOD mice produced more IFN- $\gamma$  than their littermate controls, presumably from other CD4<sup>+</sup> T cells. Thus, it is unlikely that lack of IFN- $\gamma$  secreted by NK T cells would contribute significantly to the exacerbation of IDDM in CD1KO mice. Second, NK T cells have been shown to have a cytotoxic effect on immature thymocytes (15), indicating that NK T cells may regulate T cell selection in the thymus. Therefore, lack of NK T cells might affect the repertoire of autoreactive T cells. Third, NK T cells or the expression of CD1 might be involved in maintaining the survival and function of some regulatory T cells. We performed FACS<sup>®</sup> analysis and found that CD1KO NOD and heterozygous controls have comparable numbers of CD4<sup>+</sup>CD25<sup>+</sup> T cells (data not shown), a subset of T cells that has been shown to be critical in controlling spontaneous autoimmune diabetes (7). However, it remains possible that other regulatory T cells might be affected by CD1 deficiency.

Treatment with  $\alpha$ -GalCer has been shown to modulate several immune responses in vivo, such as enhancing the antitumor (34) and antiviral activity (35), mediating protection against murine malaria (36), and regulating intestinal inflammation (37). Here we showed that  $\alpha$ -GalCer exerts a protective effect on autoimmune diabetes in NOD mice. The precise mechanism of how  $\alpha$ -GalCer activates NK T cells to protect IDDM is not clear. Previous studies showed that in vivo administration of  $\alpha$ -GalCer polarizes adaptive immune response for production of Th2 cytokines (20, 21). However, we did not detect a polarized Th2 response in  $\alpha$ -GalCer-treated NOD mice (data not shown). It is possible that activation of NK T cells by  $\alpha$ -GalCer leads to bystander activation of cell types (i.e., regulatory T cells,

NK cells, and dendritic cells) that are critical in regulating the autoreactive T cells in NOD mice. Similar to mouse NK T cells,  $\alpha$ -GalCer can also activate human NK T cells in a CD1d-dependent manner (38, 39). Thus, our results suggest that activation of NK T cells by  $\alpha$ -GalCer may represent a novel approach for therapeutic intervention of IDDM in humans.

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