

# Invariant Natural Killer T-Cell Control of Type 1 Diabetes: A Dendritic Cell Genetic Decision of a Silver Bullet or Russian Roulette

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**OBJECTIVE**—In part, activation of invariant natural killer T (iNKT)-cells with the superagonist  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer) inhibits the development of T-cell-mediated autoimmune type 1 diabetes in NOD mice by inducing the downstream differentiation of antigen-presenting dendritic cells (DCs) to an immunotolerogenic state. However, in other systems iNKT-cell activation has an adjuvant-like effect that enhances rather than suppresses various immunological responses. Thus, we tested whether in some circumstances genetic variation would enable activated iNKT-cells to support rather than inhibit type 1 diabetes development.

**RESEARCH DESIGN AND METHODS**—We tested whether iNKT-conditioned DCs in NOD mice and a major histocompatibility complex-matched C57BL/6 (B6) background congenic stock differed in capacity to inhibit type 1 diabetes induced by the adoptive transfer of pathogenic AI4 CD8 T-cells.

**RESULTS**—Unlike those of NOD origin, iNKT-conditioned DCs in the B6 background stock matured to a state that actually supported rather than inhibited AI4 T-cell-induced type 1 diabetes. The induction of a differing activity pattern of T-cell costimulatory molecules varying in capacity to override programmed death-ligand-1 inhibitory effects contributes to the respective ability of iNKT-conditioned DCs in NOD and B6 background mice to inhibit or support type 1 diabetes development. Genetic differences inherent to both iNKT-cells and DCs contribute to their varying interactions in NOD and B6.*H2g7* mice.

**CONCLUSIONS**—This great variability in the interactions between iNKT-cells and DCs in two inbred mouse strains should raise a cautionary note about considering manipulation of this axis as a potential type 1 diabetes prevention therapy in genetically heterogeneous humans. *Diabetes* 59:423–432, 2010

**I**nvariant natural killer T (iNKT)-cells are a small regulatory lymphocyte subset characterized by their unique ability to recognize glycolipid antigens presented by the major histocompatibility complex (MHC) class I-like CD1d molecule (1). Activated iNKT-cells induce profound multiple effects on innate and adaptive immune responses, primarily through rapid se-

cretion of various cytokines and other agents (1,2). Among the downstream events regulated by activated iNKT-cells is the maturation of dendritic cells (DCs) that subsequently induce various adaptive immune responses (1). Activating iNKT-cells with various agonists has shown promise in modulating DC functions for both stimulating immunological responses against tumors and infectious agents as well as inducing tolerogenic responses for inhibiting autoimmune syndromes (1,3,4). Unfortunately, in regard to possible iNKT-mediated therapeutic approaches in humans, too little is currently known about how iNKT-cells promote differentiation of immunogenic versus tolerogenic DCs. Without an ability to regulate desired downstream DC responses, the danger exists of iNKT-directed protocols exacerbating the disease being treated (3,4). Therefore, much attention is currently focused on characterizing particular courses of iNKT-cell-induced DC differentiation.

Mouse-based studies assessing the ability of iNKT-cells activated by the superagonist  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer) to enhance antitumor immunity have almost exclusively used the C57BL/6J (B6) or BALB/c strains (5–10). More strains have been evaluated for  $\alpha$ -GalCer-induced suppression of various autoimmune syndromes (4). Hence, strain-dependent factors may be important in determining whether activated iNKT-cells induce immunogenic or tolerogenic events. Indeed, there is great strain variability in iNKT-cell numbers that also differ in cytokine production profiles (11). However, it remains unknown to what extent genetic variability may determine whether activated iNKT-cells subsequently induce immunogenic versus tolerogenic differentiation of DCs.

The ability of  $\alpha$ -GalCer-activated iNKT-cells to inhibit autoimmune type 1 diabetes development in NOD mice may be at least partly due to downstream maturation of tolerogenic DCs (2). iNKT-conditioned DCs in NOD mice preferentially accumulated in pancreatic lymph nodes where some diabetogenic T-cells subsequently underwent apoptotic deletion, but with a larger proportion becoming functionally anergized (2). However, before  $\alpha$ -GalCer-based type 1 diabetes intervention approaches could be considered in humans, it would be important to know whether patient genetic heterogeneity may result in variable types of downstream DC responses. Indeed, in the current study, we found that unlike in NOD mice, in a B6 background stock iNKT-conditioned DCs not only failed to mature to a type 1 diabetes-protective state, but actually support disease development due to differing expression of various T-cell costimulatory and inhibitory molecules.

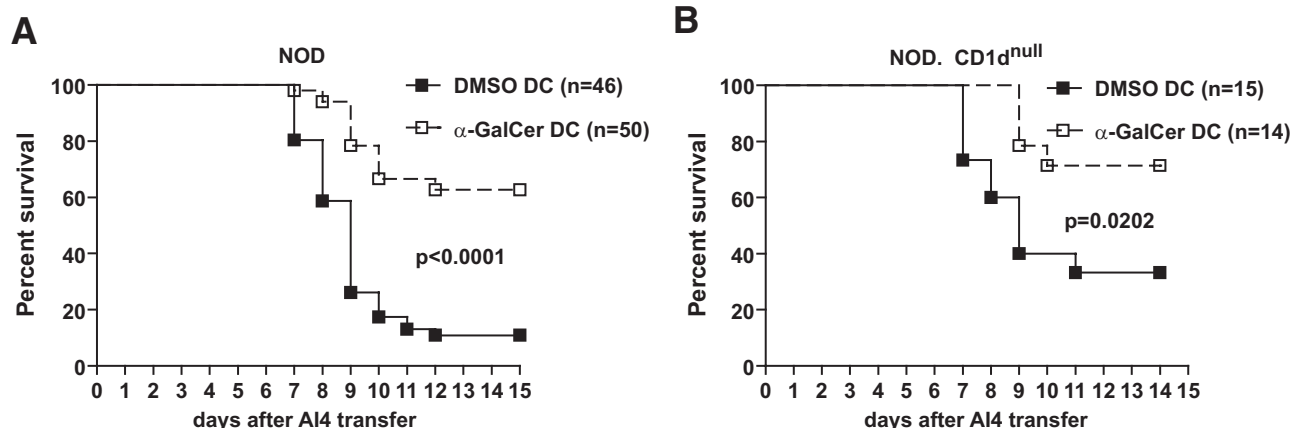
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Received 29 July 2009 and accepted 20 October 2009. Published ahead of print at <http://diabetes.diabetesjournals.org> on 10 November 2009. DOI: 10.2337/db09-1116.

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**FIG. 1. DCs from  $\alpha$ -GalCer-treated NOD donor mice protect NOD and iNKT-cell-deficient NOD.CD1d<sup>null</sup> recipients from type 1 diabetes induced by adoptively transferred AI4 T-cells.** Type 1 diabetes incidence for sublethally irradiated (600R) 6- to 8-week-old (A) NOD and (B) NOD.CD1d<sup>null</sup> recipients of  $1\text{--}4 \times 10^5$  splenic DCs isolated from NOD donors treated 24 h previously with  $\alpha$ -GalCer (2  $\mu$ g) or DMSO. NOD.Rag1<sup>null</sup>.AI4 splenocytes ( $1 \times 10^7$ ) were injected into all DC recipients 24 h later. Survival curves were compared according to the log-rank test.

## RESEARCH DESIGN AND METHODS

**Mice.** NOD/ShiLtDvs mice are maintained by sibling matings in a specific pathogen-free research colony at The Jackson Laboratory. B6 mice congenic for the NOD-derived  $H2^g^d$  MHC (B6.H2<sup>g^d</sup>) are maintained at the N8 backcross generation (12). N12 backcross generation NOD mice congenic for a disrupted CD1d gene (NOD.CD1d<sup>null</sup>) have been described (13). NOD mice transgenically expressing the T-cell receptor (TCR) from the diabetogenic AI4 CD8<sup>+</sup> T-cell clone and also carrying a functionally inactivated Rag1 gene (NOD.Rag1<sup>null</sup>.AI4) have been described (14).

**Flow cytometric analyses and reagents.**  $\alpha$ -GalCer was purchased from Alexis Biochemicals (San Diego, CA). Splenic DCs from NOD and B6.H2<sup>g^d</sup> mice treated 24 h previously with a single intraperitoneal injection of  $\alpha$ -GalCer (2  $\mu$ g) or vehicle (DMSO) were characterized by flow cytometry. Cell suspensions were Fc receptor blocked with rat (r)IgG and stained with the indicated fluorochrome-conjugated antibodies at 4°C. DCs were identified using a CD11c-specific antibody (N148). Expression levels of various DC surface markers were assessed using antibodies specific for CD80 (16-10A1), CD86 (GL1), CD70 (FR70), CD40 (3/23), pan-MHC I (M1/42), A<sup>g7</sup>-MHC II (10-2.16), programmed death-ligand-1 (PD-L1; MIH6), and OX40 ligand (OX40L; RM134L), all purchased from BD Biosciences (San Jose, CA) or eBiosciences (San Diego, CA). In mixed splenocyte studies, anti-CD45.2 (1042.1) was used to distinguish B6.H2<sup>g^d</sup> DCs from those of NOD origin. Viable stained cells were detected using LSR II or FACSCalibur flow cytometers (BD Biosciences). Data were analyzed using FlowJo software (TreeStar, Palo Alto, CA).

**Adoptive transfer of DCs and type 1 diabetes.** DCs were purified from Collagenase D (Roche) digested spleens of female NOD or B6.H2<sup>g^d</sup> mice injected intraperitoneally 24 h previously with  $\alpha$ -GalCer (2  $\mu$ g) or DMSO using CD11c-conjugated magnetic beads following the manufacturer's directions (Miltenyi Biotec). Between  $1 \times 10^5$  and  $4 \times 10^5$  CD11c<sup>+</sup> DCs were injected intravenously into sublethally irradiated (600 R from a <sup>137</sup>Cs source) 6- to 8-week-old syngeneic NOD or B6.H2<sup>g^d</sup> female recipients. DC recipients were injected intravenously 24 h later with  $1 \times 10^7$  NOD.Rag1<sup>null</sup>.AI4 splenocytes to induce type 1 diabetes. Type 1 diabetes onset in recipient mice was defined by glycosuria development assessed daily with Ames Diastix (Bayer, Diagnostics Division).

In some experiments, 6- to 8-week-old NOD and B6.H2<sup>g^d</sup> females received a single intraperitoneal injection of  $\alpha$ -GalCer (2  $\mu$ g) or vehicle, and 18 h later were sublethally irradiated and infused intravenously with  $1 \times 10^7$  NOD.Rag1<sup>null</sup>.AI4 splenocytes. Another experiment used NOD and B6.H2<sup>g^d</sup> females receiving four once-weekly intraperitoneal injections of  $\alpha$ -GalCer (2  $\mu$ g) or vehicle starting at 3–4 weeks of age. Two days after the last treatment, mice were sublethally irradiated and injected intravenously with  $1 \times 10^7$  NOD.Rag1<sup>null</sup>.AI4 splenocytes. Recipients received an additional  $\alpha$ -GalCer or vehicle injection 5 days after AI4 T-cell transfer and were monitored for type 1 diabetes. In some experiments, recipients were given additional intraperitoneal injections of the PD-L1-blocking MIH6 antibody (250  $\mu$ g/mouse) or polyclonal rat IgG, at the same time as and 2 days after AI4 T-cell transfer. **T-cell proliferation and IFN $\gamma$  enzyme-linked immunosorbent spot assays.** Splenic DCs purified from 6- to 8-week-old male NOD and B6.H2<sup>g^d</sup> mice injected 24 h previously with either  $\alpha$ -GalCer or DMSO and fixed in 0.75% paraformaldehyde (15) to prevent further maturation were assessed for ability

to activate magnetic bead-purified AI4 T-cell responses to the YFIENYLEL mimotope peptide using both the previously described enzyme-linked immunosorbent spot IFN $\gamma$  production (16) and [<sup>3</sup>H]thymidine incorporation proliferation assays (12).

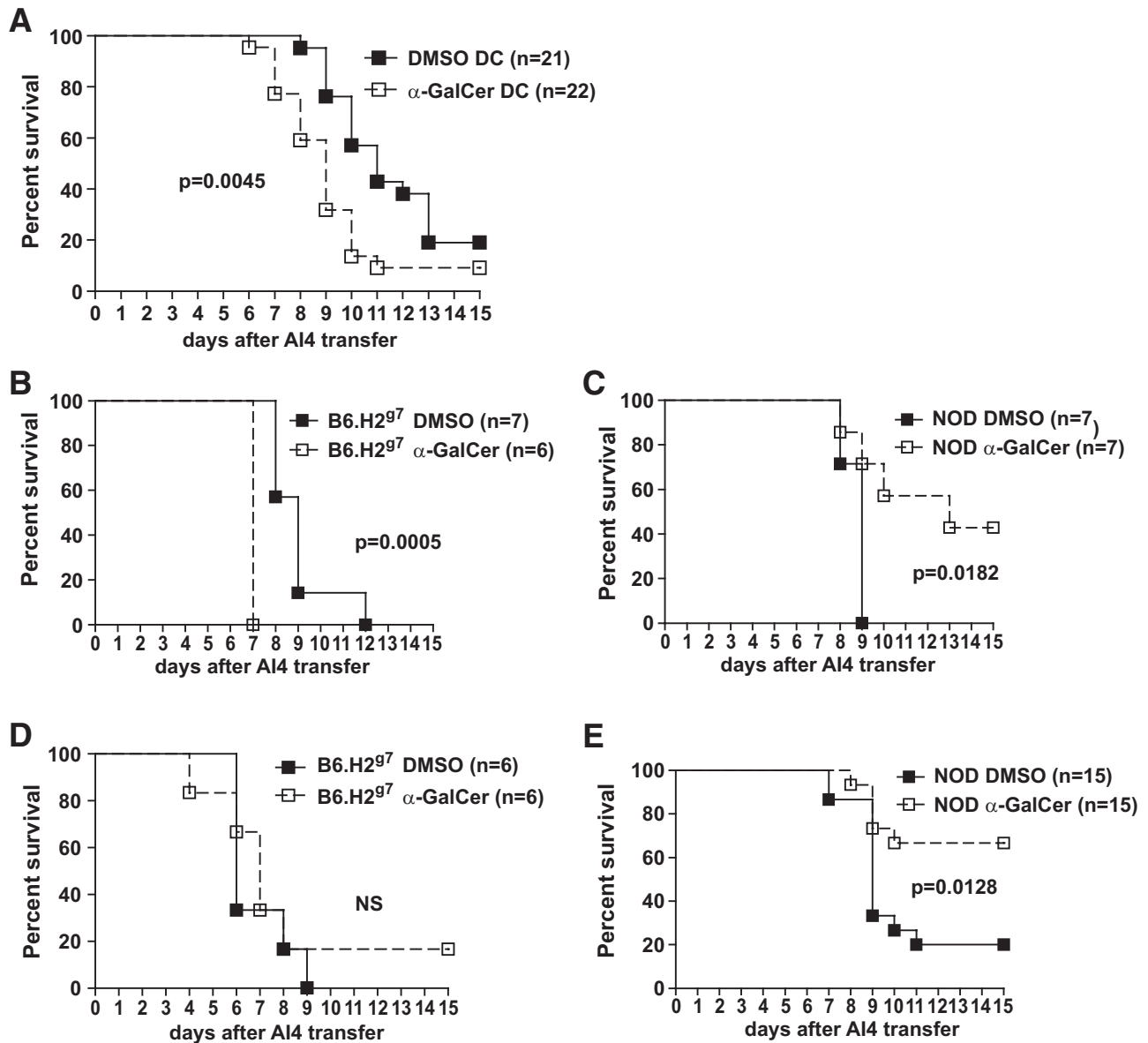
**Dendritic cell cytokine analysis.** Splenic DCs were purified as described above from male 6- to 8-week-old NOD or B6.H2<sup>g^d</sup> mice injected intraperitoneally 16 h previously with  $\alpha$ -GalCer (2  $\mu$ g) or DMSO. DCs were cultured at a concentration of  $5 \times 10^5$ /ml in tissue culture media for an additional 18 h with or without the addition of anti-CD40 (clone HM40-3; 10  $\mu$ g/ml) and lipopolysaccharide (LPS; 1  $\mu$ g/ml), after which supernatants were collected for interleukin-12 (IL-12) p70, tumor necrosis factor- $\alpha$ , and IL-10 cytokine detection via ELISA kits (BD Biosciences).

**PD-L1- and CD70-blocking assays.** Splenic DCs were purified and fixed as described above from NOD and B6.H2<sup>g^d</sup> mice treated with  $\alpha$ -GalCer (2  $\mu$ g) and either PD-L1-blocking antibody (clone 10F.9G2; 400  $\mu$ g/mouse) or an equivalent amount of rIgG 24 h and 16 h previously. A subset of fixed DCs was assessed for the extent of PD-L1 blockade by staining with fluorochrome-conjugated MIH6 antibody. For the CD70-blocking experiment, anti-CD70 or rIgG (10  $\mu$ g/ml) was added to tissue culture media containing splenic DCs purified and fixed from NOD and B6.H2<sup>g^d</sup> mice treated with  $\alpha$ -GalCer 24 h previously. In both blocking experiments, treated iNKT-conditioned DCs ( $2.5 \times 10^4$  per well) were used to induce antigenic mimotope-mediated (6.25 and 12.5 nmol/l for the CD70- and PD-L1-blocking experiments, respectively) proliferation of purified carboxyfluorescein succinimidyl ester (CFSE)-labeled NOD.Rag1<sup>null</sup>.AI4 T-cells ( $5 \times 10^4$  per well). After 3 days, AI4 T-cell proliferation was assessed by flow cytometric analyses of CFSE dilution.

**Cell culture.** For the mixed splenocyte culture, a 1:1 ratio of NOD and B6.H2<sup>g^d</sup> splenocytes was seeded at a concentration of  $5 \times 10^5$ /ml in RPMI 1640 with or without  $\alpha$ -GalCer (200 ng/ml) and cultured at 37°C for 2 days. Cells were harvested and stained with fluorochrome-conjugated antibodies to CD11c and CD45.2 as well as CD86 or PD-L1 for flow cytometric analyses. Another experiment involved coculture of purified iNKT-cells and DCs. Splenic DCs were purified to >90% purity from 6- to 8-week-old female NOD and B6.H2<sup>g^d</sup> mice as described above. iNKT-cells were isolated from splenocytes by staining with a phycoerythrin-conjugated CD1d tetramer loaded with the  $\alpha$ -GalCer PBS57 analog provided by the National Institutes of Health Tetramer Facility. iNKT-cells were enriched to >70% purity by positive selection using an MS Column (Miltenyi Biotec). NOD or B6.H2<sup>g^d</sup> DCs ( $1 \times 10^5$  cells/well) were cocultured with syngeneic or allogeneic iNKT-cells ( $1.5 \times 10^4$  per well) in round-bottomed 96-well tissue culture plates in the presence of vehicle or  $\alpha$ -GalCer (200 ng/ml) for 18 h. Cells were then harvested and stained with fluorochrome-conjugated antibodies to CD11c, CD86, and PD-L1 for flow cytometric analyses.

## RESULTS

**iNKT-conditioned NOD DCs prevent type 1 diabetes.** Although their presence has been associated with the induction of type 1 diabetes resistance, it remained unclear whether iNKT-conditioned DCs in NOD mice directly trigger disease-protective effects. Thus, we determined



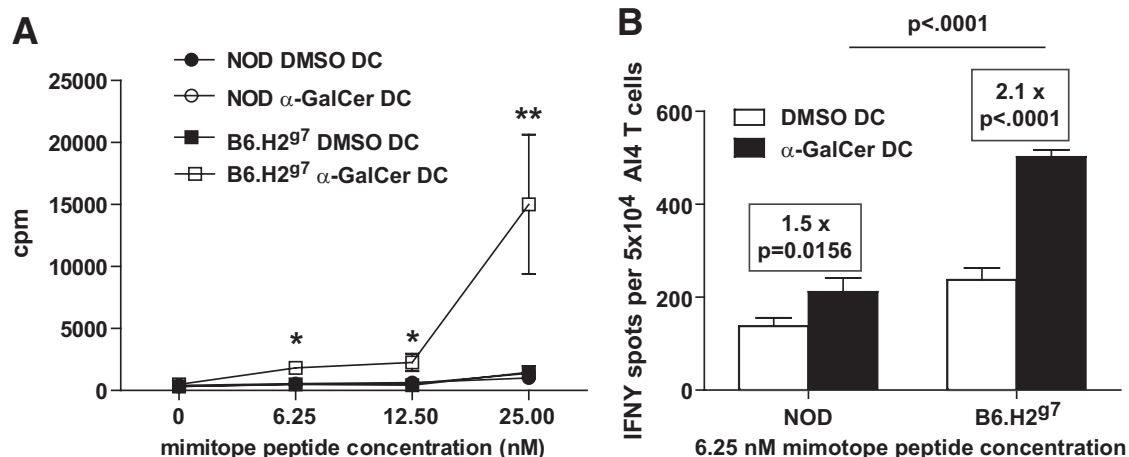
**FIG. 2.** Unlike those of NOD origin, iNKT-conditioned B6.H2<sup>g7</sup> DCs functionally support diabetogenic T-cell activation. **A:** Type 1 diabetes incidence for sublethally irradiated 6- to 8-week-old female B6.H2<sup>g7</sup> recipients of  $2 \times 10^5$  splenic DCs isolated from syngeneic donors treated 24 h previously with  $\alpha$ -GalCer (2  $\mu$ g) or vehicle. NOD.Rag1<sup>null</sup>.AI4 splenocytes ( $1 \times 10^7$ ) were injected into all DC recipients 24 h later. **B** and **C:** Type 1 diabetes incidence for **(B)** B6.H2<sup>g7</sup> and **(C)** NOD recipients pretreated with a single intraperitoneal injection of  $\alpha$ -GalCer (2  $\mu$ g) or vehicle followed 18 h later by sublethal irradiation (600 R) and intravenous infusion of  $1 \times 10^7$  NOD.Rag1<sup>null</sup>.AI4 splenocytes. **D** and **E:** Type 1 diabetes incidence for **(D)** B6.H2<sup>g7</sup> and **(E)** NOD recipients pretreated from 3–4 weeks of age with four once-weekly injections of  $\alpha$ -GalCer (2  $\mu$ g) or vehicle followed by sublethal irradiation (600 R) and intravenous infusion of  $1 \times 10^7$  NOD.Rag1<sup>null</sup>.AI4 splenocytes. Recipients received an additional dose of  $\alpha$ -GalCer or vehicle 5 days later. Survival curves in **(A–E)** were compared according to the log-rank test.

whether DCs purified from NOD females treated with  $\alpha$ -GalCer or vehicle 24 h previously and adoptively transferred into young (6–8 weeks old) syngeneic recipients differed in ability to protect them from type 1 diabetes normally induced by subsequently infused NOD.Rag1<sup>null</sup>.AI4 TCR transgenic CD8 T-cells (due to the Rag1<sup>null</sup> mutation all donor T-cells are the AI4 clonotype) (2). AI4 T-cells induced a significantly lower and slower rate of type 1 diabetes in NOD mice previously infused with DCs from  $\alpha$ -GalCer than vehicle-pretreated donors (Fig. 1A). A concern was whether type 1 diabetes protection mediated by DCs from  $\alpha$ -GalCer-treated donors actually resulted from their carrying over some amount of this glycolipid antigen eliciting a second round of iNKT-cell activation in recipient mice, triggering a non-DC-mediated mode of disease resistance.

We addressed this issue by repeating the DC transfer experiment, but using NOD.CD1d<sup>null</sup> recipients lacking iNKT-cells (17). Again, AI4 T-cells induced a significantly lower rate of type 1 diabetes in NOD.CD1d<sup>null</sup> recipients previously infused with DCs from  $\alpha$ -GalCer than vehicle-pretreated NOD donors (Fig. 1B). Hence, adoptively transferred iNKT-conditioned DCs do not elicit type 1 diabetes resistance by reactivating recipient iNKT-cells. These collective data indicate iNKT-conditioned DCs in NOD mice mature to a state capable of inhibiting diabetogenic T-cells.

**iNKT-conditioned DCs from B6.H2<sup>g7</sup> mice support type 1 diabetes development.** We next asked whether iNKT-conditioned DCs still suppress diabetogenic T-cell responses if derived from a strain such as B6 in which





**FIG. 3.** iNKT-conditioned DCs from NOD and B6.H2<sup>g7</sup> mice differ in ability to activate AI4 T-cell effector function. **A** and **B**: DCs were purified from NOD and B6.H2<sup>g7</sup> mice treated 24 h previously with  $\alpha$ -GalCer or vehicle and lightly fixed in 0.75% paraformaldehyde to prevent further maturation. DCs ( $2 \times 10^4$  per well) were then used to induce antigenic mimitope-mediated (**A**) proliferation and (**B**) IFN $\gamma$  production by purified NOD.Rag1<sup>mut</sup>.AI4 T-cells. Results represent the mean cpm and IFN $\gamma$  spots per  $5 \times 10^4$  AI4 T-cells  $\pm$  SE of three mice per treatment. \* $P < 0.001$  as determined by one-way ANOVA. Boxed values in (**B**) indicate the fold change in IFN $\gamma$  production by NOD.Rag1<sup>mut</sup>.AI4 T-cells activated by DCs purified from  $\alpha$ -GalCer- compared with vehicle-treated mice.

$\alpha$ -GalCer treatment has been associated with enhancing rather than diminishing immunological effector functions (5–10). B6 background mice congenically expressing the NOD H2<sup>g7</sup> MHC (B6.H2<sup>g7</sup>) are inherently type 1 diabetes resistant, but susceptible to AI4 T-cell-induced disease (18). Thus, we assessed whether splenic DCs purified from B6.H2<sup>g7</sup> donors treated 24 h previously with  $\alpha$ -GalCer or vehicle differed from those of NOD origin in ability to protect syngeneic recipients from AI4 T-cell-induced type 1 diabetes. Unlike those of NOD origin (Fig. 1A), iNKT-conditioned DCs from B6.H2<sup>g7</sup> mice actually accelerated AI4 T-cell-induced type 1 diabetes in syngeneic recipients (Fig. 2A). Similarly, type 1 diabetes was, respectively, accelerated and inhibited in B6.H2<sup>g7</sup> and NOD mice receiving a single intraperitoneal injection of  $\alpha$ -GalCer 18 h prior to AI4 T-cell infusion (Fig. 2B and C). These results indicate that after single-dose  $\alpha$ -GalCer treatment, iNKT-conditioned DCs in NOD and B6.H2<sup>g7</sup> mice differentiate to tolerogenic versus immunogenic states, respectively, either inhibiting or supporting AI4 T-cell-induced type 1 diabetes. However, it has been reported that B6 background mice must receive a minimum of three consecutive  $\alpha$ -GalCer injections for iNKT-cells to achieve a maturation state supporting the generation of tolerogenic DCs that inhibit development of experimental allergic encephalomyelitis (19). Similarly, NOD mice pretreated with four once-weekly injections of  $\alpha$ -GalCer are rendered resistant to AI4 T-cell-induced type 1 diabetes (2). Thus, we tested whether four once-weekly pretreatments with  $\alpha$ -GalCer also protected B6.H2<sup>g7</sup> mice from subsequent AI4 T-cell-induced type 1 diabetes. Unlike in NOD, multiple pretreatments with  $\alpha$ -GalCer failed to protect B6.H2<sup>g7</sup> mice from AI4 T-cell-induced type 1 diabetes (Fig. 2D and E). Hence, even after multiple  $\alpha$ -GalCer injections, iNKT-conditioned DCs from NOD and B6.H2<sup>g7</sup> inhibit or support, respectively, type 1 diabetes development. Furthermore, these collective results suggest the experimental allergic encephalomyelitis-protective DCs engendered in B6 mice pretreated with multiple  $\alpha$ -GalCer injections must functionally do so in a way differing from those maturing to a type 1 diabetes inhibitory state in NOD mice after only a single round of iNKT-cell activation.  $\alpha$ -GalCer-activated iNKT-cells reportedly inhibit type 1 diabetes in NOD mice

in a regulatory T-cell (Treg)—dependent manner (20). However, we could not detect any proportional or functional differences in Tregs from NOD or B6.H2<sup>g7</sup> mice pretreated with single or multiple injections of  $\alpha$ -GalCer (data not shown). Hence, we assessed whether iNKT-conditioned DCs from NOD and B6.H2<sup>g7</sup> mice differed in ability to activate AI4 T-cell effector function. DCs were purified from NOD and B6.H2<sup>g7</sup> mice treated with  $\alpha$ -GalCer or vehicle 24 h earlier and fixed in 0.75% paraformaldehyde to prevent further maturation in culture (15). They were then used to induce antigenic mimitope-mediated activation and proliferation of NOD.Rag1<sup>mut</sup>.AI4 T-cells. Compared with those from vehicle-treated syngeneic controls, iNKT-conditioned DCs from B6.H2<sup>g7</sup> mice drove higher levels of antigen-stimulated AI4 T-cell proliferation and IFN $\gamma$  production (Fig. 3A and B). Conversely, compared with those from vehicle-treated syngeneic controls, iNKT-conditioned DCs from NOD mice did not demonstrate an enhanced ability to support AI4 T-cell proliferation and only marginally increased their IFN $\gamma$  production (Fig. 3A and B). Hence, iNKT-conditioned NOD and B6.H2<sup>g7</sup> DCs differentiate to very different states characterized by a low or potent ability, respectively, to activate diabetogenic AI4 T-cells. These results would also be consistent with our previous findings (2) that although there is an increase in the number of AI4 T-cells that undergo apoptotic deletion in  $\alpha$ -GalCer-treated NOD mice, a larger proportion of such effectors is driven to an anergic state.

**iNKT-conditioned NOD and B6.H2<sup>g7</sup> DCs express different patterns of costimulatory molecules.** The capacity of DCs to stimulate T-cells is the net result of MHC and costimulatory molecule as well as proinflammatory and anti-inflammatory cytokine interactions. To determine possible cytokine contributions, iNKT-conditioned DCs were purified from NOD and B6.H2<sup>g7</sup> mice 16 h after  $\alpha$ -GalCer treatment and cultured for a further 18 h with or without a combination of anti-CD40 and LPS. Supernatants were then assessed for various cytokine levels by enzyme-linked immunosorbent assay. After activation, iNKT-conditioned DCs from B6.H2<sup>g7</sup> mice produced higher levels of IL-12 p70 and tumor necrosis factor- $\alpha$  and lower quantities of IL-10 than those of NOD

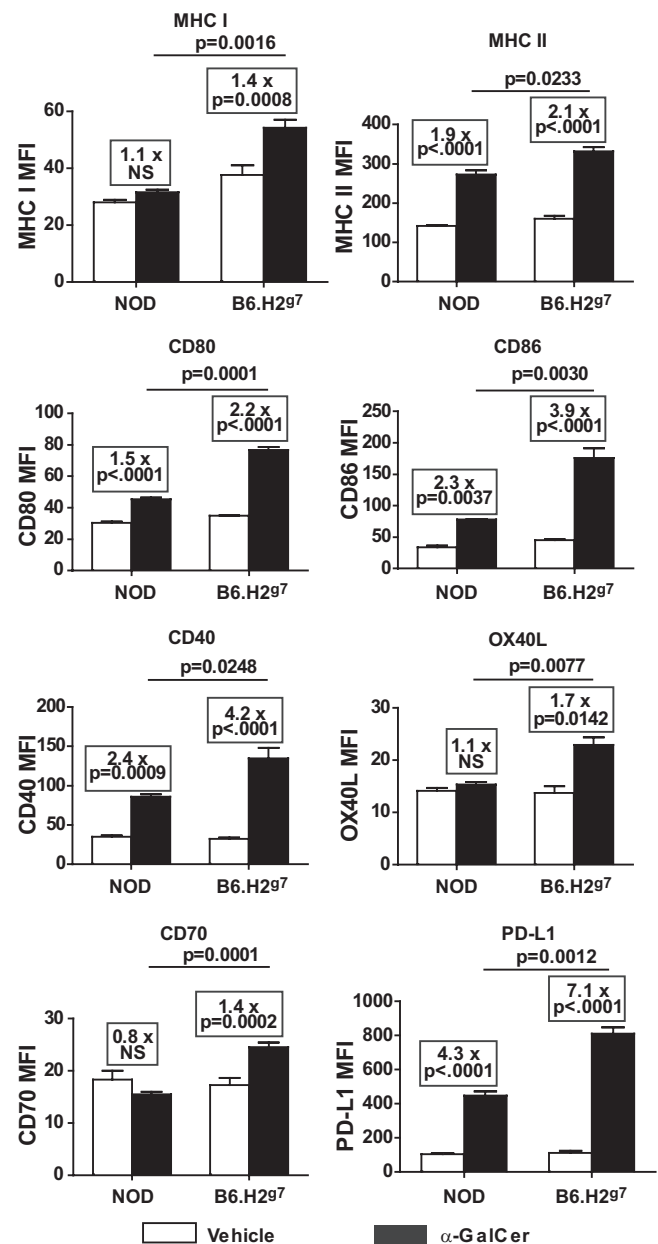
origin (supplementary Fig. 1, available in an online appendix at <http://diabetes.diabetesjournals.org/content/early/2009/10/31/db09-1116/suppl/DC1>). However, although they may contribute to some extent *in vivo*, it is unlikely that these cytokines are primary factors in the varying ability of NOD and B6.*H2g7* iNKT-conditioned DCs to activate diabetogenic T-cells. This is because, unless further activated with anti-CD40 and LPS, neither strain's iNKT-conditioned DCs produced detectable amounts of the aforementioned cytokines. Furthermore, the fixed NOD and B6.*H2g7* DCs found in the previously described *in vitro* assays (Figs. 2 and 3) to vary in ability to activate diabetogenic AI4 T-cells would lack cytokine production capacity. For these reasons, attention was focused on the possibility that varying expression levels of important T-cell costimulatory and inhibitory molecules are responsible for the divergent effects of NOD and B6.*H2g7* iNKT-conditioned DCs on diabetogenic AI4 T-cells.

As determined by assessing the mean fluorescence intensity (MFI) of specific antibody binding, at 24 h after  $\alpha$ -GalCer treatment, levels of MHC class I and II as well as CD80 and CD86 expression were significantly higher on iNKT-conditioned DCs from B6.*H2g7* than NOD mice (Fig. 4). Surface CD70 and OX40L levels are upregulated on activated DCs and play important roles in initiating and sustaining T-cell activation through respective interactions with the CD27 and OX40 lymphocyte receptors (22–24). After iNKT-cell activation, expression of both CD70 and OX40L was completely unaltered on NOD but upregulated significantly on B6.*H2g7* DCs (Fig. 4).

CD40 engagement by T-cell-expressed CD40 ligand reportedly contributes to DCs subsequently upregulating expression of CD80 and CD86 costimulatory molecules (25,26). Again, CD40 expression was significantly higher on iNKT-conditioned DCs from B6.*H2g7* than NOD mice (Fig. 4). However, this CD40 disparity could not explain strain differences in expression of other T-cell costimulatory molecules on iNKT-conditioned DCs. This was demonstrated by the finding that iNKT-conditioned DCs from CD40-deficient B6 mice still efficiently upregulated expression of other T-cell costimulatory molecules (data not shown).

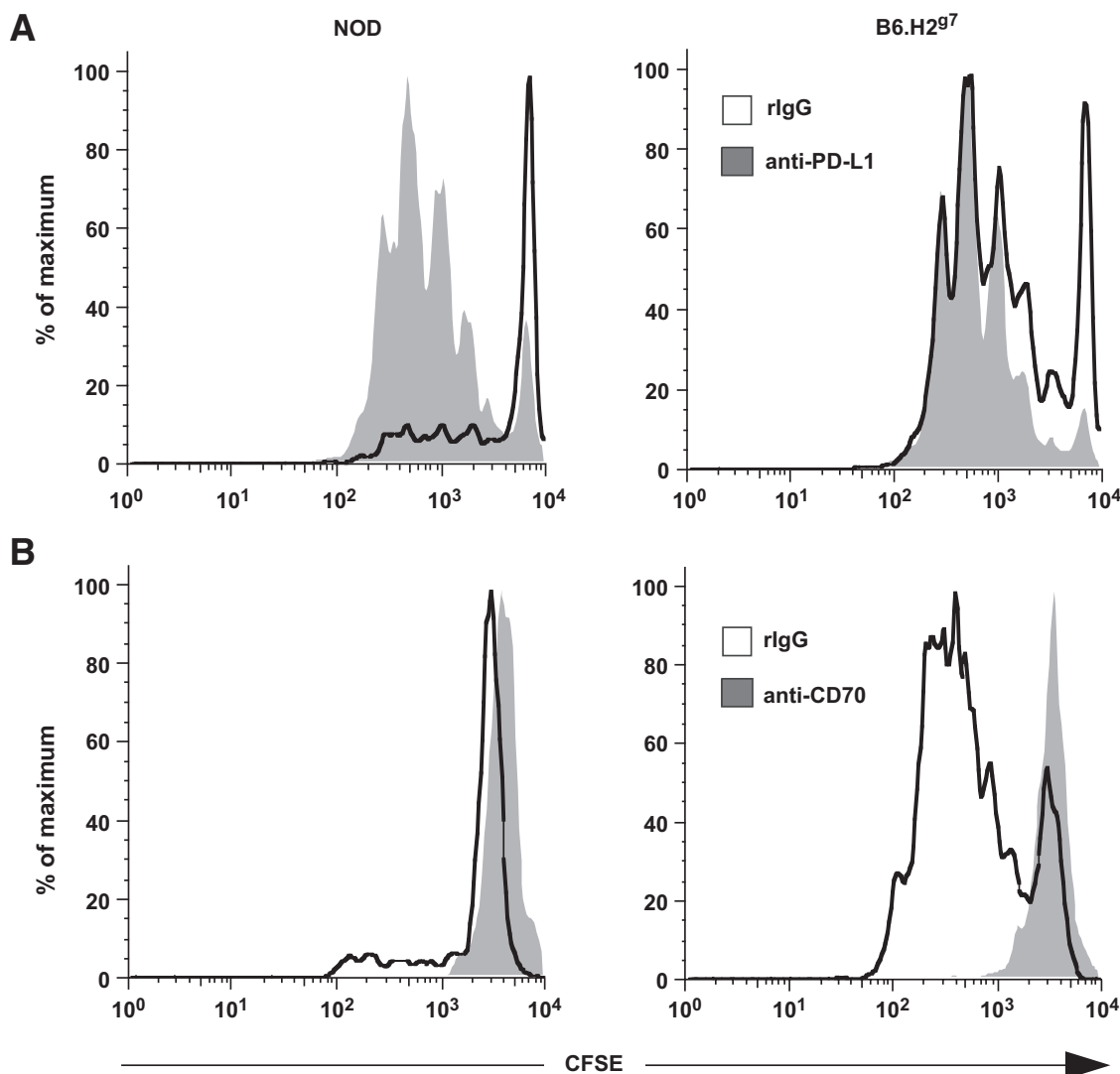
Levels of the important T-cell-inhibitory PD-L1 molecule on iNKT-conditioned DCs from NOD and B6.*H2g7* mice were also compared. PD-L1 can be expressed on mature DCs and inhibits activated T- and B-cells when engaging PD-1 (27,28). This interaction regulates type 1 diabetes in NOD mice as antibody blockade of either the ligand or receptor accelerates disease onset by inhibiting apoptosis of pathogenic lymphocytes (29). PD-L1 was upregulated on iNKT-conditioned DCs in both strains, but again a more robust response was observed in B6.*H2g7* mice (Fig. 4). However, based on these collective results, we hypothesized that the induction of differing activity patterns of T-cell costimulatory molecules varying in capacity to override PD-L1 inhibitory effects contributes to the respective ability of iNKT-conditioned DCs from NOD and B6.*H2g7* mice to inhibit or support type 1 diabetes development.

**Tolerogenic and immunogenic properties of NOD and B6.*H2g7* iNKT-conditioned DCs are abrogated by respective treatment with PD-L1- or CD70-blocking antibodies.** The T-cell costimulatory molecule we initially chose to test the above hypothesis was CD70 because its expression is unchanged or strongly upregulated on iNKT-conditioned DCs in NOD and B6.*H2g7* mice, respectively



**FIG. 4.** iNKT-cell-conditioned DCs from NOD and B6.*H2g7* mice express different patterns of T-cell costimulatory and inhibitory molecules. Flow cytometry was used to determine the mean fluorescence intensity (MFI) of antibody staining of T-cell costimulatory and inhibitory molecules on the surface of naive and iNKT-matured CD11c<sup>+</sup> DCs resident within splenocytes of 6- to 8-week-old female NOD and B6.*H2g7* mice ( $n = 3$ ) injected intraperitoneally 24 h previously with  $\alpha$ -GalCer (2  $\mu$ g) or DMSO. Boxed values indicate the within-strain fold change in expression levels of the indicated surface molecule on splenic DCs from  $\alpha$ -GalCer- compared with DMSO-treated mice. Differences across strains in absolute expression levels of surface molecules and the fold change induction within strains above baseline between iNKT-conditioned DCs from NOD and B6.*H2g7* mice were compared by one-way ANOVA.

(Fig. 4). Hence, we determined whether either PD-L1 or CD70 blockade altered the ability of iNKT-conditioned DCs from NOD or B6.*H2g7* mice to stimulate AI4 T-cells. In one experiment, DCs were purified from NOD and B6.*H2g7* mice treated with  $\alpha$ -GalCer and either a PD-L1-blocking antibody or polyclonal IgG 24 h and 16 h previously and then fixed in 0.75% paraformaldehyde to prevent further maturation in culture (15). PD-L1 blockade was confirmed on DCs directly after fixation (supplementary Fig. 2). DCs



**FIG. 5.** The tolerogenic and immunogenic properties of NOD and B6.*H2<sup>g7</sup>* iNKT-conditioned DCs are abrogated by their respective treatment with PD-L1- and CD70-blocking antibodies. **A:** DCs were purified from NOD and B6.*H2<sup>g7</sup>* mice treated with  $\alpha$ -GalCer and either anti-PD-L1 (400  $\mu$ g/mouse) or rIgG 24 h and 16 h previously and fixed to prevent further maturation. DCs ( $2.5 \times 10^4$  per well) were then used to induce antigenic mimotope-mediated proliferation of purified CFSE-labeled NOD.*Rag1<sup>null</sup>.AI4* T-cells ( $5 \times 10^4$  per well) after 3 days in culture. **B:** DCs were purified from NOD and B6.*H2<sup>g7</sup>* mice treated with  $\alpha$ -GalCer 24 h previously and fixed to prevent further maturation. DCs ( $2.5 \times 10^4$  per well) were then used to induce antigenic mimotope-mediated proliferation of purified CFSE-labeled NOD.*Rag1<sup>null</sup>.AI4* T-cells ( $5 \times 10^4$  per well) in tissue culture media containing 10  $\mu$ g/ml of anti-CD70 or rIgG. CFSE dilution of AI4 T-cells was assessed after 3 days in culture.

were also purified and fixed from NOD and B6.*H2<sup>g7</sup>* mice treated 24 h previously with  $\alpha$ -GalCer and placed in culture together with rIgG or CD70-blocking antibody (the CD70 antibody showed very poor binding persistence in vivo). DCs from each treatment group were used to induce antigenic peptide-mediated proliferation of CFSE-labeled AI4 T-cells.

As previously observed, PD-L1 intact iNKT-conditioned DCs from rIgG-treated NOD mice did not efficiently support AI4 T-cell proliferation (Fig. 5A). Conversely, AI4 T-cells proliferated vigorously when PD-L1-blocked iNKT-conditioned DCs from NOD mice were used for antigen presentation. Unlike NOD, PD-L1 intact iNKT-conditioned DCs from B6.*H2<sup>g7</sup>* mice promoted robust AI4 T-cell activation. Furthermore, PD-L1 blockade of iNKT-conditioned B6.*H2<sup>g7</sup>* DCs only marginally increased their AI4 T-cell stimulatory capacity (Fig. 5A).

The greater ability of rIgG control-treated iNKT-conditioned DCs from B6.*H2<sup>g7</sup>* than NOD mice to activate AI4

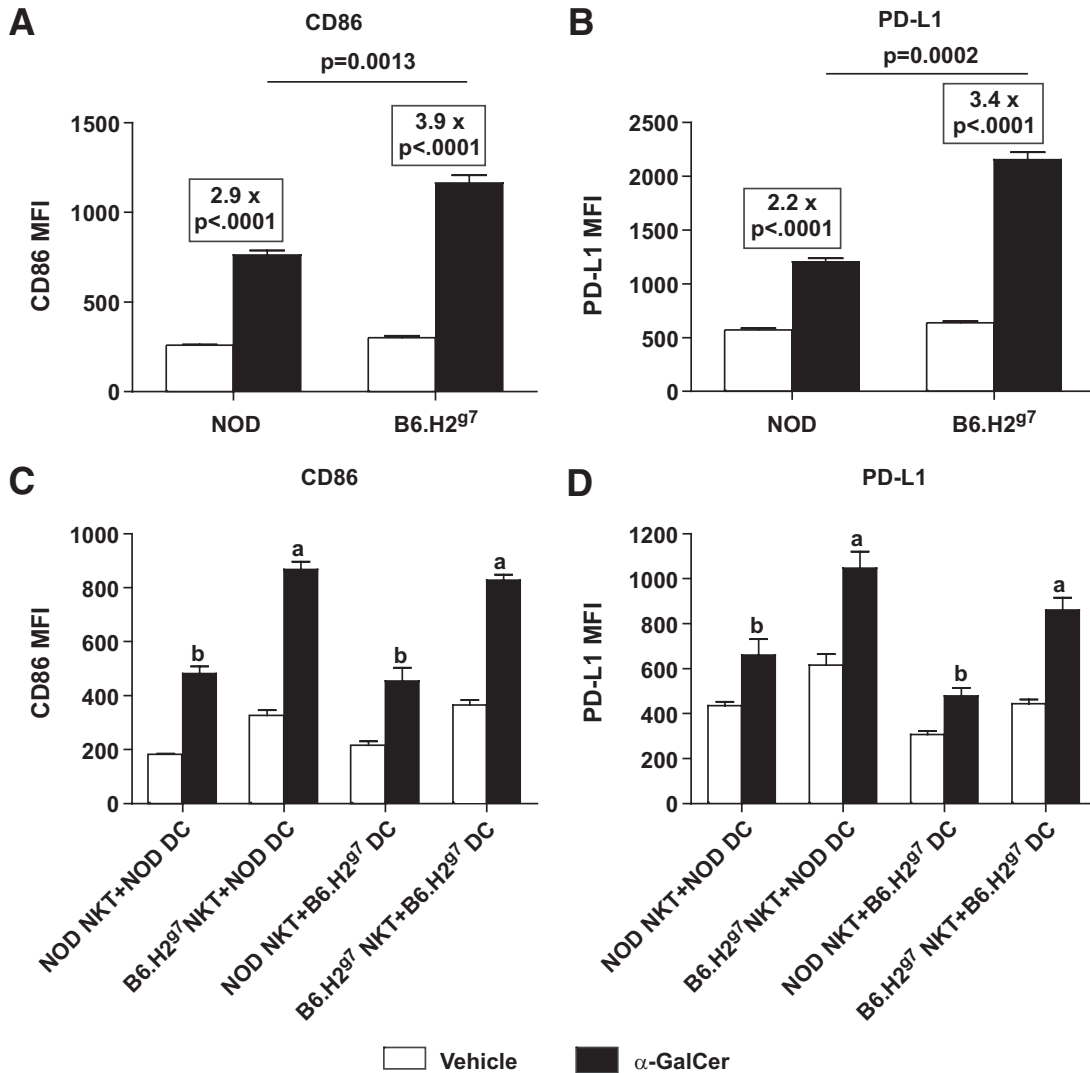
T-cells correlated with their CD70 expression levels (Fig. 5B). However, addition of the CD70-blocking antibody completely abrogated the ability of iNKT-conditioned DCs from either strain to induce antigen-mediated AI4 T-cell proliferation (Fig. 5B).

These collective data indicate AI4 T-cell activation is sensitive and insensitive to inhibition by PD-L1 signaling from NOD and B6.*H2<sup>g7</sup>* iNKT-conditioned DCs, respectively. This variability is likely due to much higher levels of costimulatory molecules, including CD70, provided to AI4 T-cells by iNKT-conditioned DCs from B6.*H2<sup>g7</sup>* than NOD mice.

**PD-L1 blockade inhibits the ability of NOD but not B6.*H2<sup>g7</sup>*-activated iNKT-cells to mediate type 1 diabetes-protective effects.** Given the above results, we hypothesized in vivo PD-L1 blockade would abrogate the earlier described ability of  $\alpha$ -GalCer-activated iNKT-cells to inhibit AI4 T-cell-induced type 1 diabetes in NOD but not B6.*H2<sup>g7</sup>* mice. To test this hypothesis, NOD.*Rag1<sup>null</sup>.AI4*







**FIG. 7.** Differences inherent to NOD and B6.H2<sup>g7</sup> iNKT-cells and DCs contribute to their varying responses to iNKT-cell activation. MFI of (A) CD86 and (B) PD-L1 antibody staining on CD11c<sup>+</sup> DCs resident in mixed cultures of NOD and B6.H2<sup>g7</sup> splenocytes (*n* = 3) incubated in the presence or absence of  $\alpha$ -GalCer (200 ng/ml) for 48 h. The two populations of NOD and B6.H2<sup>g7</sup> DCs were distinguished from each other by respective expression of the CD45.1 versus CD45.2 isoform. Boxed values indicate the within-strain fold change in CD86 and PD-L1 MFI on DCs from  $\alpha$ -GalCer– compared with vehicle-treated splenocytes. Differences across strains in absolute expression levels of surface molecules and the within-strain fold change induction above baseline between iNKT-conditioned NOD and B6.H2<sup>g7</sup> DCs were compared by one-way ANOVA. MFI of (C) CD86 and (D) PD-L1 antibody staining on purified NOD and B6.H2<sup>g7</sup> DCs ( $1 \times 10^5$  per well) cultured with  $1.5 \times 10^4$  syngeneic or allogeneic iNKT-cells (*n* = 3 for each cell type) in the presence of vehicle or  $\alpha$ -GalCer (200 ng/ml) for 18 h. Average MFI values of CD86 and PD-L1 antibody binding between  $\alpha$ -GalCer–treated cultures with no common superscripts differed significantly (*P* < 0.05) when analyzed by the Wilcoxon test after one-way ANOVA.

different expression levels of T-cell costimulatory and inhibitory molecules.

A series of T-cell costimulatory molecules were upregulated to a much greater extent on iNKT-conditioned DCs from B6.H2<sup>g7</sup> than NOD mice. In particular, CD70 and OX40L expression levels were unchanged or strongly upregulated on iNKT-conditioned DCs from NOD and B6.H2<sup>g7</sup> mice, respectively. This could be significant given a report that iNKT-cells promote CD8<sup>+</sup> cytotoxic T-cell responses by inducing CD70 expression on DCs (30). Indeed, without ruling out possible contributions from other costimulatory molecules, CD70 blockade abrogated the ability of iNKT-conditioned B6.H2<sup>g7</sup> DCs to support diabetogenic AI4 T-cell activation.

Lower levels of costimulatory molecule expression on NOD iNKT-conditioned DCs may explain why they do not support type 1 diabetes development like those of B6.H2<sup>g7</sup> origin, but do not account for their disease-protective

capacity. An explanation for this protection appears to lie in the observation that iNKT-cell–conditioned DCs also upregulate expression of the T-cell–inhibitory PD-L1 molecule. Absolute levels of PD-L1 were higher on iNKT-conditioned DCs from B6.H2<sup>g7</sup> than NOD mice. However, PD-L1 blockade only marginally enhanced the already strong ability of iNKT-conditioned B6.H2<sup>g7</sup> DCs to support diabetogenic AI4 T-cell activation. In contrast, PD-L1 blockade significantly enhanced the normally poor ability of iNKT-conditioned NOD DCs to support AI4 T-cell activation. PD-L1 blockade also abrogated the ability of  $\alpha$ -GalCer–activated iNKT-cells to induce type 1 diabetes–protective effects in NOD mice. These collective results indicate the relative activity of inhibitory PD-L1 versus various T-cell costimulatory molecules is greater in iNKT-conditioned DCs in NOD than B6.H2<sup>g7</sup> mice.

The finding that PD-L1 expression plays an important role in the type 1 diabetes–protective capacity of NOD



iNKT-conditioned DCs provides a likely mechanistic explanation for our previous observation that such antigen-presenting cells directly mediate the apoptotic deletion of some AI4 T-cells (31). However, the ability of activated iNKT-cells to inhibit type 1 diabetes development in NOD mice is also at least partly Treg dependent (20). Tregs are also reportedly less susceptible than conventional T-cells to PD-1-mediated deletion (32). iNKT-conditioned DCs from NOD and B6.*H2g7* mice maintain diabetogenic AI4 CD8 T-cells in a relatively quiescent and a highly activated state, respectively. Hence, iNKT-conditioned DCs in NOD mice may mediate PD-1-dependent apoptotic deletion of at least some diabetogenic T-cells that become activated, but keeping most of such effectors in a quiescent state also allows them to be more easily suppressed by Tregs than in the B6.*H2g7* strain. This latter possibility will be the subject of future investigations.

In contrast to our current findings, it has been reported that NOD mice must be pretreated with multiple, rather than a single, injections of  $\alpha$ -GalCer to induce type 1 diabetes-protective tolerogenic DCs (33). However, this study used a model system in which new diabetogenic T-cells could be continuously generated from hematopoietic precursors, whereas our studies evaluated the effects of various iNKT-conditioned DC populations on a single bolus of infused AI4 effectors.

Inferences for human type 1 diabetes intervention, based upon the ability of iNKT-conditioned DCs from two inbred mouse strains to differentially alter disease development, must be drawn with caution. There is likely to be comparable heterogeneity in iNKT-induced DC responses in humans. Compared with those in other strains, NOD DCs do not mature normally (34,35). This defect may be a pathogenic component of autoimmunity, as NOD DCs matured through *ex vivo* manipulation become type 1 diabetes protective (36). Similar DC maturation abnormalities reportedly characterize some human type 1 diabetic patients (37–39). If DCs from type 1 diabetic patients are analogous to those of NOD mice, then iNKT-cell activation may also restore their maturation to a tolerogenic state. However, unlike the case in inbred mice, there is likely to be more heterogeneity in the pathogenic basis of type 1 diabetes development in humans, including potential variability in DC functions. Such a scenario should preclude consideration of iNKT-cell agonists as potential type 1 diabetes therapeutic agents until reliable biomarkers are identified to predict how such compounds affect DC maturation in individual patients.

Current data indicate that NOD versus B6.*H2g7* strain differences inherent to iNKT-cells contribute to their variable induction of downstream DC maturation responses. NOD and B6.*H2g7* mice are further characterized by strain differences inherent to DCs, but are also dependent on contributions from another leukocyte population(s), which underlies their variable iNKT-cell-induced maturation patterns. NOD mice are reportedly characterized by both iNKT-cell and DC anomalies (34,35,40–43). Treatment with  $\alpha$ -GalCer may protect NOD mice from type 1 diabetes by compensating for both pathogenic iNKT-cell and DC impairments. Conversely, B6.*H2g7* mice have unimpaired iNKT-cells and DCs. This may account for why in  $\alpha$ -GalCer-treated B6.*H2g7* mice, DCs mature to an immunogenic rather than a tolerogenic state.

In summary, iNKT-conditioned DCs in NOD and B6.*H2g7* mice mature to a type 1 diabetes-protective or an immunogenic disease-inducing state, respectively. These vary-

ing DC maturation states are characterized by differing activity balances of T-cell-inhibitory and costimulatory molecules. This heterogeneity of iNKT-cell and DC interactions in inbred mouse strains raises concerns that should be considered before trying to manipulate this axis as a possible type 1 diabetes intervention approach in genetically heterogeneous humans.

#### ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants DK-46266 and DK-51090, Cancer Center Support Grant CA34196, and grants from the Juvenile Diabetes Research Foundation International.

No potential conflicts of interest relevant to this article were reported.

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