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Activated NKT Cells Inhibit Autoimmune Diabetes through Tolerogenic Recruitment of Dendritic Cells to Pancreatic Lymph Nodes[1]

Yi-Guang Chen,* Caroline-Morgane Choisy-Rossi,* Thomas M. Holl,* Harold D. Chapman,* Gurdyal S. Besra,† Steven A. Porcelli,‡ Daniel J. Shaffer,* Derry Roopenian,* S. Brian Wilson,§ and David V. Serreze[2]*

NKT cell activation by α-galactosylceramide (α-GalCer) inhibits autoimmune diabetes in NOD mice, in part by inducing recruitment to pancreatic lymph nodes (PLNs) of mature dendritic cells (DCs) with disease-protective effects. However, how activated NKT cells promote DC maturation, and what downstream effect this has on diabetogenic T cells was unknown. Activated NKT cells were found to produce a soluble factor(s) inducing DC maturation. Initially, there was a preferential accumulation of mature DCs in the PLNs of α-GalCer-treated NOD mice, followed by a substantial increase in T cells. Adoptive transfer of a diabetogenic CD8 T cell population (AI4) induced a high rate of disease (75%) in PBS-treated NOD recipients, but not in those pretreated with α-GalCer (8%). Significantly, more AI4 T cells accumulated in PLNs of α-GalCer than PBS-treated recipients, while no differences were found in mesenteric lymph nodes from each group. Compared with those in mesenteric lymph nodes, AI4 T cells entering PLNs underwent greater levels of apoptosis, and the survivors became functionally anergic. NKT cell activation enhanced this process. Hence, activated NKT cells elicit diabetes protection in NOD mice by producing a soluble factor(s) that induces DC maturation and accumulation in PLNs, where they subsequently recruit and tolerize pathogenic T cells. ***The Journal of Immunology,*** **2005, 174: 1196–1204.**

Similar to the case in humans, type 1 diabetes (T1D)[3] in NOD mice results from T cell-mediated autoimmune destruction of insulin-producing pancreatic β cells (1). Most self Ag-reactive T cells are normally eliminated by negative selection in the thymus (2). Autoreactive T cells escaping thymic negative selection can also be deleted or functionally suppressed in the periphery (3). Several lines of evidence indicate the autoreactive T cells causing T1D in NOD mice result from defects in both negative selection and peripheral tolerance induction (4–7).

Dendritic cells (DCs) are professional APC that play a key role in initiating effector T cell responses in secondary lymphoid organs (8, 9). Equally important to the initiation of effector responses, DCs also participate in the presentation of self Ags during the course of central and peripheral T cell tolerance induction (8, 9). This includes an ability of DCs to cross-present β cell Ags in a tolerogenic fashion to autoreactive T cells in pancreatic lymph nodes (PLNs) (10, 11). CD1d-restricted T cells (NKT cells) are a lymphoid population distinct from conventional T cells because they share some characteristics with NK cells (12). The majority of murine NKT cells use an invariant Vα14Jα18 TCR chain paired preferentially with a Vβ8, Vβ2, or Vβ7 chain. NKT cells recognize lipid Ags presented by the MHC class I-like CD1d molecule and can modulate both innate and adaptive immune responses (12). The interplay between NKT cells and DCs contributes to the outcome of immune responses (13). Coculture of NKT cells and DCs promotes maturation of the latter in the absence of exogenous ligands (12). NKT cells activated with the superagonist α-galactosylceramide (α-GalCer) induce IL-12 production by DCs through a CD40-CD40L interaction-dependent mechanism (14). Conversely, DCs can modulate NKT cell function by altering their cytokine production and activation status (13). In addition, administration of α-GalCer at the time of immunization greatly enhances both $CD4^+$ and $CD8^+$ T cell responses as a result of DC maturation (15). Both DCs and NKT cells are defective in NOD mice. NKT cells in NOD mice are numerically decreased and functionally impaired (16–18). Likewise, NOD DCs do not mature normally, and as a result express lower levels of CD86 than those from nonautoimmune-prone mice (19, 20). This may be significant, as DC maturation is required for tolerance induction in some experimental settings (21, 22). Therefore, the initiation of autoimmune T1D may result from inappropriate communication between NKT cells and DCs.

An increase in NKT cell numbers and/or function inhibits T1D development in NOD mice. Transfer of NKT cells protects NOD mice from T1D (16). CD1d-deficient NOD mice lacking NKT cells showed accelerated disease onset (23, 24). Several studies

*The Jackson Laboratory, Bar Harbor, ME 04609; †School of Biosciences, University of Birmingham, Birmingham, United Kingdom; ‡Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY 10461; and §Diabetes Research Laboratories, Massachusetts General Hospital, Cambridge, MA 02138

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[2] Address correspondence and reprint requests to Dr. David V. Serreze, The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609. E-mail address: dvs@jax.org

[3] Abbreviations used in this paper: T1D, type 1 diabetes; α-GalCer, α-galactosylceramide; AICD, activation-induced cell death; β_2m, β_2-microglobulin; BM, bone marrow; CM, conditioned medium; DC, dendritic cell; LN, lymph node; MLN, mesenteric LN; PLN, pancreatic LN.

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demonstrated that activating NKT cells by α-GalCer treatment inhibits T1D development in NOD mice (25–27). One of these studies reported that NKT cell activation induced T1D resistance by increasing the recruitment of mature tolerogenic myeloid DCs into PLNs (26). In addition, when transferred into young female NOD mice, mature myeloid DCs isolated from PLNs, but not other anatomical sites, blocked T1D development (26, 28). These results indicate an involvement of DCs in NKT cell-mediated T1D protection. Others attributed the protective effect of α-GalCer to its ability to shift the cytokines produced by β cell-autoreactive T cells from a Th1 (IFN-γ) to a Th2 (IL-4/IL-10) profile (25, 27). However, we do not favor this latter mechanism based on a finding that α-GalCer treatment initiated at 4 wk of age elicited equivalent levels of T1D protection in standard NOD mice and stocks genetically deficient in IL-4 and/or IL-10 (M. Clare-Salzer, Y. Naumov, S. Abrol, E. Paek, M. Osborne, Y.-G. Chen, D. Serreze, and S. B. Wilson, manuscript in preparation). Thus, in the current study, we determined whether modulation of the NKT-DC axis in NOD mice altered the survival or function of a normally highly diabetogenic β cell-autoreactive $CD8^+$ T cell population.

Materials and Methods

Mice and reagents

NOD/Lt mice are maintained by brother-sister mating in a specific pathogen-free research colony at The Jackson Laboratory. A MHC class I and CD8 T cell-deficient NOD.β_2-*microglobulin*^{null} (NOD.β_2m^{null}) stock is maintained at the N11 backcross generation (29). N8 and N9 NOD congenic stocks respectively deficient in CD4 or CD8 T cells have been previously described (19, 30). An NOD stock lacking B lymphocytes (31) is maintained at the N10 backcross generation. NOD mice transgenically expressing the TCR from the diabetogenic $CD8^+$ T cell clone AI4 (Vα8/Vβ2) have been previously described (designated NOD.*AI4*) (30). An NOD.*AI4* substock congenic for a functionally inactivated *Rag1* gene (NOD.*Rag1*^{null}.*AI4*) has also been described (32). mAbs used for flow cytometry were fluorochrome-conjugated anti-CD11c (HL3), anti-CD80 (16-10A1), anti-CD86 (GL-1), anti-CD69 (H1.2F3), anti-B220 (RA3-6B2), anti-CD3 (145-2C11), anti-CD8 (53-6.72), and anti-TCR Vα8 (B21.12). These mAbs and annexin V were purchased from BD Pharmingen. α-GalCer (KRN7000) was kindly provided by Kirin Brewery or synthesized using a method developed by the authors that will be described elsewhere (P. Illarionov, S. Porcelli, and G. Besra, manuscript in preparation). The structure of our synthetic α-GalCer was identical with that previously published for the compound designated KRN7000 (33), except that the fatty acid chain length was two carbons shorter (C24:0 instead of C26:0). Synthetic α-GalCer was purified to homogeneity, and the structure was validated by mass spectrometry and nuclear magnetic resonance spectroscopy. The synthetic α-GalCer and the KRN7000 reagents were found not to differ in their ability to induce cytokine production (IFN-γ, IL-12, IL-4) or the downstream maturation of DC in the sorts of experiments depicted in Fig. 1. The only difference is that the synthetic α-GalCer is slightly less potent (~4-fold) than the KRN7000 reagent. RPMI 1640 tissue culture medium was prepared, as previously described (34).

Splenocyte culture

Splenocytes were seeded at a concentration of 2.5×10^6/ml in RPMI 1640 with or without α-GalCer (200 ng/ml) and cultured at 37°C for 2 days. Cells were harvested and stained with fluorochrome-conjugated Abs to CD11c as well as CD86 or CD80 for flow cytometric analyses. Conditioned medium (CM) was collected, filtered, and assessed for ability to induce DC maturation. To do this, NOD.β_2m^{null} splenocytes were incubated for 24 h with CM samples diluted to a concentration of 10% in RPMI 1640. Flow cytometry was used to identify DCs ($CD11c^+$) recovered from the culture and their expression levels of the indicated markers.

Short-term α-GalCer treatment effects

Female NOD mice (4–5 wk old) were injected i.p. with α-GalCer (2 μg/recipient) or the same volume of PBS. Single cell suspensions were prepared from PLNs and mesenteric lymph nodes (MLNs) on days 1, 2, 4, and 8, and stained with Abs to CD11c and CD86 or CD3 and B220 for flow cytometric analyses.

Gene expression analysis

PLNs and MLNs were individually collected from four NOD female mice (7 wk of age) that had received an i.p. injection of α-GalCer (2 μg) 24 h previously or were untreated. Total RNA was purified using the RNAqueous-4PCR kit (Ambion), then treated with DNase, and its quality was verified by Agilent gels. cDNA synthesis was conducted using the RETROscript kit (Ambion). ImmunoQuantArray analysis to determine the quantitative expression levels of 48 genes (list and primers at ⟨www.jax.org/staff/roopenian/labsite/gene_expression.html#notes⟩) in each PLN and MLN was performed essentially as described (35), using an ABI 7900 thermocycler (Applied Biosystems). The global pattern recognition algorithm, which circumvents the ambiguities caused by reliance on a single normalizer, was used to identify significant changes in gene expression (35). Only genes showing a conservative global pattern recognition cutoff score of ≥0.4 and a statistical threshold of $p \leq 0.05$ were considered to have significantly different expression levels. Fold changes of expression were calculated by the previously described algorithm (36).

Adoptive transfer of T1D

Female NOD mice received three to four once weekly i.p. injections of α-GalCer (2 μg) or the same volume of PBS. Two days after the last treatment, mice were sublethally irradiated (600 R from a ^{137}Cs source) and injected i.v. with 5×10^6 NOD.*Rag1*^{null}.*AI4* splenocytes. In some experiments, donor cells were prelabeled with 5 μM CFSE before transfer. Recipient mice were either euthanized at the indicated times posttransfer or monitored for T1D development over a 3-wk period. For studying T1D development, recipients received one additional α-GalCer or PBS treatment at 5 days after splenocyte transfer. T1D was assessed by daily monitoring of glycosuric levels with Ames Diastrix (Bayer, Diagnostics Division), with disease onset defined by two consecutive values of ≥3.

Partial bone marrow (BM) chimeras

Sublethally irradiated (500 R) 4- to 5-wk-old NOD females were injected i.v. with $2.5–5 \times 10^6$ BM cells from female NOD.*AI4* or NOD.*Rag1*^{null}.*AI4* mice. At 6–8 wk postreconstitution, chimerization was confirmed by increased frequency of AI4 T cells (coexpression of TCR Vα8 element and CD8) among peripheral blood leukocytes compared with the basal level in unmanipulated female NOD mice (<0.5%).

T cell proliferation and IFN-γ production

PLN and MLN suspensions containing equal numbers of AI4 T cells were seeded in 96-well plates in 200 μl of RPMI 1640 and stimulated with the indicated concentrations of an AI4 mimotope peptide (YFIENYLEL; kindly provided by T. Dilorenzo, Albert Einstein College of Medicine, Bronx, NY). Irradiated (2000 rad) NOD splenocytes (5×10^5 cells/well) were added as APCs. Cells were incubated at 37°C for 3 days, and 1 μCi/well [^{3}H]thymidine was added during the last 18 h. Supernatants were collected from parallel plates and assessed for IFN-γ concentrations by ELISA (BD Biosciences).

Flow cytometric analyses

Single cell suspensions were stained with the indicated Abs at 4°C for 30 min. Stained cells were washed and analyzed on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest software. Propidium iodide was used to gate out dead cells.

Results

Activated NKT cells induce DC maturation through a soluble factor(s)

It has been proposed that α-GalCer-stimulated NKT cells elicit T1D resistance in NOD mice through a mechanism involving enhanced DC maturation and immigration to PLNs (26). To further test this possibility, we assessed the ability of α-GalCer stimulation to induce the maturation of DC resident among NOD or NOD.β_2m^{null} splenocytes. Compared with untreated control samples, based on elevated expression of the CD86 ligand for the CD28 T cell costimulatory receptor, NOD splenic DCs exhibited a more mature phenotype following α-GalCer-induced NKT cell activation (Fig. 1). Although lacking NKT cells, mice genetically deficient in β_2m (β_2m^{null}) mice do produce DCs (37). Following incubation in the presence of α-GalCer, DCs resident among NOD.β_2m^{null} splenocytes failed to up-regulate CD86 expression

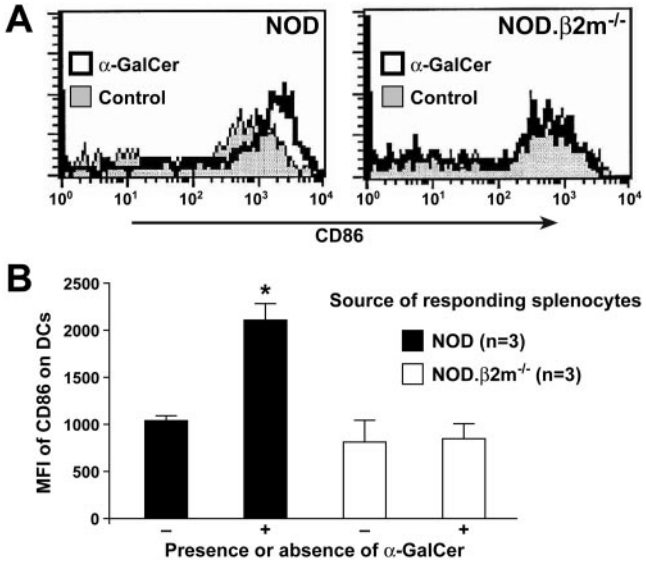

FIGURE 1. Activated NKT cells induce maturation of NOD DC. NOD and NOD.β_2m^{null} splenocytes were cultured with or without α-GalCer for 48 h. *A*, Representative flow cytometry histograms depicting CD86 expression on DCs ($CD11c^+$). *B*, Summary of CD86 Ab-staining intensity (mean ± SEM) of DCs resident among NOD and NOD.β_2m^{null} splenocytes cultured with or without α-GalCer. ∗, CD86 expression significantly higher ($p < 0.05$, Student's *t* test) than in control sample cultured without α-GalCer.

(Fig. 1). This latter finding indicated α-GalCer does not directly induce DC maturation. CM from α-GalCer-stimulated NOD, but not NKT cell-deficient NOD.β_2m^{null} splenocytes, induced higher CD86 expression levels on NOD.β_2m^{null} DCs (Fig. 2). Finally, α-GalCer-activated NKT cells from NOD stocks lacking conventional $CD4^+$ or $CD8^+$ T cells, or B cells, also induced DC maturation (data not shown). This indicated activated NKT cells do not have to interact with other lymphoid subsets to induce DC maturation. Taken together, these data indicate a soluble factor(s) released by activated NKT cells directly induces DC maturation. Through the use of NOD genetic knockout stocks, blocking Abs, and ELISA analyses of active CM samples, we have ruled out IL-4, IL-10, IFN-γ, IL-13, TGF-β, and TNF-α as being factors produced by NKT cells that drive DC maturation.

Accumulation of T cells, B cells, and DCs in PLNs of α-GalCer-treated NOD mice

It has been previously demonstrated that the inhibition of T1D in α-GalCer-treated NOD mice entails the accumulation of NKT cells and mature myeloid DCs in PLNs (26). However, the kinetics by which DCs mature and enter the PLNs following α-GalCer treatment, and what effects this has on other cell types were not fully understood. To initially address this issue, standard NOD mice were treated once with α-GalCer or PBS, and over the next 8 days the proportions, numbers, and CD86 expression levels of DCs in PLNs were assessed. Numbers of T and B cells in PLNs were also evaluated. As comparisons, cells isolated from MLNs were also examined.

In α-GalCer-treated mice, total PLN cell numbers increased significantly on day 2, peaked on day 4, and were returning to basal level afterward (Fig. 3*A*). In contrast, no substantial expansion was observed in MLNs. The numerical changes of DCs, B cells, and T cells within PLNs and MLNs of α-GalCer-treated NOD mice followed the same pattern (Fig. 3, *B–D*, respectively). More DCs already accumulated in the PLNs of α-GalCer-treated NOD mice on day 1, while T cell numbers remained unchanged at this time point (Fig. 3, compare *B* and *D*). A previous study demonstrated it is primarily myeloid DCs ($CD11c^+CD8\alpha^-$) that are preferentially recruited to the PLNs of α-GalCer-treated NOD mice (26). Accompanied with their increased number on day 1, based on higher levels of CD86 expression, DCs within PLNs of α-GalCer-treated NOD mice were more mature than those from controls (Fig. 3*E*). These collective results indicate α-GalCer treatment of NOD mice rapidly induces DC maturation and migration into PLNs, followed by a cascade of events leading to a much greater expansion of total cells on day 4. DCs within secondary lymphoid organs are capable of attracting naive T cells (38). In addition, T cell levels in lymph nodes (LNs) have been correlated to the number of residing DCs (39). Hence, our results suggest that in α-GalCer-treated NOD mice, mature DCs first preferentially migrate into PLNs, where they further recruit, and may also promote the proliferation of T cells.

Enhanced recruitment and subsequent nonpathogenic proliferation of $CD8^+$ T cells in the PLNs of NOD mice protected from T1D by α-GalCer treatment

As illustrated above, following the influx of mature DCs, T cell numbers increase in the PLNs of α-GalCer-treated NOD mice. However, it was not clear whether T cells expanding within the PLNs of α-GalCer-treated NOD mice included β cell-autoreactive clonotypes. A resource that allowed us to address this issue is a NOD stock transgenically expressing the TCR of the β cell-autoreactive $CD8^+$ T cell clone AI4 (NOD.*AI4*), and as a result it develops T1D at an accelerated rate (30). T1D development remains accelerated in NOD. *AI4* mice that are also homozygous for the *Rag1*^{null} mutation (NOD.*Rag1*^{null}.*AI4*) that blocks the development of any nontransgenic T cells (40). The use of this latter stock as donors in an adoptive transfer system allowed us to assess

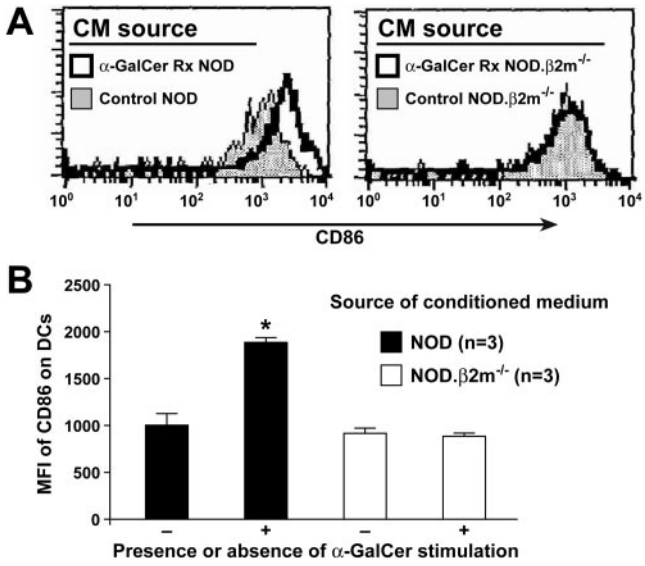

FIGURE 2. A soluble factor(s) in CM from α-GalCer-stimulated NOD splenocytes induces DC maturation. NOD.β_2m^{null} splenocytes were incubated for 24 h with 10% CM derived from NOD or NOD.β_2m^{null} splenocytes that had been precultured in the presence or absence of α-GalCer. *A*, Representative flow cytometry histograms showing on CD86 expression on NOD.β_2m^{null} DCs exposed to each CM source. *B*, Summary of CD86 Ab-staining intensity (mean ± SEM) of NOD.β_2m^{null} DCs exposed to each CM source. ∗, CD86 expression significantly higher ($p < 0.05$, Student's *t* test) than in control sample stimulated with CM derived from NOD splenocytes cultured in the absence of α-GalCer.

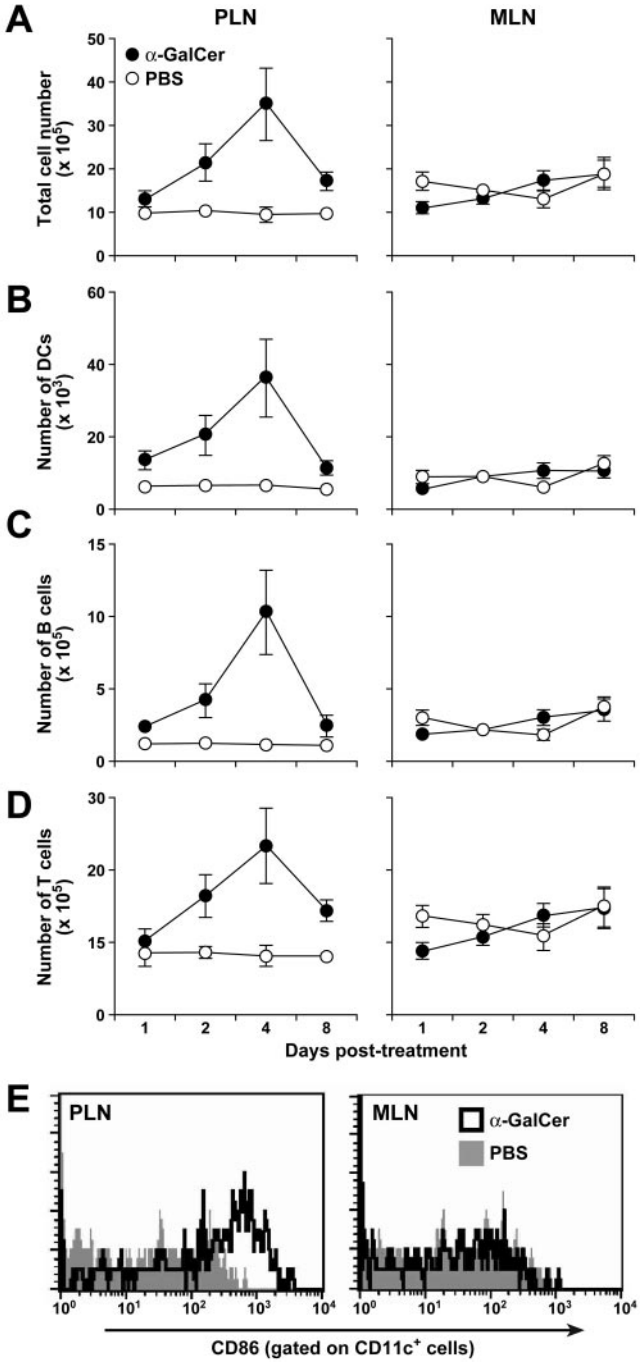

FIGURE 3. Accumulation of mature DCs, followed by T and B lymphocytes in PLNs of α-GalCer-treated NOD mice. Female NOD mice received a single i.p. injection of α-GalCer or PBS on day 0. PLN and MLN cells were counted and analyzed by flow cytometry 1, 2, 4, and 8 days later with Abs delineating T cells (CD3), B cells (B220), or DC (CD11c). Levels of CD86 expression on DCs were also assessed. *A*, Total cell numbers per LN. *B–D*, Numbers of DCs, B cells, and T cells per LN, respectively. Each value represents the mean ± SEM of six to eight mice. *E*, Expression levels of CD86 on DCs on day 1.

the anatomical sites to which diabetogenic CD8 T cells home and become activated, and how this may be altered when DC maturation is enhanced by α-GalCer-stimulated NKT cells. CFSE-labeled NOD.*Rag1*^{null}.*AI4* T cells were transferred into sublethally irradiated NOD mice. At 3 days posttransfer, the numbers and activation status of AI4 T cells in recipient spleens, PLNs, and MLNs were assessed. AI4 T cells were identified by coexpression of CD8, the TCR Vα8 element, and CFSE labeling. In the absence of α-GalCer, little proliferation of AI4 T cells was observed in the spleens and MLNs (Fig. 4*A*). Conversely, AI4 T cells in PLNs had undergone several rounds of division, indicating Ag recognition had occurred. Most likely, the pancreatic β cell autoantigen recognized by AI4 T cells is cross-presented by DCs to these pathogenic effectors in the PLNs.

To study the effects of NKT cell activation on β cell-autoreactive AI4 T cells, we established an adoptive transfer system that accelerated T1D development in NOD recipients. Splenocytes from female NOD.*Rag1*^{null}.*AI4* mice adoptively transferred T1D to sublethally irradiated NOD female recipients within a 2-wk period in a dose-dependent fashion (data not shown). A cell dose of 5×10^6 splenocytes ($\sim 1 \times 10^6$ AI4 T cells) was chosen for subsequent transfer experiments. In contrast, age-matched female NOD mice given the same dose of irradiation, but no NOD.*Rag1*^{null}.*AI4* splenocytes, did not develop T1D over the same period (data not shown). This indicated accelerated T1D development in the splenocyte recipients is due to the transferred AI4 T cells rather than homeostatic expansion of host lymphocytes, which can cause autoimmune disease in certain situations (41). We next tested whether α-GalCer pretreatment blocked the ability of adoptively transferred AI4 T cells to induce T1D. Most PBS-treated control recipients (75%) developed T1D within 3 wk after AI4 T cell transfer (Fig. 4*B*). In contrast, α-GalCer-pretreated recipients developed a significantly lower incidence of T1D (8.3%) over the same period. It should be noted that the T1D-protective mechanism induced by the course of α-GalCer treatments used for these studies was not abrogated by the low dose of irradiation that the recipients received before AI4 T cell infusion.

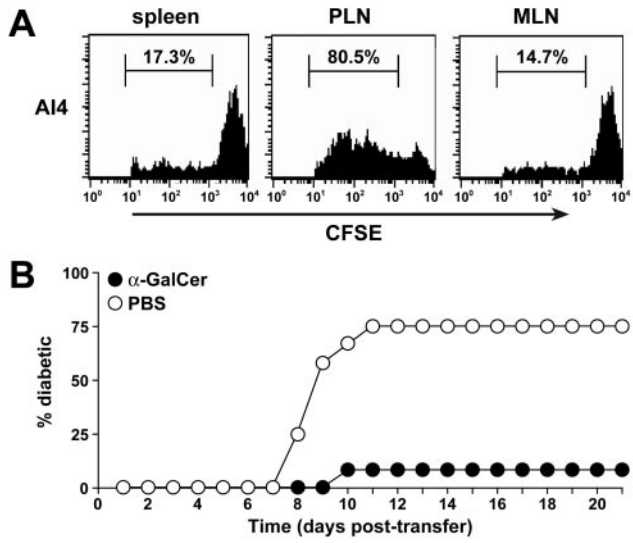

FIGURE 4. α-GalCer treatment blocks the adoptive transfer of T1D by AI4 T cells. *A*, Diabetogenic AI4 CD8$^+$ T cells encounter their cognate Ag in PLNs. A total of 5×10^6 CFSE-labeled splenocytes isolated from NOD.*Rag1*^{null}.*AI4αβ* mice were transferred into sublethally irradiated NOD mice. Three days later, cells from spleens, PLNs, and MLNs were isolated and analyzed by flow cytometry. Histograms show staining of CD8/CFSE double-positive cells. The percentage in each histogram indicates the proportion of divided cells. Similar results were obtained in another experiment. *B*, Incidence of T1D in AI4 T cell recipient NOD mice pretreated with α-GalCer or PBS ($n = 12$ per group). Recipient female NOD mice were pretreated with α-GalCer or PBS once per week for 4 wk before being injected i.v. with NOD.*Rag1*^{null}.*AI4* splenocytes. Five days later, the recipients received an additional dose of α-GalCer or PBS. The combined results of two independent experiments are shown.

To determine the early events associated with T1D protection in α-GalCer-pretreated mice, we isolated PLNs and MLNs from the recipients at 3 and 6 days posttransfer, and determined the numbers of AI4 T cells that they contained. More AI4 T cells were present in the PLNs than MLNs of both α-GalCer- and PBS-treated mice (Fig. 5*A*). Interestingly, in PLNs, significantly higher numbers of AI4 T cells were observed in α-GalCer- than PBS-treated recipients on day 3 and to a lesser extent on day 6. In contrast, no difference was noticed in the MLNs of α-GalCer- and PBS-treated mice. Increased numbers of AI4 T cells in the PLNs of α-GalCer-treated recipients could be due to more efficient recruitment and/or a higher proliferation rate. To test these possibilities, CFSE-labeled NOD.*Rag1*^{null}.AI4αβ splenocytes were transferred into sublethally irradiated α-GalCer- or PBS-pretreated NOD mice. As expected, in both groups, AI4 T cells underwent robust division in the PLNs, but not MLNs, at 3 days posttransfer. Comparable proportions of AI4 T cells underwent division in PLNs of both α-GalCer- and PBS-treated recipients (data not shown). However, a significantly higher total number of both nondivided and divided AI4 T cells was observed in PLNs of α-GalCer- than PBS-pretreated recipients (Fig. 5, *B* and *C*). These results indicated that increased AI4 T cell levels in the PLNs of α-GalCer-treated NOD mice result from enhanced recruitment and subsequent proliferation.

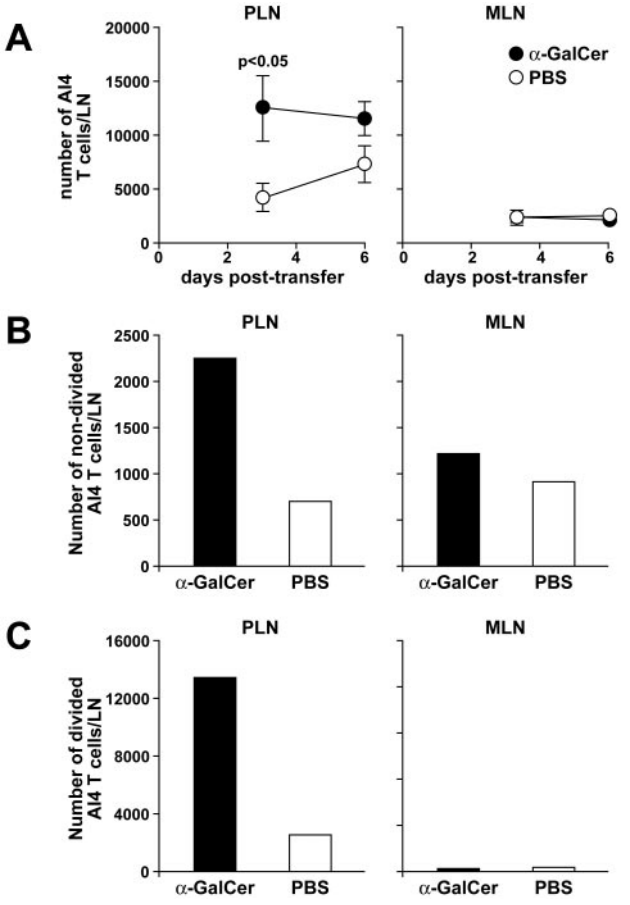

FIGURE 5. Increased recruitment and subsequent nonpathogenic proliferation of adoptively transferred AI4 T cells in the PLNs of α-GalCer-treated NOD recipient mice. *A*, Numbers of AI4 T cells in PLNs and MLNs of recipient NOD mice treated with α-GalCer or PBS. Recipient female NOD mice were treated with α-GalCer or PBS once per week for 3 wk before being sublethally irradiated and injected i.v. with 5×10^6 NOD.*Rag1*^{null}.*AI4* splenocytes. PLN and MLN cells from three to four mice were pooled, and numbers of AI4 T cells were determined by coexpression of CD8 and the TCR Vα8 element. Each value represents the mean ± SEM of five cell pools from four independent experiments. Statistical analyses were performed using Student's *t* test. *B* and *C*, Recipient female NOD mice were treated as described above, except that CSFE-labeled NOD.*Rag1*^{null}.*AI4* splenocytes were transferred. PLN and MLN cells were pooled from seven mice, and the numbers of AI4 T cells were determined by coexpression of CD8 and the TCR Vα8 element 3 days posttransfer. The numbers of nondivided (*B*) and divided (*C*) AI4 T cells were respectively calculated by multiplying the total cell count by the percentage of cells in which CFSE dilution had not or did occur. Similar results were obtained in another experiment.

The question remained why systemic α-GalCer treatment elicited the migration of mature DCs and T cells into the PLNs, but not the MLNs of NOD mice. Thus, we compared the expression levels by real-time PCR of genes encoding 19 different chemokines and 11 of their receptors in the PLNs and MLNs of NOD mice that were either untreated or injected 24 h previously with α-GalCer (complete gene list and primers at ⟨www.jax.org/staff/roopenian/labsite/gene_expression.html#notes⟩). There were no significant differences between PLNs and MLNs from untreated mice. However, CCL17 (5.3-fold), CCL19 (2.4-fold), CCL5 (1.8-fold), and CXCL16 (1.6-fold) gene expression levels were significantly higher in the PLNs than MLNs of α-GalCer-treated NOD mice. Conversely, CCR4 (3.3-fold) and CCR6 (1.7-fold) were expressed at higher levels in the MLNs of the α-GalCer-treated NOD mice. This differential level of chemokine expression may underlie the migration of DCs and T cells to the PLNs, but not the MLNs, of α-GalCer-treated NOD mice.

AI4 T cells are tolerized in PLNs

Our results indicate that following NKT cell activation, AI4 T cells are preferentially recruited to and proliferate in the PLNs of NOD mice, where they may be tolerized rather than functionally activated. AI4 T cells may be tolerized in α-GalCer-treated mice through two possible mechanisms. It is possible α-GalCer treatment alters the PLN microenvironment in a way that forces AI4 T cells to undergo an abortive activation, rather than being primed to become functional effectors. Alternatively, it is possible that even in the PBS-treated controls, any AI4 T cells and perhaps other diabetogenic clonotypes entering the PLNs are tolerized, but α-GalCer treatment quantitatively increases and accelerates this process. Enhancing either of these possible T cell tolerance induction processes could represent the T1D-protective mechanism elicited by the increased number of mature DCs entering PLNs as a downstream consequence of NKT cell activation in NOD mice.

The number of AI4 T cells remained unchanged from 3 to 6 days posttransfer in PLNs of α-GalCer-treated NOD recipients (Fig. 5*A*), despite the fact they were undergoing extensive proliferation on day 3 (Figs. 4*A* and 5*C*). In contrast, AI4 T cell numbers increased between days 3 and 6 in PLNs of PBS-treated mice. Because α-GalCer treatment clearly inhibited T1D development, it is unlikely AI4 T cell numbers remained unchanged in PLNs as a consequence of their migration to the pancreatic islets. They could cease to proliferate or alternatively be deleted through activation-induced cell death (AICD). To partially answer this question, we determined whether α-GalCer treatment induced apoptosis of AI4 T cells in the PLNs of recipient mice. When compared with MLNs, we observed more apoptotic AI4 T cells in the PLNs of both α-GalCer- and PBS-treated recipient mice (Fig. 6). This indicated apoptosis of adoptively transferred AI4 T cells in PLNs was an Ag-driven process. On day 3 posttransfer, the numbers of apoptotic AI4 T cells were significantly higher in PLNs of α-GalCer- than PBS-pretreated recipient mice (Fig. 6, *left panel*). There was no statistically significant difference on day 6 due to an increased number of apoptotic AI4 T cells in the PLNs of control recipients.

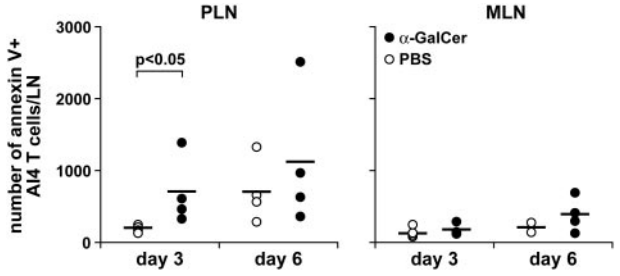

FIGURE 6. Apoptosis of adoptively transferred AI4 T cells in PLNs. Recipient female NOD mice were treated with α-GalCer or PBS once per week for 3 wk before being sublethally irradiated (600 R) and injected i.v. with NOD.*Rag1*^{null}*.AI4* splenocytes. PLN and MLN cells from three to four mice were pooled and stained by annexin V and Abs against CD8 and the TCR Vα8 element. Apoptotic AI4 T cells were defined by coexpression of CD8 and the TCR Vα8 element, positive staining of annexin V, and lack of propidium iodide. Each value represents the mean ± SEM of four cell pools from three independent experiments. Statistical analysis was performed using Wilcoxon rank sum test.

These results favor the idea that even in the controls, AI4 T cells are normally deleted in PLNs, but not at levels preventing T1D development. However, this already ongoing process is enhanced and accelerated to a T1D-protective level when activated NKT cells elicit the entry of larger numbers of mature tolerogenic DCs into the PLNs.

To further test the possible tolerogenic mechanisms described above, we determined whether PLNs represent a site in NOD mice in which AI4 T cells are inactivated upon entry, and whether this process is enhanced following NKT cell activation. We first compared the ability of AI4 T cells within PLNs and MLNs of untreated NOD.*AI4* mice to proliferate in response to an antigenic mimotope peptide. Unlike those within MLNs, PLN-residing AI4 T cells responded very poorly to antigenic stimulation (Fig. 7*A*). T cells in AI4 TCR transgenic NOD mice are skewed toward the CD8$^+$ compartment. Thus, the hyporesponsiveness of PLN AI4 T cells could have resulted from a previous Ag encounter in the absence of proper CD4$^+$ T cell help (42). To test this possibility, we generated partial AI4 BM chimeras that retain high levels of host type (standard NOD) CD4$^+$ and CD8$^+$ T cells. The AI4 clonotype (identified by CD8 and TCR Vα8 coexpression) represents 30–50% of total CD8$^+$ T cells in these partial chimeras (data not shown). In agreement with the adoptive transfer results described earlier, AI4 T cells in the PLNs, but not the MLNs, of these chimeras expressed the CD69 activation marker (Fig. 7*B*). However, as shown in Fig. 7, *C* and *D*, a previous Ag encounter in an environment in which CD4 T cell help could be available did not endow a memory phenotype to AI4 T cells in the PLNs that now allowed them to respond more vigorously to mimotope stimulation than those in MLNs. Rather, they continued to show reduced proliferation and IFN-γ production compared with AI4 T cells recovered from MLNs of the same chimeras.

The results described above indicate that even in the absence of α-GalCer treatment, the AI4 T cells and perhaps other β cell-autoreactive clonotypes entering the PLNs of NOD mice can be tolerized, rather than functionally activated. In untreated NOD mice, this tolerogenic process clearly does not occur at a sufficient level to prevent T1D. Deletion of autoreactive T cells in the PLNs through cross-presentation of β cell Ags by DCs has been indicated (10, 11). However, unlike nonautoimmune control strains, NOD mice do not efficiently delete peripheral CD8$^+$ T cells that recognize a neo-Ag expressed in pancreatic β cells (6). These results suggest a quantitative tolerance induction defect in PLNs is,

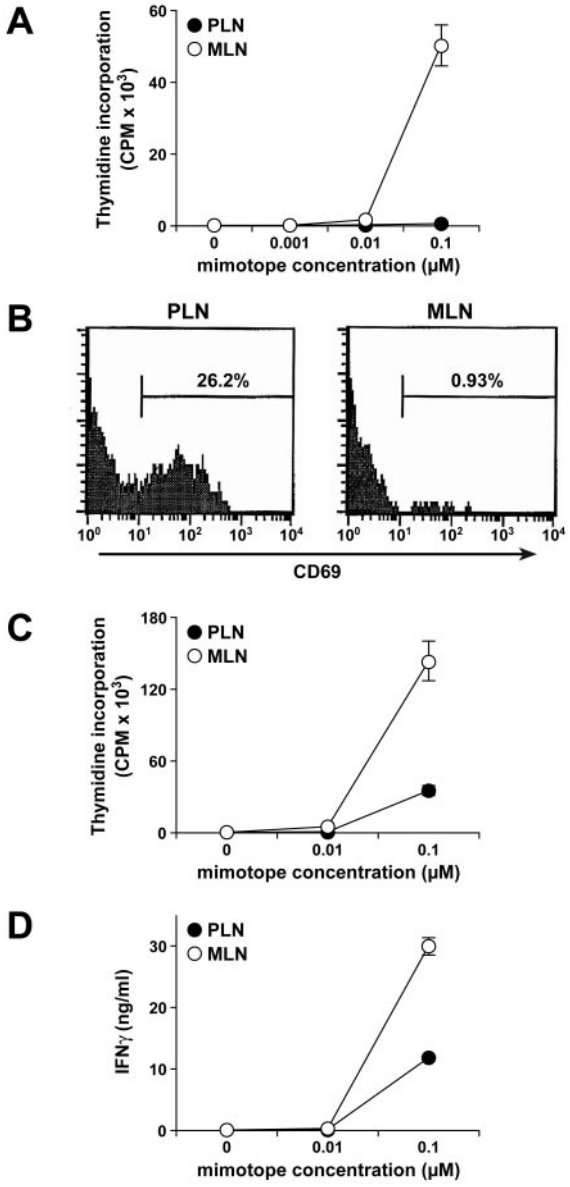

FIGURE 7. PLN-residing AI4 T cells are hypofunctional. *A*, Pooled PLN and MLN cell suspensions containing equal number of AI4 T cells (5×10^4/well) were generated from five NOD.*AI4* mice. In vitro proliferative responses of AI4 T cells stimulated for 72 h with the indicated concentrations of mimotope peptide were determined, as described in *Materials and Methods*. Results represent the mean cpm ± SEM of triplicate wells. *B–D*, Partial AI4 BM chimeras were generated, as described in *Materials and Methods*. AI4 T cells in PLNs and MLNs were analyzed 8 wk after BM transfer. Cells from three mice were pooled for the assays. *B*, CD69 expression on AI4 T cells (gated on CD8$^+$ and TCR Vα8$^+$) in PLNs (*left*) and MLNs (*right*). Percentages represent the proportions of CD69$^+$ cells. *C*, PLN and MLN suspensions containing equal numbers of AI4 T cells (1.7×10^4/well) were prepared from the partial BM chimeras and stimulated with the indicated concentrations of mimotope peptide for 72 h. Proliferation results represent the mean cpm ± SEM of triplicate wells. Similar results were obtained in another experiment. *D*, PLN and MLN suspensions containing equal number of AI4 T cells (1.5×10^4/well) were prepared from the partial BM chimeras and stimulated with the indicated concentrations of mimotope peptide for 72 h. Supernatants from three wells of each source of AI4 T cells cultured with the indicated concentration of mimotope peptide were pooled and assessed in triplicate for IFN-γ content. Results represent the mean ± SEM of the triplicates. Similar results were obtained in another experiment.

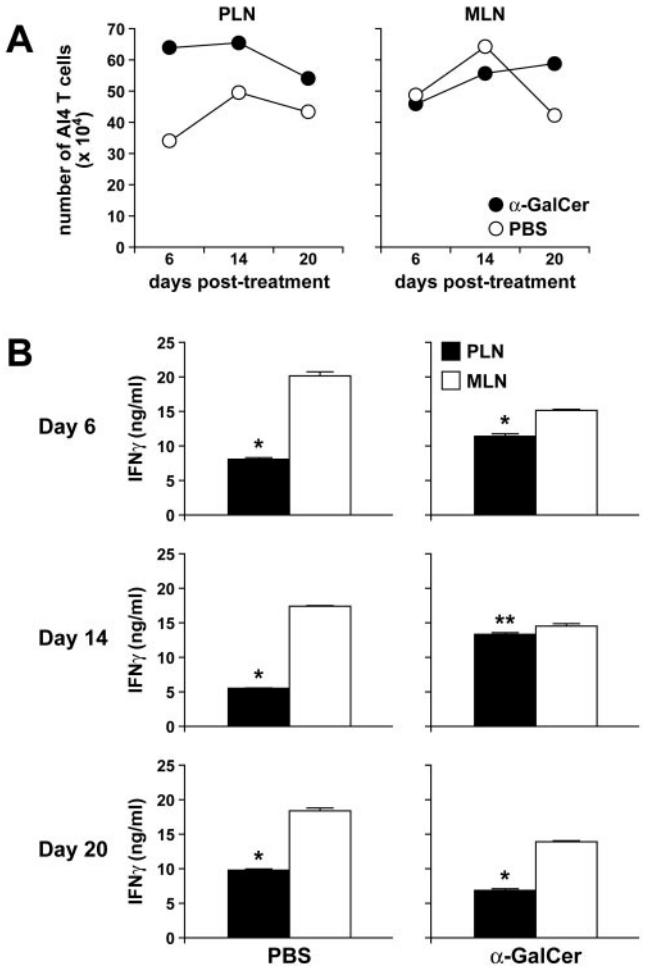

FIGURE 8. α-GalCer treatment induces the tolerogenic recruitment of AI4 T cells to PLNs. AI4 BM chimeras were generated, as described in Fig. 7. The percentages of AI4 T cells were determined in peripheral blood leukocytes 8 wk after BM transfer. Mice were grouped together so that in average each group contained ~7% AI4 T cells in peripheral blood leukocytes. AI4 BM chimeras received 2 μg of α-GalCer or equal volume of PBS on day 0. PLN and MLN cells were recovered and pooled from three to four mice on days 6, 14, and 20 to determine the number of AI4 T cells/LN (*A*) and their IFN-γ production in response to mimotope stimulation in vitro (*B*). The IFN-γ production assay was performed, as described in Fig. 7*D*, using LN suspensions containing matched numbers of AI4 T cells (1.5×10^4) and the mimotope peptide at a concentration of 0.1 μM. *, $p < 0.005$; **, $p = 0.04$, when compared with MLN-residing AI4 T cells (Student's *t* test). Similar results were obtained in another experiment.

at least in part, responsible for T1D development in NOD mice. BM-derived DCs from NOD mice have been reported to not mature normally (19). Such maturation defects may impair the tolerogenic capacity of PLN-residing DCs. Nevertheless, previous studies demonstrated the minority of DCs that do manage to mature normally in untreated NOD mice can enter the PLNs, and are capable of mediating T1D-protective effects (26, 28). Taken together, these findings suggest impaired maturation of DCs in NOD mice limits their migration to PLNs. As a result, the number of mature DCs in PLNs is normally insufficient to cross-tolerize enough β cell-autoreactive T cells to prevent T1D. Therefore, the apparent T1D-protective function of NKT cell activation in NOD mice is to quantitatively increase the already ongoing process of mature DCs entering the PLNs to levels in which they can now recruit and cross-tolerize larger numbers of pathogenic T cells than normally occurs. To further test this possibility, we treated partial AI4 BM chimeras with a single injection of α-GalCer and analyzed the subsequent numbers and antigenic responsiveness of AI4 T cells in the PLNs.

Similar to α-GalCer-treated standard NOD mice, a single injection significantly increased the cellularity of PLNs in the partial AI4 BM chimeras within 6 days (data not shown). Although not differing on a percentage basis, larger numbers of PLN-residing AI4 T cells were observed in chimeras injected with α-GalCer than PBS 6 days previously (Fig. 8*A*, *left panel*). In contrast, no numerical or proportional differences were observed for MLN-residing AI4 T cells between α-GalCer- and PBS-treated chimeras (Fig. 8*A*, *right panel*, and data not shown). After 20 days, numbers of AI4 T cells within PLNs remained slightly higher in α-GalCer- than PBS-treated chimeras (Fig. 8*A*, *left panel*). As expected, in PBS-treated BM chimeras, AI4 T cells in the PLNs responded poorly to antigenic stimulation compared with those in MLNs, regardless of the time they were examined (Fig. 8*B*, *left panels*). However, because they should arrive in a nontolerized state, we hypothesized that any AI4 T cells that are newly recruited at an enhanced frequency to the PLNs as a consequence of NKT cell activation should demonstrate a transient ability to respond to antigenic stimulation. This was indeed the case, as the mimotope-stimulated response of AI4 T cells within PLNs of α-GalCer-treated BM chimeras was elevated over a 2-wk period, but returned to a low level on day 20 (Fig. 8*B*, *right panels*). In contrast, AI4 T cells in the MLNs of α-GalCer-treated chimeras responded strongly to mimotope stimulation at all time points.

It should be noted that the total numbers of AI4 T cells in the partial BM chimeras were significantly greater than in the recipients of already differentiated splenic AI4 T cells used for the experiments depicted in Figs. 4–6. This may be why it took a longer period of time following α-GalCer treatment to detect tolerogenic effects in the PLNs of the partial BM chimeras (6–20 days) than in the recipients of differentiated splenic AI4 T cells (3–6 days). However, the numerical and functional evidence from all the systems we used indicates α-GalCer activation of NKT cell inhibits T1D in NOD mice by enhancing the migration of mature Ag-presenting DCs into the PLNs, where they recruit and cross-tolerize larger numbers of pathogenic T cells than normally occurs.

Discussion

It is widely believed that, in NOD mice, diabetogenic T cells are pathogenically primed in the PLNs before migrating to the islets. Most studies documented proliferation or up-regulation of activation markers by adoptively transferred β cell-autoreactive T cells in PLNs. However, these results should be interpreted with caution. It is now clear that activation of T cells does not always lead to their acquisition of effector functions. Indeed, several reports demonstrated T cells proliferate and express activation markers before they are tolerized (11, 43, 44). Particularly, autoreactive T cells exhibit activation characteristics when first encountering their cognate Ags in the PLNs, which subsequently leads to a protective tolerance induction rather than a destructive response (11, 44). The results obtained from our adoptive transfer and partial BM chimera experiments are in line with these observations. However, we cannot rule out the possibility that effector T cells capable of mediating β cell destruction in NOD mice normally quickly leave the PLNs upon their activation, and are only tolerized when retained at this site for a more prolonged period. In this case, it could be argued that tolerogenic retention of diabetogenic T cells in the PLNs of NOD mice is enhanced when increased numbers of mature DCs enter this site as a consequence of NKT cell activation.

Our results would seem to conflict with a previous report in which α-GalCer activation of NKT cells also induced DC maturation, but this switched them to an immunogenic rather than a tolerogenic status (15). However, it should be noted while CFA is normally used to boost immune responses to coadministered Ags in part through the induction of DC maturation, it efficiently blocks T1D development in NOD mice (45). In addition, ex vivo manipulated mature NOD DCs possess higher T1D-protective capacity over their immature counterparts (46, 47). It is possible mature DCs respond to self and foreign Ags differently depending on the microenvironment in which they reside and the signals they receive. Although NKT cell activation enhances immune responses to foreign Ags, it may also suppress autoimmune responses. This may be due to a differential ability of foreign and self Ags to induce Toll receptor signaling. The beneficial effects of α-GalCer on the prevention of autoimmune diseases may also be restricted to certain genetic backgrounds, such as NOD mice. In fact, it has been shown that α-GalCer administration to New Zealand Black/New Zealand White mice, a strain that develops spontaneous lupus, exacerbated their disease progression (48). NOD mice possess multiple defects in the innate immune system (18, 19). α-GalCer-mediated T1D protection in NOD mice appears to result from functional improvement of both DCs and NKT cells, two important populations bridging innate and adaptive immunity. In contrast, it has been reported that NKT cell activation at 10 wk of age is unable to inhibit T1D in IL-4-deficient NOD mice, and hence, protection at this late prodromal stage of disease development requires the induction of Th2 cytokines (49). However, we have found α-GalCer treatment initiated at 4 wk of age effectively inhibits T1D in the same stock of IL-4-deficient NOD stock used by the authors of this recent study (M. Clare-Salzer, Y. Naumov, S. Abrol, E. Paek, M. Osborne, Y.-G. Chen, D. Serreze, and S. B. Wilson, manuscript in preparation).

The roles of DCs in maintaining both central and peripheral tolerance are well established (8). Thymic DCs are sufficient to negatively select autoreactive T cells (50). In the periphery, DCs can cross-tolerize Ag-specific T cells in the absence of danger signals (8). Different DC subsets may carry out distinct functions (8). In contrast, it has been proposed that the developmental stages of DCs determine the outcome of Ag presentation, with mature DCs being immunogenic and immature DCs being tolerogenic (8). DCs in NOD mice do not mature normally (19, 20), but yet they develop spontaneous autoimmune T1D. The stimulation strength needed to induce T cell deletion by AICD is higher than that required to provoke immunological effector functions (51). Therefore, one plausible explanation is the majority of DCs in NOD mice are still capable of inducing immune responses, but lack an ability to trigger the deletion of diabetogenic T cells by AICD. Recently, it has been demonstrated that high expression levels of costimulatory molecules on DCs, a phenotype associated with their maturation, are required for the cross-tolerization of Ag-specific T cells (21). One major factor enabling DCs to induce immunity rather than tolerance is the presence of CD40 signaling (21). Taken together, the impairment of DC maturation in NOD mice might hinder their tolerogenic capacity. However, following NKT cell activation, the subsequently enhanced maturation state of NOD DCs could restore their ability to suppress β cell-autoreactive T cells.

In summary, we have demonstrated a cascade of events by which NKT cell activation leads to T1D protection in NOD mice. Activated NKT cells promote DC maturation and accumulation in the PLNs of NOD mice in part through a soluble factor(s). This is followed by a flux of T cells moving into PLNs, including diabetogenic clonotypes. Upon arrival in the PLNs, these pathogenic T cells undergo an abortive activation and are tolerized.

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