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Failure of α-Galactosylceramide to Prevent Diabetes in Virus-inducible Models of Type 1 Diabetes in the Rat

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Abstract. *Background: α-Galactosylceramide (α-GalCer) is an invariant natural killer T (iNKT) cell ligand that prevents type 1 diabetes in NOD mice. However, α-GalCer can activate or suppress immune responses, raising concern about its potential use in human diabetes. Materials and Methods: To evaluate this therapeutic issue further, BBDR and LEW.1WR1 rats were treated with Kilham rat virus (KRV) plus polyinosinic-polycytidylic acid, with or without α-GalCer, and followed for onset of diabetes. Results: α-GalCer did not prevent diabetes in inducible rat models. To investigate this discrepancy, we analyzed iNKT cell function. Splenocytes stimulated with α-GalCer produced similar levels of IFNγ in all rat strains, but less than mouse splenocytes. Rat splenocytes stimulated with α-GalCer preferentially produced IL-12, whereas mouse splenocytes preferentially produced IL-4. Conclusion: α-GalCer elicits species-specific cytokine responses in iNKT cells. In humans with type 1 diabetes, differences in iNKT cell responses to stimulation with α-GalCer due to age, genetic variability and other factors may influence its therapeutic potential.*

Invariant natural killer T (iNKT) cells are a subset of T-cells that express both T-cell receptor (TCR) molecules and specific NK cell receptors (1-4). The TCR alpha and beta chains expressed by iNKT cells do not undergo random variable region recombination (4, 5); their invariant TCRs recognize glycolipid antigens presented by a major histocompatibility complex (MHC)-class I type molecule, CD1d, on antigen-presenting cells (6-9). Functional analyses of iNKT cells have largely been based on stimulation by α-galactosylceramide (α-GalCer), a synthetic glycolipid that specifically stimulates iNKT cells (4, 10, 11). iNKT cells produce both Th1- (IFNγ) and Th2-type (IL-4, IL-10) cytokines within hours of activation by α-GalCer. These cytokines, in turn, influence the outcome of subsequent adaptive immune responses by polarizing T-cells towards a Th1 (cytotoxic) or Th2 (immunosuppressive) phenotype (4, 5).

The early activation response, an invariant TCR, and the ability to recognize bacterial, viral and parasitic pathogens suggest that iNKT cells play a role in both the innate and adaptive immune responses (4, 5). Studies in mice have shown that iNKT cells also recognize self-antigens and play a role in maintaining self-tolerance and preventing autoimmunity in disease models including experimental allergic encephalomyelitis (EAE) and type 1 diabetes (T1D) (2, 4, 9, 12, 13). Not surprisingly, based on their dual cytokine response, activated NKT cells can also exacerbate disease as observed in models of asthma and lupus (2, 4, 9, 12-14). Such studies raise concerns as to the potential of α-GalCer as a safe and effective inmmunotherapy.

The NOD mouse is a widely studied animal model of spontaneous autoimmune T1D (15-18). In this model, α-GalCer has been reported to prevent the development of diabetes (19-21). Other animal models of T1D include BBDR (22, 23) and LEW.1WR1 (24) rats, which readily become diabetic after immunological perturbation. They are useful models because human T1D is thought to occur through a complex interaction of genetic susceptibility and exposure to environmental 'perturbants' such as virus (25). BBDR rats do not develop spontaneous diabetes when housed in viral antibody-free conditions. However, after infection with Kilham rat virus (KRV), ~10-30% of BBDR rats develop autoimmune diabetes (26, 27). When pre-treated with the TLR-3 and MDA5 agonist, polyinosinic-polycytidylic acid (poly I:C), diabetes occurs in 100% of KRV-infected BBDR rats (26, 27). The LEW.1WR1 rat also

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develops diabetes (>90%) within two weeks following injection of poly I:C and infection with KRV (22, 23). Here we report on the ability of α-GalCer to modulate autoimmune diabetes in virus-inducible rat models of T1D.

Materials and Methods

Animals. Viral antibody-free BBDR/Wor and LEW.1WR1 rats were from BRM, Inc. (Worcester, MA, USA), LEW and F344/Crl from Charles River Laboratories (Wilmington, DE, USA), and WF from Harlan Sprague Dawley (Indianapolis, IN, USA). NOD and C57BL/6 mice were from The Jackson Laboratory (Bar Harbor, ME, USA). Animals were maintained in accordance with guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School and the recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences).

Treatment protocols. To induce diabetes, 21- to 24-day old BBDR and LEW.1WR1 rats of either sex were injected intraperitoneally (*i.p.*) with one dose of KRV (10^7 pfu/rat) alone or preceded by three consecutive daily doses of poly I:C (Sigma, St. Louis, MO, USA, 1 μg/g body weight). For diabetes protection studies, 14- to 17-day-old rats were given 4 consecutive weekly *i.p.* injections of 5 μg or 10 μg per rat of α-GalCer in 200 μl vehicle (PBS plus 0.05% Tween) or vehicle alone beginning one week prior to the first poly I:C injection (or KRV infection in rats treated with KRV alone). The α-GalCer was provided by Dr. Wilson (Harvard Medical School, Boston, MA, USA) or from Alexis/Axxora LLC (San Diego, CA, USA). Diabetes was confirmed by two consecutive readings of plasma glucose concentration >250 mg/dl using an Accuchek Active glucometer (Hoffmann-La Roche, Basel, Switzerland). Animals were tested twice weekly for glycosuria until diabetes onset or until 40 days after KRV injection at which point the animals were terminated.

Cell preparation and flow cytometric analyses. Single cell lymphocyte suspensions were made from isolated rat spleens; hepatic lymphocytes were isolated as described (28). iNKT cells were analyzed by flow cytometry using FACScan, FACScalibur or LSRII instruments (BD Biosciences, San Jose, CA, USA). Antibodies included: αβ-TCR-PerCp (clone R73), CD4-FITC (clone OX-35), CD8-FITC (clone OX-8) and NKR-P1A-PE (clone 10/78) (BD Biosciences). CD4-Pacific blue (W3/25) was from Serotec (Raleigh, NC, USA).

In vitro α-GalCer stimulation. Rat splenocytes (5×10^6 cells/ml) were stimulated in RPMI-1640 medium supplemented with 10% FBS, 50 μM 2-β-ME, and α-GalCer or vehicle (DMSO) at the indicated concentrations. Cultures stimulated with PMA plus ionomycin (50 ng/ml and 500 ng/ml, respectively) were used as positive controls. Rat IFNγ and IL-4 (BD Biosciences), and IL-12p40 (Biosource, Carlsbad, CA, USA) in culture supernatants were quantified by ELISA as per manufacturers' instructions. NOD and C57BL/6 mice splenocytes were similarly cultured and cytokines assayed by ELISA (BD Biosciences).

Statistical analyses. Diabetes-free survival was analyzed by the Kaplan and Meier method; differences between groups were computed using the log-rank statistic. Comparisons of three or more means used one-way analyses of variance and the least significant differences (LSD) method for *a posteriori* contrasts; $p<0.05$ was considered statistically significant.

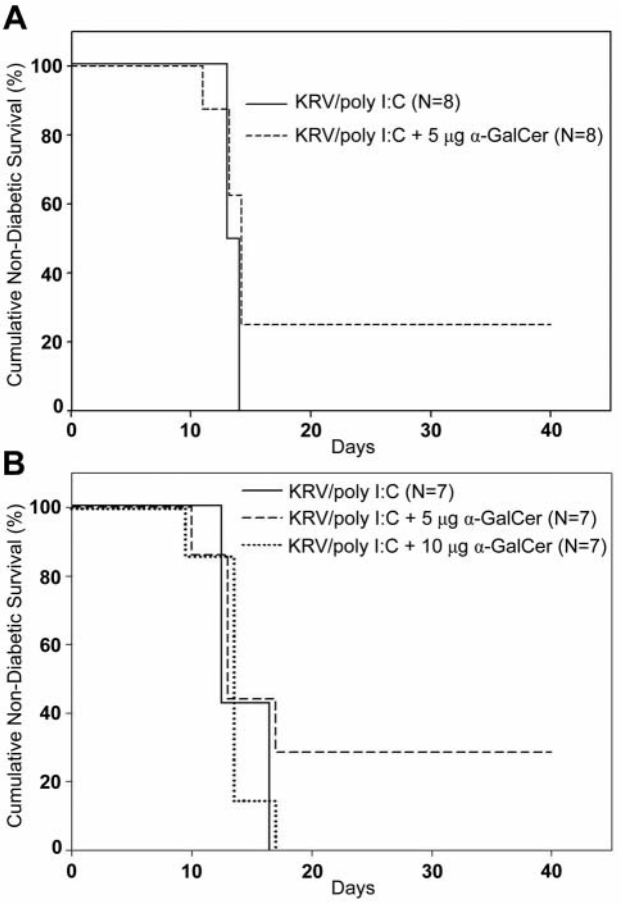

Figure 1. *α-GalCer treatment does not prevent diabetes in rats treated with KRV plus poly I:C. A, BBDR rats were injected with α-GalCer (5 μg/dose) or vehicle in the KRV plus poly I:C induction protocol. B, LEW.1WR1 rats were treated with α-GalCer (5 μg/dose or 10 μg/dose) or vehicle in the KRV plus poly I:C induction protocol. All animals were followed for 40 days after KRV injection for diabetes onset.*

Results

α-GalCer does not prevent or delay diabetes in rats treated with KRV plus poly I:C. α-GalCer has been shown to prevent diabetes in NOD mice (16, 19, 21). To test its comparative effectiveness in a different model system, we first used BBDR rats treated with KRV plus poly I:C; these animals received either vehicle or α-GalCer (5 μg/dose). We observed no significant difference in the frequency or time to onset of diabetes in the α-GalCer-treated group compared to the vehicle control group (Figure 1A). The median times to diabetes onset for KRV plus poly I:C-treated rats in the absence or presence of α-GalCer were 13.5 and 14 days, respectively.

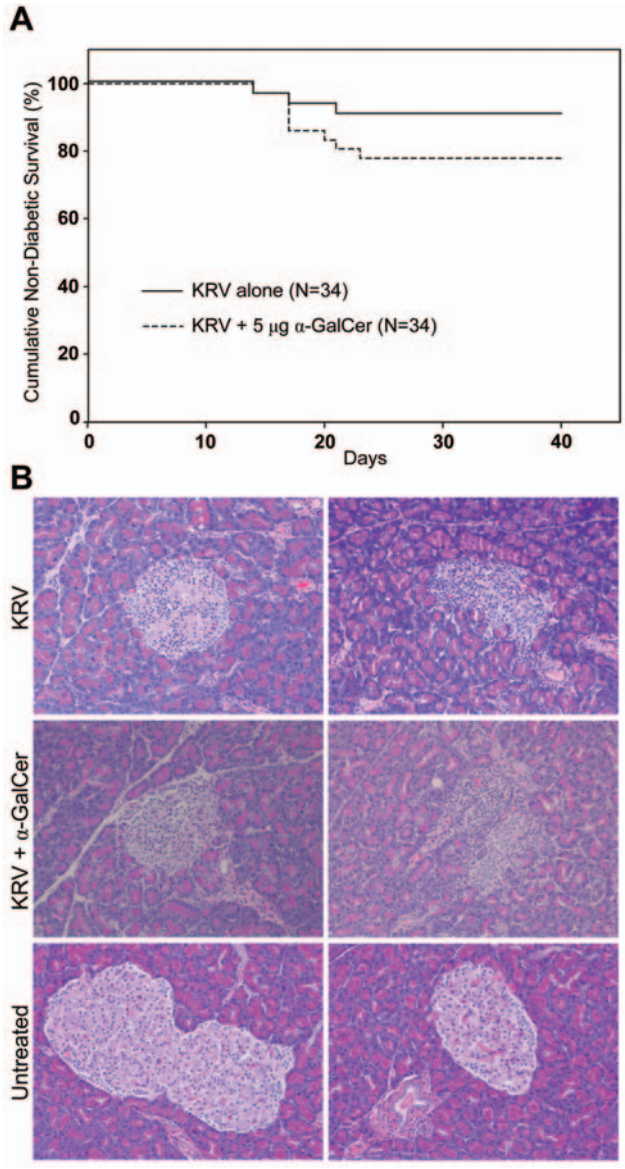

Figure 2. *α-GalCer treatment does not delay or exacerbate diabetes in KRV-infected BBDR rats. A, BBDR rats were injected with α-GalCer (5 μg/dose) or vehicle in the KRV alone induction protocol. The animals were followed for 40 days after KRV injection for diabetes onset. B, Representative pancreas sections from KRV-infected rats at time of diabetes onset showing extensive insulitis in both vehicle control (KRV) and α-GalCer-treated (KRV + α-GalCer) rats. Pancreatic sections were stained with hematoxylin and eosin; sections from an untreated BBDR rat of the same age are shown for comparison.*

To determine whether this lack of diabetes prevention by α-GalCer was restricted to the BBDR strain, we tested the LEW.1WR1 rat, which is also susceptible to virus-induced diabetes (24). We treated LEW.1WR1 rats using the same KRV plus poly I:C protocol. In this study, we tested both 5 μg and 10 μg doses of α-GalCer. There were no differences in the frequency or latency to diabetes onset among the three groups (Figure 1B). Median time to diabetes onset was 13 days for all groups.

Table I. *NK, T and iNKT cell populations in spleen and liver lymphocytes isolated from diabetes-sensitive and -resistant rat strains.*

	Spleen[a]			Liver[a]		
Rat strain	NK	T	iNKT	NK	T	iNKT
BBDR	16.5±3.3	11.6±1.8	4.0±1.0	34.6±10.5	13.7±7.7	6.7±5.2
LEW.1WR1	30.2±14.0	15.0±6.1	5.2±0.6	21.2±3.3	26.5±4.4	4.5±1.0
LEW	31.6±12.0	13.7±4.8	5.6±1.0	32.2±6.2	24.5±7.4	5.8±1.6
WF	9.5±1.7	14.3±4.2	5.0±0.5	33.3±4.8	13.0±6.6	6.7±5.6

Values are mean±SD for each rat strain (21- to 35-day-old, n=6 for each strain). [a]Gated on lymphocytes.

α-GalCer did not alter diabetes outcome in rats treated with KRV alone. Treatment with KRV alone generally induces diabetes in only ~10-30% of BBDR rats (22, 23). We investigated the possibility that this less 'potent' diabetes induction protocol might be more amenable to preventive therapies than was the KRV plus poly I:C protocol. Rats were treated with α-GalCer or vehicle (4 weekly injections) beginning one week prior to infection with KRV. There was no statistically significant difference between the two groups (p=0.13) in the percentage of rats that became diabetic. Among rats that developed diabetes, time to onset was 14-21 and 14-23 days for the KRV-treated rats in the absence or presence of α-GalCer, respectively (Figure 2A). In addition, the degree of insulitis observed in pancreas sections from diabetic rats was similar in both groups (Figure 2B). Pancreatic sections from KRV-treated rats that did not become diabetic showed no difference in the percent of infiltrated islets in pancreas sections from animals that received α-GalCer or vehicle control (<5% in each group).

iNKT cell frequency was normal in diabetes-inducible rat strains. We explored mechanisms that might explain the lack of efficacy of α-GalCer to prevent diabetes in rats as compared with NOD mice. To determine if iNKT cells may be deficient in the rat strains used for diabetes induction, we performed flow cytometric analyses and measured the frequency of iNKT cells in spleen and liver from our test strains, BBDR and LEW.1WR1, and two controls strains, WF and LEW. Flow cytometric analyses revealed that iNKT cells comprised 4-6% of the total lymphocytes in the spleens of the four rat strains analyzed (Table I, left panel). These percentages are similar to those in F344 rat splenic lymphocytes (~6%, data not shown and (29)). iNKT cells also comprised 4-6% of the total lymphocytes in the livers of the four rat strains analyzed (Table I, right panel). The

Table II. *CD4 and CD8 phenotype of spleen and liver iNKT cells isolated from diabetes-sensitive and -resistant rat strains.*

	Spleen[a]				Liver[a]			
Rat Strain	$CD4^+CD8^-$	$CD8^+CD4^-$	$CD4^+CD8^+$	$CD4^-CD8^-$	$CD4^+CD8^-$	$CD8^+CD4^-$	$CD4^+CD8^+$	$CD4^-CD8^-$
BBDR	24.9±5.5	44.3±20.8	5.1±2.5	25.6±21.5	22.3±10.6	16.6±22.1	14.7±17.8	46.5±27.5
LEW.1WR1	23.0±5.8	32.9±21.8	7.0±3.3	37.1±29.0	23.0±9.9	37.7±23.0	12.2±6.1	27.1±24.3
LEW	23.7±3.1	24.9±23.1	5.0±1.7	46.5±27.2	19.2±4.5	28.1±28.1	5.0±2.2	47.7±26.8
WF	16.3±6.6	57.8±15.0	3.4±1.0	23.5±10.0	12.7±11.7	48.2±34.6	10.9±8.7	28.2±23.7

Values are mean±SD for each rat strain (21- to 35-day-old, n=6 for each strain). [a]Gated on NKT cells.

percentages of splenic and liver iNKT cells were statistically similar in the four strains (p=N.S.).

The majority of rat iNKT cells were $CD8^+$ or $CD4^-CD8^-$. In the mouse, iNKT cell subpopulations phenotypically consist of single positive $CD4^+$ and double negative $CD4^-CD8^-$ cells (30). To investigate the phenotypic distribution of iNKT subsets among rat strains, we analyzed iNKT cells for their expression of CD4 and CD8. In the spleen, the majority of iNKT cells in the four rat strains analyzed were $CD8^+CD4^-$ (25-57%) or $CD4^-CD8^-$ (23-47%, Table II, left panel). The remainder of the iNKT cells consisted of $CD4^+CD8^-$ (16-25%) or $CD4^+CD8^+$ (3-7%) cells. iNKT cells from the liver showed similar profiles of CD4 and CD8 expression (Table II, right panel). The iNKT cell profiles of splenic and hepatic lymphocytes showed no statistically significant differences among rat strains.

a-GalCer-stimulated rat splenocytes produced less IL-4 than did mouse splenocytes. Normal mouse iNKT cells when activated by α-GalCer rapidly produce both Th1 (IFNγ) and Th2 (IL-4) cytokines (1, 2, 4, 5, 31), but the response of rat iNKT cells to α-GalCer has not been studied in detail. We hypothesized that the immunosuppressive Th2 response of iNKT cells from diabetes-inducible rats might be deficient. To investigate this, splenocytes were isolated from age-matched BBDR, LEW.1WR1, and F344 rats and stimulated with α-GalCer at concentrations up to 1,000 ng/ml. As a comparison, splenocytes isolated from diabetes-prone NOD and normal C57BL/6 mice were similarly stimulated with α-GalCer.

After 48 h of stimulation with α-GalCer, C57BL/6 splenocyte culture supernatants showed a dose-dependent increase in IL-4 production (Figure 3A). At this same time point, production of IL-4 by α-GalCer-stimulated NOD or rat splenocytes was much less (~1 to 4 pg/ml) and not greater than that of the vehicle (DMSO) control; PMA plus ionomycin-stimulated IL-4 levels were also low. At 72 h of α-GalCer stimulation, IL-4 production in both the NOD and C57BL/6 splenocyte cultures had increased dramatically. In contrast, IL-4 levels remained low in all rat strains analyzed (Figure 3B).

IFNγ production by a-GalCer-stimulated splenocytes was comparable among rat strains and less than in the mouse. We next measured the ability of rat iNKT cells to produce the Th1 cytokine, IFNγ, in response to stimulation with α-GalCer. IFNγ production by α-GalCer-stimulated spleen cells from both the diabetes-inducible rat strains and control F344 rats was comparable at both 48 (Figure 4A) and 72 h (Figure 4B). In contrast, IFNγ production by C57BL/6 splenocytes stimulated with α-GalCer appeared to be higher than that of NOD splenocytes (and the three rat strains) at both time points. As expected, all splenocytes analyzed showed a robust IFNγ response to PMA plus ionomycin stimulation (Figure 4). Our data suggest that, irrespective of strain, rat iNKT cells have a lower IFNγ response to α-GalCer stimulation than do mouse iNKT cells.

IL-12 production by a-GalCer-stimulated splenocytes was greater in rats than in mice. Exogenous administration of α-GalCer has been reported to cause IL-12 production in splenocytes, possibly as a consequence of iNKT-mediated DC activation (32, 33). To determine whether α-GalCer stimulates rat splenocytes to produce IL-12, we measured IL-12 in the culture supernatants of BBDR, LEW1.WR1, and F344 splenocytes following stimulation with 0 to 1,000 ng/ml α-GalCer. IL 12 production by splenocytes in response to α-GalCer stimulation was readily detectable at 24 h in all rat strains analyzed (Figure 5). In addition, the amount of IL-12 produced was greater than that produced in similarly treated C57BL/6 or NOD mouse splenocyte cultures. IL-12 levels in the supernatants of all splenocytes analyzed were maximal at 24 h (data not shown), in agreement with a previous report of IL-12 production by C57BL/6 splenocytes (33).

Discussion

Treatment of NOD mice with the iNKT superagonist, α-GalCer, is effective in preventing diabetes (16, 19, 21). In contrast, a similar α-GalCer protocol neither delayed (nor accelerated) the onset of diabetes in BBDR or LEW1.WR1 rats treated with KRV with or without poly I:C pre-

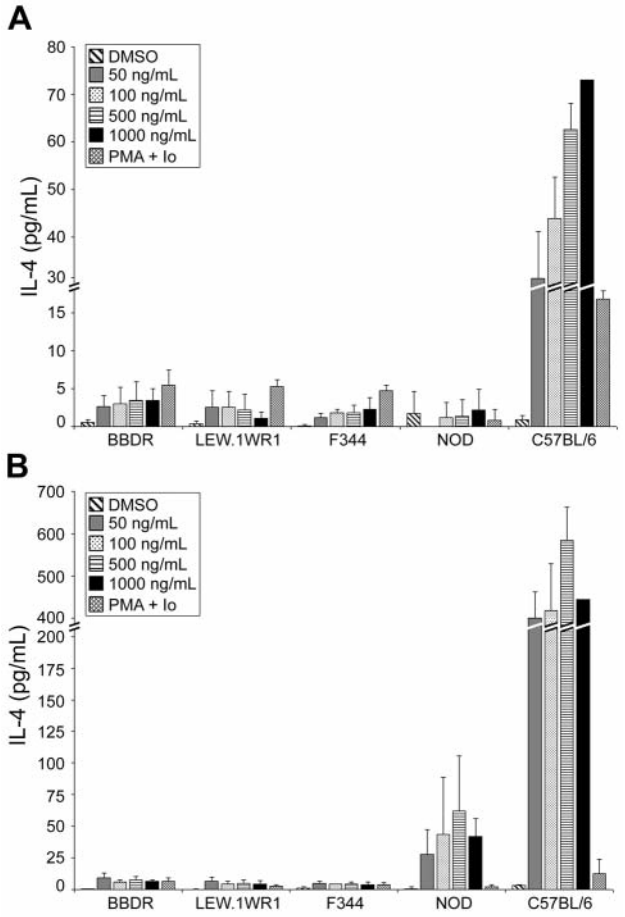

Figure 3. *IL-4 production by rat and mouse spleen cells cultured in the absence of presence of α-GalCer. Spleen cells from the indicated rodent strains were cultured for (A) 48 h or (B) 72 h with vehicle (DMSO), the indicated concentrations of α-GalCer, or PMA plus ionomycin (PMA + Io). The concentration of IL-4 in culture supernatants was measured in triplicate by ELISA. Data are presented as means±S.D. (n=3 animals for each strain).*

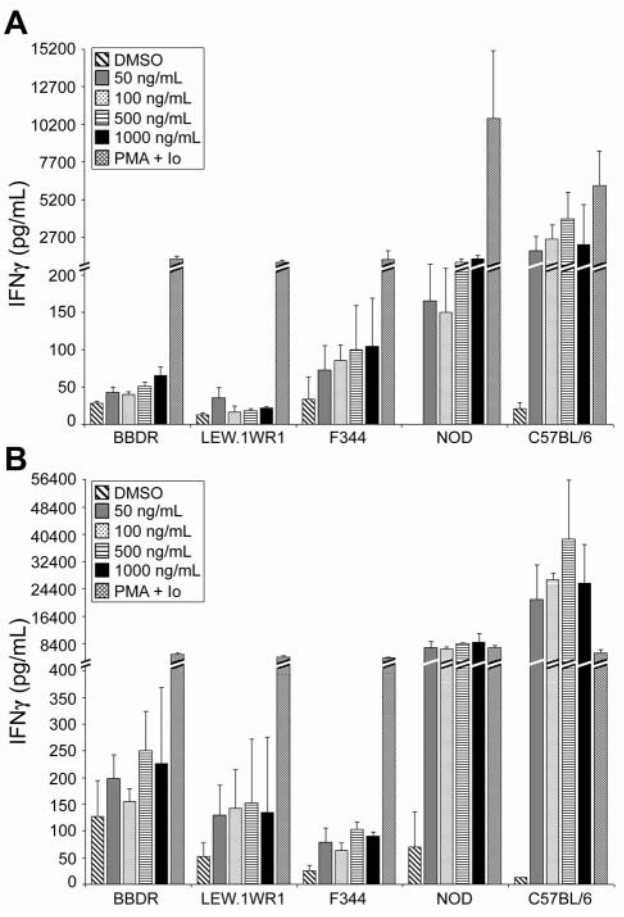

Figure 4. *IFNγ production by rat and mouse spleen cells cultured in the absence or presence of α-GalCer. Spleen cells from the indicated rodent strains were cultured for (A) 48 h or (B) 72 h with vehicle (DMSO), the indicated concentrations of α-GalCer, or PMA plus ionomycin (PMA + Io). The concentration of IFNγ in culture supernatants was measured in triplicate by ELISA. Data are presented as means±S.D. (n=3 animals for each strain).*

treatment, nor did it alter the degree of insulitis in those animals that remained diabetes-free. This failure of α-GalCer treatment to modulate diabetes penetrance in the rat could not be attributed to lack of iNKT cells in these animals. We found no difference in the frequency or phenotype of iNKT cells in either diabetes-inducible BBDR and LEW1.WR1 rat strains or diabetes-resistant WF and LEW rats. Similarly, we found no substantial difference in the quantitative amount of α-GalCer-stimulated cytokines produced by splenocytes isolated from the various rat strains. In contrast, our studies did reveal a substantial qualitative difference in iNKT phenotype between rat and mouse, and in the cytokine response of rat *vs*. mouse splenocytes to α-GalCer. Collectively, these differences could account for the species-specific inability of α-GalCer treatment to alter diabetes in rat models of T1D.

The phenotype of rat iNKT cells has been reported to be different from that of mouse iNKT cells in terms of co-expression of CD4 and CD8 molecules, raising the possibility that iNKT subsets may play a role in a differential response to α-GalCer. Rat iNKT cells have been reported to be predominately $CD8^+$ (2, 4) as opposed to mainly $CD4^+$ or $CD4^-CD8^-$ in the mouse (5). Our analyses revealed that a significant proportion of rat iNKT cells were $CD4^-CD8^-$, in addition to the major $CD8^+$ subpopulation previously reported (2, 4). Only small subpopulations of $CD4^+$ and $CD4^+CD8^+$ iNKT cells in the rat were detected. Overall, our phenotyping studies showed that the percentages of total

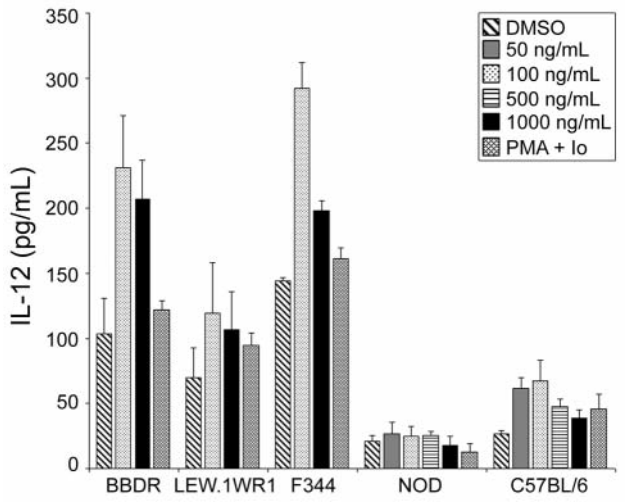

Figure 5. *IL-12 production by rat and mouse spleen cells cultured in the absence or presence of α-GalCer. Spleen cells from the indicated rodent strains were cultured for 24 h with vehicle (DMSO), the indicated concentrations of α-GalCer, or PMA plus ionomycin (PMA + Io). The concentration of IL-12 in culture supernatants was measured in duplicate by ELISA. Data are presented as means±S.D. (n=3 animals for each strain).*

iNKT cells were similar among rat strains, as were the relative proportions of the various iNKT subsets. There was, however, a substantial difference in the proportions of $CD4^+$ and $CD8^+$ iNKT cells between the mouse and rat, suggesting that a subset of iNKT cells required for diabetes prevention could be absent in the rat. Indeed, various activities of iNKT cells in tumor rejection, autoimmune disease, and microbial infections may result from activation of phenotypic subsets that differ in cytokine production and cytotoxic activity (34).

Consistent with our iNKT cell phenotyping data, the rat strains we tested were similar to each other with respect to the type and quantity of cytokines produced by splenocytes in response to α-GalCer. In contrast, our data reveal substantial differences between the rat and mouse. In general, higher levels of the Th1 cytokine, IL-12, were found in α-GalCer-stimulated rat splenocytes than in mouse splenocytes, whereas rat splenocytes appeared to be deficient in production of the Th2 cytokine, IL-4. This is of interest because a reduction in the number and functional response of this IL-4-producing $CD4^+$ iNKT cell population has been reported in both diabetic NOD mice and human diabetic patients (35, 36). Although deficient IL-4 production is thus an attractive mechanism for explaining the failure of α-GalCer therapy in the rat, recent studies dispute the requirement for IL-4 production for diabetes protection (37, 38).

In conclusion, our study demonstrates that α-GalCer is not effective in preventing disease in virus-inducible rat models of autoimmune diabetes. Our data suggest that the susceptibility of BBDR and LEW1.WR1 rats to inducible autoimmune diabetes does not appear to be a consequence of a deficiency in the number or function of iNKT cells, but rather may reflect a species-specific difference in the type and amount of cytokines produced in response to α-GalCer. This, in turn, may be a consequence of differences between the rat and mouse with respect to the proportions of $CD4^+$ and $CD8^+$ iNKT subpopulations. Our data, together with the fact that iNKT cell subpopulations and function in humans may differ due to age and genotype, indicate that additional understanding of the mechanisms that regulate iNKT cell function will be crucial before a therapeutic intervention such as α-GalCer can be considered safe and effective for autoimmune disorders such as T1D in humans.

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