Activation of CD1d-restricted T cells protects NOD mice from developing diabetes by regulating dendritic cell subsets

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CD1d-restricted invariant NKT (iNKT) cells are immunoregulatory cells whose loss exacerbates diabetes in nonobese diabetic (NOD) female mice. Here, we show that the relative numbers of iNKT cells from the pancreatic islets of NOD mice decrease at the time of conversion from peri-insulitis to invasive insulitis and diabetes. Conversely, NOD male mice who have a low incidence of diabetes showed an increased frequency of iNKT cells. Moreover, administration of α-galactosylceramide, a potent activating ligand presented by CD1d, ameliorated the development of diabetes in NOD female mice and resulted in the accumulation of iNKT cells and myeloid dendritic cells (DC) in pancreatic lymph nodes (PLN), but not in inguinal lymph nodes. Strikingly, injection of NOD female mice with myeloid DC isolated from the PLN, but not those from the inguinal lymph nodes, completely prevented diabetes. Thus, the immunoregulatory role of iNKT cells is manifested by the recruitment of tolerogenic myeloid DC to the PLN and the inhibition of ongoing autoimmune inflammation.

CD1d-restricted invariant NKT (iNKT) cells are thought to regulate or be critical effectors for an extremely diverse set of immunologic responses (1). Despite the functional importance of iNKT T cells in these various responses, their mechanism of action has remained enigmatic. Human CD161+ Vα24JαQ T cells and the murine homologue, CD161+ Vα14Jα281+ T cells, are activated specifically by the nonpolymorphic class Ib molecule CD1d through presentation of a glycolipid antigen (2). Murine iNKT cells were first suggested to play an important role in initiating Th2 responses through the burst production of IL-4 on activation (1). However, studies of IL-4 receptor α-chain knockout mice as well as nonobese diabetic (NOD) mice have indicated that some of the immunomodulatory effects of these cells were not mediated by IL-4 (3, 4). An absolute requirement for iNKT cells in the generation of Th2 responses was excluded by the observation that mice whose CD1d locus was ablated by gene targeting retained the capacity to generate antigen-specific Th2 responses (5).

Dysfunction and/or diminished frequency of iNKT cells is clearly correlated with the development of autoimmunity in both rodents and humans (6–8). In several murine models of autoimmunity, CD1d-restricted T cells were shown to be present in diminished numbers and to decrease in frequency before the onset of disease (4, 9, 10). The importance of both frequency and function of iNKT cells was directly demonstrated by the protective effect seen after passive transfer of these cells or by the generation of transgenic NOD mice expressing the T cell antigen receptor (TCR) Vα14Jα281 α-chain (4, 6, 11). Conversely, diabetes was exacerbated by the introgression of a CD1d-null phenotype onto the NOD background (12).

Despite the major role demonstrated for iNKT cells in regulating autoimmunity, the precise functions of iNKT cells are incompletely understood. In murine autoimmune models, it is currently unclear in which tissue activation of iNKT cells is important and which antigen-presenting cells (APC) are responsible for this activation. In this regard, we and others recently showed that both human and murine CD1d-restricted cells express multiple cytokines, chemokines, and surface proteins other than IL-4 and IFN-γ that are critical to the maturation of myeloid dendritic cells (DC) (13, 14), suggesting that cross talk between DC and iNKT cells is an important effector function for these T cells (15). In the present study, the effect on the development of diabetes in the NOD mouse as well as the kinetics of tissue distribution for invariant Vα14Jα281 cells and tissue-specific changes in DC frequency after treatment with α-GalCer, an activating lipid presented by CD1d, were investigated.

Materials and Methods

Mice. Colonies of NOD/LtJ and nonobese-resistant (NOR)/LtJ female mice were maintained in the animal facilities of the Beth Israel Deaconess Medical Center and the University of Florida by using specific pathogen-free conditions.

Injection of Bioactive Lipids and Diabetes Assessment. Starting at 3–4 weeks of age, female NOD mice were given weekly injections of lipid or vehicle and followed for the development of diabetes. Female NOD mice were injected with 100 μg/kg α-GalCer in 0.5% Tween/PBS (KRN7000, Kirin Brewery, Gunma, Japan) or vehicle. Diabetes was assessed by blood glucose determination with two sequential measurements of >300 mg/dl separated by 3–7 days as identifying an animal as diabetic. Quantitation of T cell frequency and cytokine analysis after lipid therapy was done by using 5–6-week-old female NOD following injections on day 0 and day 4. The mice were injected with 100 μg/kg α-GalCer, 100 μg/kg α-ManCer (AGL595, Kirin Brewery), or vehicle.

FACS Analysis. Fluorescent staining of splenocyte populations was performed by using FITC-, phycoerythrin-, or PerCP-conjugated monoclonal antibodies to TCR, CD3, CD11c, CD11b, MHC class II, CD8α, CD1d, CD4, CD161, CD80, and CD86 obtained from PharMingen. Stained cells were analyzed on a FACScan flow cytometer (Becton Dickinson).

Abbreviations: iNKT, invariant NKT; NOD, nonobese diabetic; DC, dendritic cells; LN, lymph nodes; PLN, pancreatic lymph nodes; APC, antigen-presenting cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TCR, T cell antigen receptor; NOR, nonobese-resistant; LPS, lipopolysaccharide.

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Isolation of Lymphocytes and Pancreatic Islets. Splenocytes and lymphocytes from regional lymph nodes (LN) were isolated by dissection and used for FACS and cytokine secretion or homogenized TRIZOL (GIBCO/BRL) for RNA analysis. Lymph nodes were crushed and then dissolved in TRIZOL and stored at −80°C. RNA was isolated from 10–15 × 10^6 cells/spleen after filtering through a mesh strainer and removal of erythrocytes with red cell lysis buffer (Sigma). Pancreatic islet isolation was performed as previously described (16). Briefly, the common bile duce was identified, cannulated, and then infused with a solution of collagenase P (Boehringer Mannheim) (1 mg/ml in HBSS). The optimal digestion time was empirically determined for each lot of collagenase P, generally 16–19 min at 37°C. The islets from individual mice were washed three times in HBSS/5% FCS and filtered through a 40-mesh strainer. The washed pellet was diluted in 5 ml of histopaque 1077 (Sigma), overlaid with serum-free HBSS, and centrifuged; the resuspended islets were then hand picked by using a dissecting microscope (16).

cDNA Synthesis. Total RNA was isolated from pancreatic islets (30–120 islets/mouse), lymph nodes, and splenocytes from individual mice by using TRIZOL (GIBCO/BRL) according to manufacturers' recommendations. First-strand cDNA synthesis was performed by using oligo(dT) as a primer for reverse transcription of total RNA in a reaction mixture using Moloney murine leukemia virus–reverse transcriptase (Life Technologies, GIBCO-BRL) and as described (17).

Quantitative PCR Analysis. Quantitation of mRNA levels was done by using Real-time TaqMan Gold reverse transcriptase–PCR kits and run on an ABIprism 7700 sequence detector (Applied Biosystems). PCR primer sets were either purchased [glyceraldehyde-3-phosphate dehydrogenase (GAPDH), TCR β-chain] or designed according to manufacturer specifications. Primer sets were then optimized for input cDNA and reaction conditions as recommended. The primers used for Vα14Jα281 TCR α-chain were direct 5′-TGGATGACACTGCCACCTACAT-3′, reverse 5′-TCCAAAATGCAGCCTCCCTA-3′, and probe, 6FAM 5′-GGCTGAACCTCTATCGCCCACCAGA-3′ TAMRA. C_ot values for each message were determined and normalized to internal control GAPDH levels (TaqMan rodent GAPDH control reagents, Applied Biosystems). Control experiments demonstrated that GAPDH signal was unaffected by strain, treatment, or aging.

***In Vitro* Cytokine Recall Experiments.** Mice were injected on d0 and d4 with either vehicle alone (PBS/0.5% Tween) or increasing doses 2, 4, and 8 μg of α-GalCer i.p. On day 10 or 20, mice were killed. LNs were digested with collagenase D, and RBCs were lysed. At this point, cells were analyzed by FACS or plated at 2 × 10^6 cells per 900 μl in RPMI, 10% FCS and seeded into 24-well plates. Cells were then treated with vehicle or a 10× solution of α-GalCer as 10 mg/ml solution in 10% DMSO RPMI and incubated for 5 days. Cell culture supernatants were harvested and analyzed by ELISA for IL-4 or IFN-γ using the Quantikine capture and detection antibody pairs available from PharMingen. To determine IL-12 secretion, isolated lymph node cells were cultured with LPS and anti-CD40, and IL-12 secreted into the supernatant was assayed at 48 h.

Isolation and Transfer of Dendritic Cells. Dendritic cells were isolated from the pancreatic lymph nodes (PLN) and inguinal lymph nodes of 8–10-week-old NOD female mice by metrizamide centrifugation as previously described (18). Briefly, mechanically disrupted LN were passed through steel screens with gentle pressure and washed with RPMI 1640 plus 10% FCS. The cells were gently pipetted onto a 14.5% (wt/vol) metrizamide (Sigma) gradient. The gradients were then centrifuged at 600 × *g* for 15 min at room temperature. DC were collected from the interface and washed twice in PBS. For transfer, 50,000 cells were injected into the hind footpads of 4–6-week-old female mice. Isolated islets were harvested from 3–4-week-old female NOD mice by hand picking following intraductal injection of collagenase. The hand-picked islets were then sonicated on ice for 5 min and then rapidly freeze-thawed for one cycle. Preparations without viable cells were then frozen at −80°C until use. Freshly isolated inguinal DC were cultured with islet extracts (500 μg of protein/100,000 DC) at 37°C for 2 h and washed three times with PBS before injection.

Results

The Natural History of iNKT Cell Frequency in the Islets of NOD and NOR Mice. To address the question of whether iNKT cells are specifically recruited or lost from the islets during the progression to diabetes, islets and spleens were collected from female NOD and NOR mice and male NOD mice at 5, 10, and 15 weeks of age. NOR female mice and NOD male mice were chosen as controls because they are MHC congenic with NOD female mice, develop insulitis, but have a markedly reduced incidence of diabetes (19). After isolation of islets and spleens from individual animals, the relative frequency of tissue-specific mRNA transcripts encoding the Vα14Jα 281 TCR α-chain was determined by using real-time PCR (TaqMan) assays. The real-time PCR assay was developed because it allowed for the sensitive and specific *in situ* quantitation of invariant TCR transcripts, particularly in older female NOD mice, where there is a significant loss of islet number. It is estimated that up to 80% of iNKT cells respond to α-GalCer, and the vast majority of these cells are Vα14Jα281 T cells (20). Messenger RNA for Vα14Jα281 was found in the islets of female NOD, NOR, and male NOD mice at all time points examined but not in control mice bearing a CD1d-null or Jα281-null phenotype (data not shown). Interestingly, in female NOD mice, Vα14Jα 281 transcripts in the islets were found to decline over time, particularly during the transition period when the conversion from insulitis to destructive insulitis is occurring (Fig. 1*A*). Conversely, the islets of NOD male mice continued to accumulate Vα14Jα281 TCR mRNA over the same time period, whereas those of NOR female mice remained unchanged. Importantly, TCR β-chain transcripts in the islets of all of the mice studied continued to accumulate at the same rate (Fig. 1*B*). Thus, iNKT cells were either preferentially lost or failed to accumulate relative to total T cells in the islets of female NOD mice.

The CD1d/iNK T Cell Axis Regulates Diabetes Progression. Augmentation of iNKT cell frequency partially protects NOD female mice from diabetes (6, 11). To determine whether activation of iNKT cells *in vivo* could protect female NOD mice from diabetes, 3–4-week-old female NOD and CD1d-null NOD mice were treated with injections of 100 μg/kg α-GalCer or vehicle once a week. Treatment with α-GalCer significantly reduced the incidence and delayed the onset of diabetes only in the wild-type mice (Fig. 2). Diabetes was exacerbated in CD1d-null NOD females, a finding consistent with previous data (12), and importantly, α-GalCer treatment was ineffective in the CD1d-null mice. Thus, iNKT cells in NOD mice are important regulators of autoimmunity, and treating these mice with the activating glycolipid α-GalCer was protective.

Treatment with α-GalCer Specifically Recruits iNKT Cells to Pancreatic Islets and Lymph Nodes. To determine whether therapy with α-GalCer resulted in the recruitment of invariant Vα14Jα281 T cells to the islets and draining lymph nodes, their relative frequency in these tissues was determined. Six-week-old NOD mice were treated with α-GalCer, α-ManCer, or vehicle on day 0 and day 4, and then islets, spleens, and inguinal and pancreatic

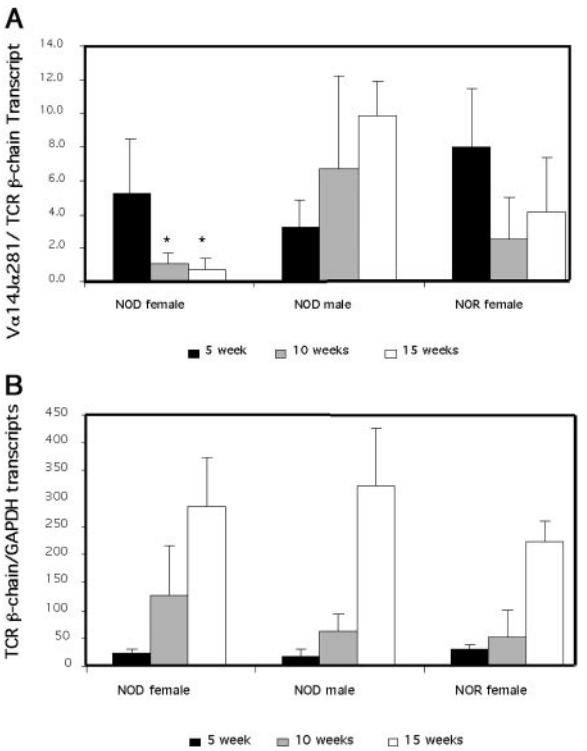

Fig. 1. Natural history of invariant iNKT cells. (*A*) Quantitation of Vα4Jα281 TCR transcripts in individual NOD/LtJ female, NOD/LtJ male, and NOR/LtJ female mice. cDNA prepared from pancreatic islets and spleens of mice aged 5, 10, and 15 weeks were assayed for TCR content using primer sets specific for the invariant TCR α-chain by Taqman assay and are reported as a ratio to total TCR β-chain content. (*B*) Quantitation of total TCR β-chain in islets isolated from NOD/LtJ female, NOD/LtJ male, and NOR/LtJ female mice that are reported relative to GAPDH internal controls. No invariant Vα14Jα281 TCR transcript was detected in RNA prepared from CD1d$^{(-/-)}$/NOD, or Ja281$^{(-/-)}$/BALB/c mice (data not shown).

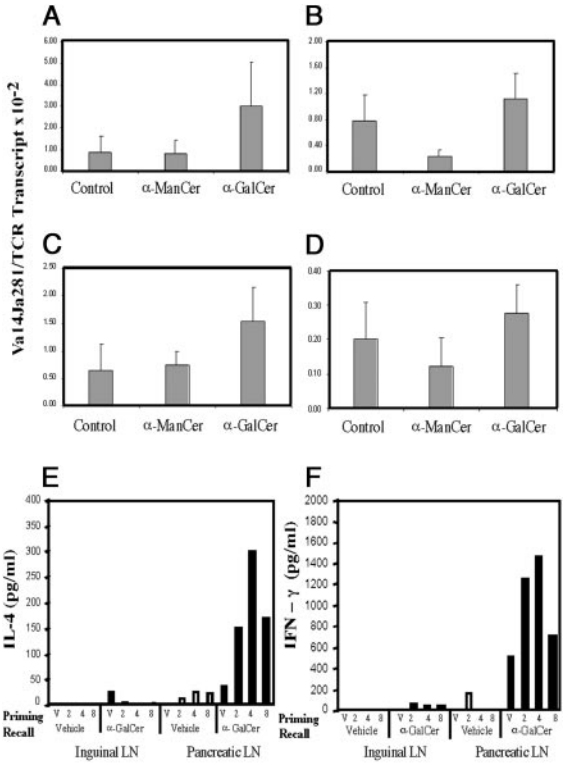

Fig. 3. Treatment with α-GalCer recruits iNKT cells to the islets and pancreatic lymph nodes. Relative frequency of invariant Vα14Jα281 TCR transcripts in pancreatic islets (*A*), spleen (*B*), pancreatic LN (*C*), and inguinal LN (*D*). Starting at 5–6 weeks of age, Female NOD/LtJ mice were treated with α-GalCer (100 μg/kg), α-ManCer (100 μg/kg), or vehicle as 2 i.p. at day 0 and day 4 (n = 10–13/treatment). On d10, islets and spleens were harvested from individual mice, and the relative frequency of invariant Vα14Jα281 TCR transcripts was determined as in Fig. 1. The frequency of invariant Vα14Jα281 TCR transcripts in islets ($P < 0.05$, Student's t test) and pancreatic LN ($P < 0.05$) was significantly increased relative to vehicle control after α-GalCer injection. (*A* and *C*) Priming of mice *in vivo* with α-GalCer augments the *in vitro* secretion of IFN-γ and IL-4 by α-GalCer. Female NOD mice were treated as above with vehicle or increasing doses, 2, 4, or 8 μg of α-GalCer. After 10 days, inguinal and pancreatic lymph nodes were then harvested and cultured *in vitro* with either vehicle or α-GalCer for 5 days, and levels of IL-4 (*E*) or IFN-γ (*F*) present in the culture supernatants were determined by ELISA. Similar results were seen on day 5 and day 20 (data not shown).

lymph nodes from individual mice were isolated on day 10. It should be noted that the islet isolation protocol, cannulation of the common bile duct with infusion of collagenase, and subsequent dilation and digestion of the pancreas *in situ* precluded the harvesting of both islets and pancreatic lymph nodes from the same animal. Therefore, both spleens and islets, or inguinal and pancreatic lymph node samples, were obtained from separate animals. Treatment with α-GalCer (but not α-ManCer) resulted in the accumulation of invariant Vα14Jα281 TCR transcripts only in the islets and pancreatic LN (Fig. 3 *A–D*). Furthermore, *in vivo* priming with α-GalCer augmented α-GalCer-induced *in vitro* IL-4 and IFN-γ secretion (Fig. 3 *E* and *F*). Paradoxically, injection of α-ManCer resulted in a decrease of iNK T cell frequency ($P < 0.01$) in the spleens relative to vehicle and α-GalCer-treated animals (Fig. 3*B*). The effect of α-GalCer therapy was specific for the islets and the lymph nodes draining the pancreas, as no treatment-related changes were seen in inguinal or mesenteric lymph nodes (Fig. 3 and data not shown). Therefore, the protective effect of α-GalCer therapy in NOD mice was associated with tissue-specific accumulation of iNKT cells in the islets and pancreatic LN.

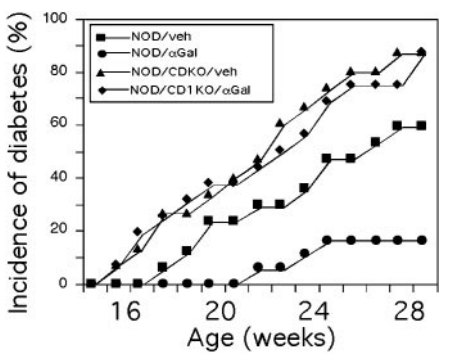

Fig. 2. Diabetes progression in NOD mice is regulated by iNK T cells. Female NOD/LtJ and CD1d$^{(-/-)}$/NOD mice (age 3–4 weeks) were treated with weekly injections of α-GalCer (100 μg/kg i.p., NOD/LtJ, n = 18; CD1d$^{(-/-)}$/NOD, n = 16) or vehicle (NOD/LtJ, n = 17; CD1d$^{(-/-)}$/NOD, n = 15). Treatment with α-GalCer significantly protected wild-type NOD females (P = 0.001, Kaplan–Meier method) from diabetes, whereas the introgression of the CD1d$^{(-/-)}$ phenotype exacerbated disease and rendered therapy with α-GalCer ineffective (P = 0.0002 for vehicle, P = 0.006 for α-GalCer treatment).

Treatment with α-GalCer Alters Dendritic Cell Subsets in Pancreatic Lymph Nodes. In the mouse, CD1d is preferentially expressed on CD11c+ CD8α+ dendritic cells relative to CD11c+ CD8α− subset (21). The CD8α+ subset is also thought to be a major

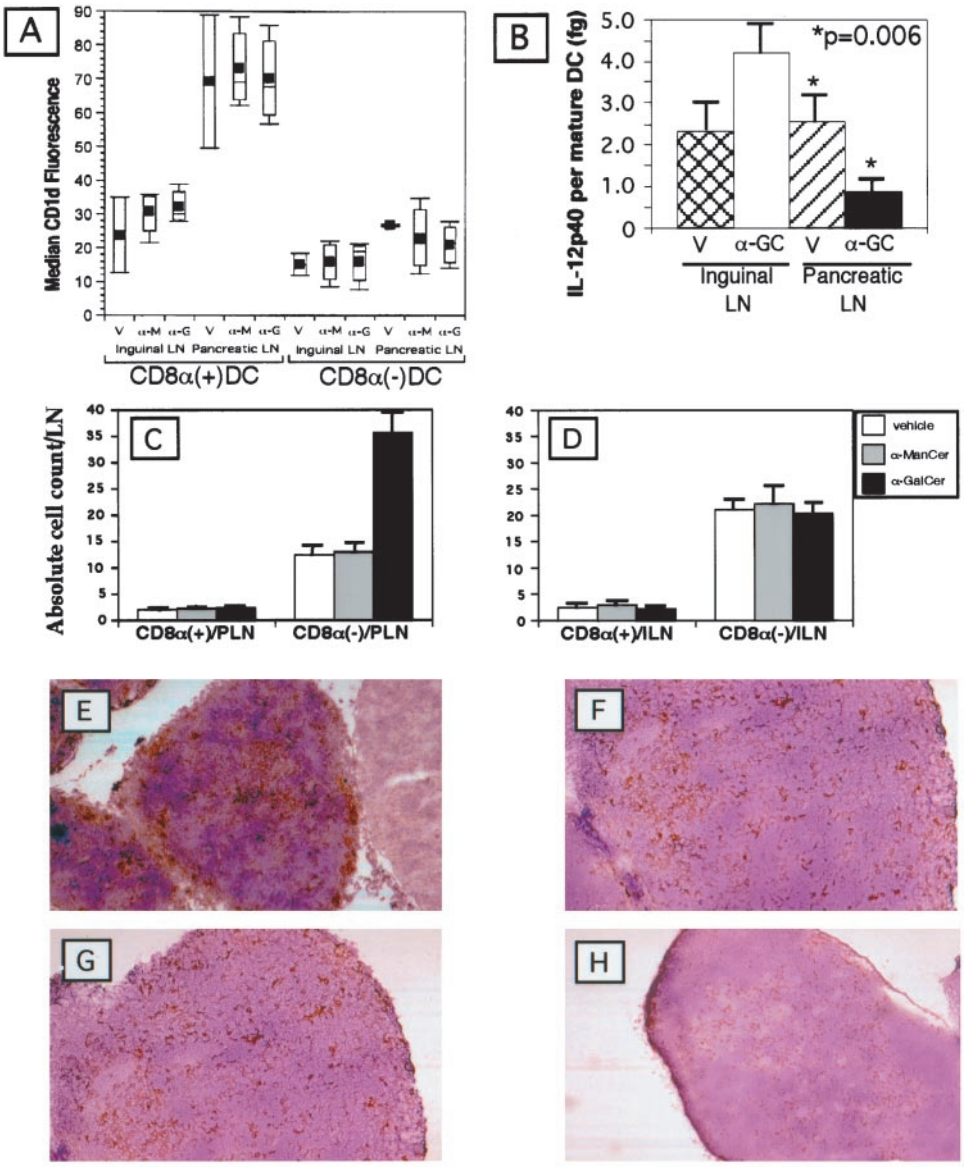

Fig. 4. Treatment with α-GalCer results in the preferential accumulation of myeloid CD8$\alpha-$/CD11c+ dendritic cells in the lymph nodes draining the pancreas. (*A*) CD1d is preferentially expressed on CD8$\alpha+$/CD11c+ dendritic cells in lymph nodes draining the pancreas and is unaffected by treatment. Five to six 6-week-old female NOD mice were treated as in Fig. 3, and inguinal and pancreatic lymph nodes were isolated. Median fluorescence for CD1d was determined on cells gated to be DC by FSC and SSC and CD11c+/MHC class II bright ($n = 6$ mice/group; data are shown as Box-and-Whisker plots; mean, $-$; median, filled bars; first and third quartiles, empty bars; error bars, standard deviation). (*B*) *In vitro* LPS-induced IL-12 secretion by PLN DC is suppressed by prior *in vivo* α-GalCer treatment. Mice were treated with vehicle, or α-GalCer (100 μg/kg), i.p. on day 0 and day 4 ($n = 6$/treatment, for a total of six experiments). On day 10, inguinal and pancreatic LN were harvested. Isolated cells were challenged with LPS/anti-CD40 (32) or LPS (data not shown) and incubated for 48 h. The amount of IL-12p40 in the culture supernatants was determined by ELISA, and the results are reported as normalized IL-12 secreted per total DC ($P = 0.006$, student's *t* test comparing vehicle with α-GC-treated PLN). DC frequency was determined by cell count and FACS. The absolute values for IL-12 were vehicle-treated inguinal LN, 408 $\pm$ 108 pg/ml; PLN 180 $\pm$ 64 pg/ml; and α-GalCer-treated inguinal, 580 $\pm$ 80 pg/ml; PLN 210 $\pm$ 35 pg/ml. (*C* and *D*) The total number ($\times 10^3$) of CD8$\alpha+$/CD11c+ and CD8$\alpha-$/CD11c+ DC in pancreatic and inguinal lymph nodes was determined 10 or 20 (not shown) days after mice were treated with vehicle, α-ManCer (100 μg/kg), or α-GalCer (100 μg/kg), i.p. on day 0 and day 4 ($n = 6$/treatment, for a total of six experiments). Treatment with α-GalCer resulted in significantly more CD8$\alpha-$/CD11c+ DC in the pancreatic lymph node ($P = 0.005$, student's *t* test) compared with vehicle. The various treatments did not alter the frequencies of CD8$\alpha+$/CD11c+ DC. No significant differences in mean fluorescent index for MHC class II, CD80, or CD86 was observed for the various treatments, and CD11c staining was not observed on T, B, or NK cells (data not shown). (*E–H*) Immunohistochemical staining for CD11c+ cells (brown deposits) in representative lymph nodes from mice treated with α-GalCer (*E*), α-ManCer (*F*), or vehicle (*G*). A representative inguinal node from an animal treated with α-GalCer is also shown (*H*). CD11c staining for vehicle-treated mice was identical to α-ManCer-treated mice, and isotype control staining was negative (data not shown).

source of *in vivo* IL-12 important for promoting Th1-like T cell responses and to cross-prime cytotoxic T lymphocyte responses (22). Conversely, transfer of CD8$\alpha(-)$ myeloid DC has been reported to be tolerogenic and suppress autoimmunity when transferred into NOD female mice (23). Because treatment with α-GalCer protected NOD mice from diabetes, and iNKT cells have previously been reported to interact with DC (14, 15), changes in dendritic cell subsets and CD1d expression were examined after α-GalCer treatment. Six-week-old female NOD mice were injected with α-GalCer, α-ManCer, or vehicle, and dendritic cell phenotypes were determined in various regional lymph node populations. Irrespective of treatment, CD1d was

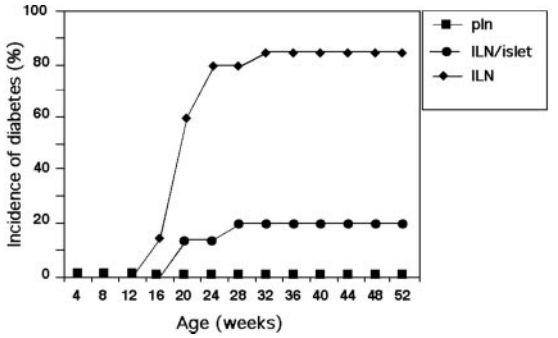

Fig. 5. Transfer of myeloid DC isolated from pancreatic LN protects female NOD mice from developing diabetes. Transfer of myeloid DC from pancreatic LN ($n = 6$ recipients) or inguinal DC cultured in media alone ($n = 15$ recipients) or loaded with sonicated islet cell preparations ($n = 15$ recipients) significantly ($P < 0.002$) protected NOD female mice from diabetes. Myeloid DC from either inguinal or pancreatic lymph nodes were isolated (18) from nondiabetic female NOD mice. Pancreatic DC were cultured in media alone, and inguinal DC were preincubated with media or sonicated islet preparations for 2 h before transfer. Individual NOD naïve female mice received 50,000 DC as a one-time injection in the footpad and were then followed for diabetes development. Control experiments demonstrated these DC preparations to be essentially pure populations of myeloid DC and that no viable cells remained after sonication of isolated islets (K.B., unpublished results, and data not shown).

expressed at levels 2–4-fold higher on the CD11c+ CD8α+ dendritic cells found in the pancreatic lymph nodes when compared with other CD8α− DC and to DC in the inguinal lymph node (Fig. 4*A*). Moreover, prior *in vivo* injection of α-GalCer significantly inhibited the capacity of pancreatic lymph node DC to secrete IL-12 in response to *in vitro* lipopolysaccharide (LPS) and anti-CD40 stimulation (Fig. 4*B*). Notably, α-GalCer injection resulted in a significant expansion of myeloid CD11c+ CD8α− DC only in the pancreatic lymph nodes (Fig. 4 *C* and *D*). The expansion of CD11c+ DC was readily observed by immunohistology, which revealed increased numbers of CD11c+ cells in the subcapsular sinus, indicative of recent migration from the tissue into the pancreatic LN (Fig. 4*E*). No expansion was seen in the inguinal lymph nodes or in pancreatic lymph nodes from α-ManCer or vehicle-treated animals (Fig. 4 *D* and *F–H*). Importantly, myeloid DC isolated from pancreatic LN of 8-week-old untreated mice completely abrogated the development of diabetes when transferred into naïve NOD prediabetic recipients (Fig. 5). Myeloid DC isolated from the inguinal LN were unable to do so unless preincubated with whole islet preparations. Therefore, mature myeloid DC previously exposed to whole islets are tolerogenic, and their accumulation in the pancreatic LN was augmented by α-GalCer therapy.

Discussion

CD1d-restricted T cells regulate a wide array of immune responses (6, 24–27). Human CD1d-restricted T cells have been suggested to regulate immune responses by controlling the maturation and function of myeloid DC subsets (13, 14). To investigate this mechanism as a potential regulatory function for iNKT cells in NOD mice, the tissue-specific frequency of invariant Vα14Jα281 T cells and DCs seen after activation of this family of T cells was determined.

Invariant Vα14Jα281 T cells were lost from the islets of Langerhans of NOD female mice, but not in NOR female or NOD male mice, during the transition to invasive insulitis. Transition through this checkpoint is thought to be a key step toward the destructive phase of autoimmunity (28). These results extend other work noting the loss of iNKT cells from spleens and thymi of NOD females and associate the loss of these cells in the islets with disease progression and their accumulation with the sex-biased resistance seen in male NODs (4, 6, 9).

The *in vivo* modulation of iNKT cells with the activating ligand α-GalCer, or by the introgression of a CD1d-null phenotype, had significant impact on the course of diabetes development and is in close agreement with other published results (12, 29). Treatment with weekly injections of α-GalCer significantly reduced and delayed the development of diabetes only in mice expressing CD1d, whereas diabetes was exacerbated in mice whose CD1d locus had been deleted (Fig. 3). The protection by α-GalCer was associated with an early α-GalCer-specific increase in the frequency of invariant Vα14Jα281 TCR transcripts and myeloid DC only in the islets and lymph nodes draining the pancreas (Fig. 4).

Injection of mice with α-GalCer results in burst secretion of IL-4 and IFN-γ by iNKT cells (2). Interleukin-4 and IFN-γ are not the only effector cytokines secreted by iNKT cells, as these cells secrete or express on their cell surface a large panel of gene products important for the recruitment and differentiation of macrophages and myeloid dendritic cells (13). An obligate interaction with dendritic cells was also demonstrated for IL-12-dependent tumor surveillance by iNKT cells (15, 27). In fact, iNKT cells of NOD mice were impaired in their ability to respond to *in vitro* activation with IL-12 (4). Notably, defective activation is a hallmark of iNKT cells derived from mice and humans with autoimmune diabetes (9, 13). Thus, α-GalCer therapy seems to overcome the hyporesponsiveness of iNKT cells in NOD mice and is sufficient to activate the regulatory functions of these cells.

Interestingly, a genetic link between the ability of self-reactive T cells to directly induce IL-12 secretion from APCs and autoimmunity was recently reported (30), and the chronic administration of IL-12 to NOD mice markedly accelerated diabetes by driving the development of Th1-biased autoreactive T cells (31). Activated dendritic cells are thought to be a major source of *in vivo* IL-12 (32). Moreover, dendritic cells infiltrate the islets very early in the inflammatory response and are important for the initiation and maintenance of disease (33, 34). However, transfer of mature myeloid dendritic cells has also been reported to prevent diabetes in the NOD mouse (23). Moreover, when transgenic mice were generated that expressed ovalbumin or influenza HA protein in pancreatic islet β cells, bone marrow-derived APC restricted to the lymph nodes draining the pancreas, presumably DC, were required for the generation of peripheral tolerance (35).

The recent identification of dendritic cell subsets that differentially regulate T cell responses may explain some of the conflicting results regarding dendritic cells and diabetes. In the mouse, the CD11c+, CD11b−, CD8α+ subset is the major source of IL-12 secreted by dendritic cells, and this subset expresses higher levels of CD1d compared with the CD11c+, CD11b+, CD8α−, CD11C+, CD11b−, CD8α− populations (21, 32). The CD8α+ DC subset promotes Th1-biased immune responses, prevents the development of peripheral tolerance, and cross-primes cytotoxic T cells *in vivo*, whereas the two CD8α− DC subsets are believed to promote Th0 or Th2-like responses and do not cross-prime cytotoxic T lymphocytes (22, 36). Correspondingly, the phenotype of the dendritic cell subsets that transferred protection in the NOD mouse was mature myeloid CD8α− DC, the same cells whose frequency was augmented in the pancreatic lymph nodes of NOD mice treated with α-GalCer (18, 23).

Two functions of iNKT cells could explain the change in the ratio of CD8α+ to CD8α− dendritic cells. First, this family of T cells secretes a panel of cytokines and chemokines that have been shown *in vitro* to be important for the recruitment and differentiation of myeloid DC, i.e., the CD8α− population (13, 37, 38). Consistent with this prediction, activation of iNKT cells with α-GalCer resulted in a preferential accumulation of this

subset of DC in the pancreatic LN. The accumulation of DC in this lymph node, particularly in the subcapsular sinus space, likely results from migration from the pancreas and islets. Second, iNKT cells express perforin and granzymes and, on activation, become potently cytolytic. Perhaps the preferential expression of CD1d on the CD8α+ subset of DC renders them more sensitive to deletion by cytolysis. A similar mechanism for the regulation of human DC1 subsets by cytolysis has been proposed (14).

The diminution of IL-12 secretion after *in vitro* LPS and anti-CD40 stimulation of the pancreatic LN DC following *in vivo* priming with α-GalCer, combined with the changes seen in the pancreatic LN DC subsets, suggests that IL-12 secretion and differentiation to mature myeloid DC are regulated events. This interpretation is consistent with emerging data demonstrating that DC produce IL-12 only for brief periods of time (32, 39). Moreover, DC effector function depends on the state of myeloid DC differentiation and the nature of the DC-activating signal, e.g., coculture of inguinal DC with islet preparations (40). Interestingly, APC/DC function in NOD mice is impaired when compared with those of NOR mice (41). Thus, in this system, control of DC subsets or activation state regulates antigen-specific tolerance.

In summary, iNKT cells can be found in significant numbers at the site of inflammation and the draining lymphatic tissue. Activation with α-GalCer or deletion of CD1d, respectively, protects or exacerbates diabetes in the NOD mouse. Therapy with α-GalCer specifically recruited iNKT cells to the islets and the draining pancreatic lymph nodes of female NOD mice. Additional studies examining the effect of the introgression of CD1d-null phenotype on the function of DC in NOR mice and male NOD mice are underway. Associated with the recruitment and activation were local changes in relative frequency of tolerogenic dendritic cell subsets such as the CD11c+, CD8α− subset. Thus, iNKT cells seem to regulate diabetes by locally controlling the frequency and function of DC subsets.

Note Added in Proof. Additional evidence for the prevention of diabetes by the administration of α-GalCer was recently reported (42, 43).

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