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J Immunol 2004; 172:5908-5916; doi: 10.4049/jimmunol.172.10.5908
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Up-Regulation of CD1d Expression Restores the Immunoregulatory Function of NKT Cells and Prevents Autoimmune Diabetes in Nonobese Diabetic Mice

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The immunoregulatory function of NKT cells is crucial for prevention of autoimmunity. The prototypical NKT cell Ag α-galactosylceramide is not present in mammalian cells, and little is known about the mechanism responsible for NKT cell recruitment and activation. Up-regulation of CD1d, the NKT cell restriction molecule, expressed on mononuclear cells infiltrating the target organ, could represent the physiological trigger for NKT cells to self-contain T cell immunity and to prevent autoimmunity. Recognition of CD1d, either by itself or bound to self-ligands (selfCD1d), could drive NKT cells toward an immunoregulatory phenotype. Hence, ineffective NKT cell-mediated immunoregulation in autoimmune-prone individuals including nonobese diabetic (NOD) mice could be related to defective signals that regulate CD1d expression at time and site of autoimmunity. To test this hypothesis, we transgenically overexpressed CD1d molecules under the control of the insulin promoter within the pancreatic islets of NOD mice (insCD1d). Recognition of overexpressed CD1d molecules rescued NKT cell immunoregulatory function and prevented autoimmune diabetes in insCD1d transgenic NOD mice. Protection from diabetes was associated with a biased IL-4-secreting cytokine phenotype of NKT cells and alteration of the cytokine microenvironment in the pancreatic lymph nodes of transgenic mice. The net effect was a reduced development of the autoimmune T cell repertoire. Our findings suggest that up-regulation of CD1d expression during inflammation is critical to maintain T cell homeostasis and to prevent autoimmunity. The Journal of Immunology, 2004, 172: 5908–5916.

N natural killer T cells represent a nonconventional T lymphocyte subset carrying markers of both T and NK cells and expressing an invariant TCR α-chain (Vα14Jα18 in mice and Vα24JαQ in humans) (1, 2). NKT cells are regulatory T cells that play a dual role in modulation of T cell immunity. On one hand, NKT cells can actively induce T cell tolerance and prevent autoimmunity. This NKT cell function was originally suggested by the observation that a defect of NKT cells correlates with susceptibility to autoimmune diseases in mice (3–8) and humans (9–13). The key immunosuppressive role of NKT cells was further proven by the finding that NKT cells are responsible for development of systemic tolerance when Ags are administered through the eye (14). Moreover, expansion of the NKT cell repertoire in Vα14 TCR transgenic mice (15) or NKT cell activation by administration of their model Ag α-galactosylceramide (αGalCer)3 protected mice against autoimmune diseases such as experimental autoimmune encephalomyelitis (16–18) and type 1 diabetes (19, 20). In contrast, there is convincing evidence that NKT cells belong to innate immune networks and play a key role in front-line defense against parasites (21, 22), bacteria (23, 24), viruses (25, 26), and antitumor responses (27, 28) by inducing an inflammatory cytokine environment. The observed dual role of NKT cells leads to speculation that their function is balanced between the necessity to build an effective inflammatory immune response against pathogens and prevention of autoimmunity. The NKT cell decision to differentiate toward an inflammatory or immunosuppressive pathway could depend upon the specifics of CD1d Ag recognition. The nonclassical MHC class I-like molecule CD1d functions as restriction molecule for NKT cells and is critical for their maturation and activation of their model Ag α-galactosylceramide (αGalCer)3 protected mice against autoimmune diseases such as experimental autoimmune encephalomyelitis (16–18) and type 1 diabetes (19, 20). In

3 Abbreviations used in this paper: αGalCer, α-galactosylceramide; NKTCR, NKT cell receptor; GAD65, glutamate dehydrogenase; IA2, anti-insulin Ab; DC, dendritic cell; PLN, pancreatic lymph node; IDDM, insulin-dependent diabetes mellitus; int, intermediate.

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The Journal of Immunology

Received for publication August 13, 2003. Accepted for publication March 1, 2004. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 M.F. is supported by a Young Faculty Grant from Fondazione Centro San Raffaele del Monte Tabor. G.C. is supported by CEF Grant QLK2-CT-2001-01205.
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3 Abbreviations used in this paper: αGalCer, α-galactosylceramide; NKTCR, NKT cell receptor; GAD65, glutamate dehydrogenase; IA2, anti-insulin Ab; DC, dendritic cell; PLN, pancreatic lymph node; IDDM, insulin-dependent diabetes mellitus; int, intermediate.
expression or lower the threshold of NKT cell receptor (NKTcr) activation. αGalCer, as a strong agonist of the NKTcr, provided a sufficient signal to rescue NKT cell function in autoimmune-prone individuals, possibly by mimicking the NKT self-ligand and restoring defective NKTcr activation (16–20). Similarly, overexpression of CD1d molecules could restore NKT cell-mediated immunoregulation. To test this hypothesis, we transgenically overexpressed murine CD1d molecules within the pancreatic islets of NOD mice under the control of the human insulin promoter (insCD1d NOD mice). Female NOD mice spontaneously develop a disease similar to human type 1 diabetes (35) and carry a functional defect of the NKT cell/CD1d immunoregulatory pathway that has been clearly linked to the genesis of autoimmune diabetes (3–5). We found that recognition of selfCD1d up-regulated within an inflammatory environment (i.e., pancreatic islets of female insCD1d NOD mice) activated NKT cells and rescued their immunoregulatory function, thus preventing autoimmune diabetes. In our model, selfCD1d-induced NKT cells showed a biased IL-4-secreting phenotype, and protection from autoimmune diabetes was associated with increased expression of regulatory cytokines within the pancreatic lymph nodes (PLN) of transgenic insCD1d NOD mice. The net effect of NKT cell immunoregulation was the inhibition of autoimmune T cells development. Our data suggest that recognition of selfCD1d molecules leads NKT cells toward an immunosuppressive pathway facilitating control of T cell responses and counterregulation of autoimmunity.

Materials and Methods
Generation of transgenic mice
Transgenic mice expressing CD1d within β cells of pancreatic islets were generated according to methods described previously (36). Briefly, a 1860-bp fragment encompassing murine CD1d cDNA was released from pHB500o and subcloned into the inspro plasmid vector to place expression under the transcriptional control of the human insulin promoter. The 4.3-kb transgene construct was linearized, purified, and microinjected into pronuclei of NOD/shi fertilized eggs at the Transgenic and Embryonic Stem Cell Facility of The Scripps Research Institute. The presence of transgene in insCD1d NOD mice was verified by PCR typing of tail DNA. Three founders had integrated copies of the transgene encoding CD1d in their genome. Of these, two transmitted the transgene to the progeny. These two lines showed similar levels of CD1d expression within pancreatic β cells and incidence of diabetes. One of those two lines was expanded and used in the experiments shown.

Animal husbandry
Transgenic insCD1d NOD mice and their negative littermates were bred and housed together in a specific pathogen-free environment in the animal facility of The San Raffaele Research Institute. NOD.Scid mice were obtained from Charles River Laboratories (Calco, Milan, Italy), handled under sterile conditions in a laminar flow hood, and housed in sterile microisolator cages. All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee of The San Raffaele Research Institute.

Adoptive cell transfer
Prediabetic 14- to 16-wk-old insCD1d and age-matched negative NOD mice were used as splenocyte donors. Spleens were meshed on a nylon screen and depleted of RBC by hypotonic lysis with NH4Cl. A total of 5 × 106 splenocytes was injected i.v. into 8- to 12-wk-old NOD.Scid recipients. Blood glucose values were determined weekly starting at 2 wk after transfer.

Clinical evaluation of diabetes
Diabetes was assessed by measuring venous blood glucose concentrations in nonfasting mice with Glucometer Esprit Strips (Bayer, Pittsburgh, PA). Mice were considered diabetic after two consecutive measurements of >250 mg/dl.

Histology
For immunohistochemical staining, mice were sacrificed, and pancreata were excised, embedded in optimal cutting temperature medium, and snap-frozen in liquid nitrogen. To determine CD1d expression, cryosections were stained with rat anti-murine CD1d mAb clone 1B1 (BD Pharmingen, La Jolla, CA) or rat IgG isotype. For insulin staining, pancreata were fixed in 10% formalin, embedded in paraffin, and sectioned (4 μm). Pancreatic sections (at five points per organ) were stained with H&E, and at least 40 islets per pancreas were analyzed and scored for insulin. Islets were ranked free of insulitis (intact islets), affected by peri-insulitis (mononuclear cells in the connective tissue around the islets), and affected by insulitis (mononuclear cell infiltration of >20% of the islet).

In vitro antigen stimulation of NKT cells
Spleens and PLN were excised and reduced to a single-cell suspension by meshing on a nylon screen. Cells were incubated either with a synthetic form of αGalCer (KRN7000; kindly provided by Kirin Brewery, Gunma, Japan) at 100 ng/ml or vehicle alone in culture medium (RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin/streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, and 50 μM 2-ME) in 96-well round-bottom microplates (Nunc, Roskilde, Denmark). For clonal expansion of NKT cells, PLN cells and splenocytes were cultured for 7 days in complete RPMI 1640 with αGalCer (100 ng/ml), IL-7, and IL-15 (R&D Systems, Minneapolis, MN), as described previously (37).

Measurement of T cell and Ab autoimmune responses
For measurement of T cell autoimmunity, spleens from female 8- to 10-wk-old insCD1d transgenic and negative NOD mice were teased through nylon mesh. Single-cell suspensions were counted and plated at 105 cells/well in well rounded-bottom 96-well microplates in HL-1 serum-free medium (BioWhittaker, Walkersville, MD) supplemented with 100 U/ml penicillin/streptomycin, 2 mM glutamine, and 50 μM 2-ME. Glutamate decarboxylase (GAD65) was produced in SF9 insect cells by a baculovirus system, affinity-purified with a Ni2+/nitrilotriacetic resin (Qiagen, Chatsworth, CA), and added to the cell cultures at 20 μg/ml. The primary cultures were kept at 37°C and 5% CO2 for 5 days; 1 μC of [3H] thymidine was added in the last 18 h of culture; and incorporation of radioactivity was measured by liquid scintillation counting. Anti-insulin Abs (IAA) were measured by radiobinding assay as previously described (38). Sera were obtained from peripheral blood collected from the tail veins of 10- to 14-wk-old NOD mice. 125I-Labeled insulin (Amersham, Buckinghamshire, U.K.) of 20,000 cpm was incubated with 5 μl of serum with or without cold human insulin in 25 μl of TBT buffer (50 mMol/L Tris and 1% Tween 20 (pH 8.0)) for 3 days at 4°C. Ab was subsequently captured by adding 1 mg of protein A-Sepharose (Pharmacia, Peapack, NJ) and 6 μl of protein G (gamma chain) and incubated at 4°C for 1 h with shaking. Tubes were then washed five times with 1 ml of cold TBT, and pellets counted in a gamma counter. The titers were calculated on the basis of the difference in cpm in parallel with and without cold insulin (Δcpm) and expressed as arbitrary units interpolated from a standard curve using human serum in the range of 3–800 arbitrary units.

Generation and stimulation of dendritic cells (DC)
To derive DC from bone marrow, femurs and tibiae of NOD and C57BL/6 mice were collected, and cells were flushed from the bone marrow cavity with cold RPMI 1640. After washing in RPMI 1640/5% FCS, cells were cultured in six-well culture plates for 6 days at 5 × 105 cells/well in the presence of GM-CSF and flt3L (10 ng/ml; R&D Systems). To determine the effect of maturation stimuli on CD1d expression of cultured DC, LPS (1 μg/ml), IL-1β (10 ng/ml), and TNF-α (10 ng/ml) were added to the bulk DC cultures for 72, 48, or 24 h before cytometric analysis.

Flow cytometric analysis
Percentages of NKT cells in PLN, pancreata, and spleens, and expression of CD1d on DC of NOD and C57BL/6 mice were determined by flow cytometry. PLN, spleens, and pancreata were collected at 6–8 wk of age and meshed through a nylon screen. Single-cell suspensions were treated with mAb against FcyR (kindly supplied by Dr. P. DellaPenna (San Raffaele Research Institute, Milan, Italy)) and stained with FITC-conjugated anti-TCR-β (BD Pharmingen) and PE-conjugated empty or αGalCer-loaded CD1d tetramers for 90 min at 23°C (39). DC cultures were extensively washed with cold PBS, 1% FCS, and 0.01% NaN3, and stained with FITC-conjugated mAbs anti-CD11c and PE-conjugated anti-CD1d (BD Pharmingen). Dead cells were excluded by cyanine nucleic acid staining with TO-PRO-3 (Molecular Probes, Eugene, OR). Data were acquired on a FACSCalibur cytometer and analyzed by CellQuest software (both from BD Biosciences, San José, CA).
Cytokine secretion by ELISA

Supernatants were collected from NKT cell cultures stimulated with αGalCer after 48 h and 7 days of culture. Supernatants from GAD65-stimulated T cells were collected after 48 h. ELISA 96-well plates (Nunc) were coated with primary mAbs (BD PharMingen) anti-murine IL-4 (clone 11B11) at 4 µg/ml and anti-murine IFN-γ (clone R46A.2) at 2 µg/ml in carbonate buffer and incubated overnight at 4°C. Wells were treated for 4 h with blocking solution (PBS/Tween, 10% FCS, and 1% BSA), and the samples were added to the wells at two 1-fold dilutions and incubated 18 h at 4°C. Biotinylated secondary Abs anti-IL-4 and anti-IFN-γ at 1 µg/ml and streptavidin-peroxidase plus H2O2-based developing system were used to detect the amount of cytokines. The detection levels were enhanced with the use of extravidin and biotinylated anti-avidin Ab (clone WC19.10; Sigma-Aldrich, St. Louis, MO). The concentrations of IL-4 and IFN-γ were interpolated from a standard curve using recombinant murine IL-4 and IFN-γ linear in the range of 20–800 pg/ml.

Tissue expression of cytokines by RT-PCR

Total RNA was extracted from spleen, lymph nodes, and pancreata of nontransgenic and transgenic mice using TRIzol reagent. RNA was reverse transcribed using Superscript II RT and oligo(dT)12-18 primer (Invitrogen, Carlsbad, CA). The amount of cDNA in each sample was normalized after PCR for murine β-actin gene. The PCR mix consisted of 10 mM Tris (pH 8.3), 1.5 mM MgCl2, 50 mM KCl, 0.2 mM dNTPs, and 1.25 U of Platinum TaqDNA Polymerase (all reagents from Invitrogen). Primers used for PCR were as follows: β-actin, sense, 5'-GTG GCC GGC TCT AGG CAC CAA-3', antisense, 5'-CTC TTT GAT TGC ACG CAC GAT TTC-3', amplifying a 539-bp product; IL-4, sense, 5'-CCA GCT AGT TGT CAT CCT GGT TTC TTT CTT-3', antisense, 5'-CAG TGA TGT GGA CTT GGA CTC ATT TCA TGG TGC-3', amplifying a 584-bp product; and IL-10, sense, 5'-ATG CAG GAC TTT AAG GGT TAC TTG GGT T-3', antisense, 5'-ATT TCG GAG AGA GGT ACA AAC GAG GTT T-3', amplifying a 455-bp product. PCR were performed on PerkinElmer (Wellesley, MA) 2400 thermal cycler as follows: 94°C for 2 min followed by 35 cycles at 94°C for 40 s, 64°C for 20 s, and 72°C for 40 s. RT-PCR were resolved by electrophoresis in a 2% agarose gel containing ethidium bromide (Amersham Pharmacia Biotech, Piscataway, NJ).

Statistical analysis

Data were expressed as mean ± SEM. Statistical analyses were performed with Student’s unpaired t tests. Incidence of diabetes was determined with Wilcoxon log rank test. Values of p < 0.05 were considered statistically significant.

Results

Up-regulation of CD1d expression within pancreatic islets protected insCD1d NOD mice from autoimmune diabetes

CD1d molecules are normally expressed on different cell subsets including T cells, B cells, DC, and macrophages. CD1d-expressing DC are able to induce NKT cell activation and expansion (40, 41). Hence, NKT cell recognition of selfCD1d expressed by DC infiltrating the target site of autoimmunity could generate an NKT cell-mediated immunoregulatory circuit to down-regulate and self-limit inflammation. DC derived from bone marrow of NOD mice either immature or matured in the presence of inflammatory stimuli such as LPS, IL-1, or TNF-α expressed lower levels of CD1d compared with DC of nonautoimmune C57Bl/6 mice (Fig. 1). This observation suggested that ineffective NKT cell activation and expansion in autoimmune diabetes-prone individuals may be related to defective regulation of CD1d expression on APCs such as DC infiltrating the target site of autoimmunity. To test whether CD1d up-regulation at the site of autoimmunity could rescue NKT cell immunoregulatory function in autoimmune-prone mice, we generated insCD1d transgenic NOD mice that ectopically expressed murine CD1d within pancreatic β cells under the control of the insulin promoter. Transgenic expression of cell surface Ags and restriction molecules within the pancreatic islets is an efficient tool to recruit specific T cell subsets and assess their role in the pathogenesis of autoimmune diabetes (36, 42). Ectopic expression of CD1d on pancreatic β cells could resemble physiological up-regulation of CD1d on DC infiltrating the site of inflammation and specifically trigger NKT cell-mediated immunoregulation. As expected, we found normal constitutive expression of CD1d in the endothelial wall of small blood vessels and collecting ducts of NOD pancreata (Fig. 2A). Fig. 2B shows that the CD1d molecule was highly expressed in the islets of Langerhans of insCD1d transgenic NOD mice. Up-regulation of CD1d was selectively induced within pancreatic islets of insCD1d transgenic mice, and constitutive levels of CD1d were found in thymus, spleen, and lymph nodes of transgenic and negative NOD mice (data not shown). To determine whether overexpression of CD1d molecules at the site of autoimmunity counterregulates the pathogenesis of autoimmune diabetes, we evaluated incidence of diabetes in female insCD1d transgenic NOD mice and sex-matched negative littermates by weekly measurements of blood glucose levels starting at 10 wk of age. The onset of diabetes was strongly delayed in the transgenic group (p < 0.01) (Fig. 3A). Moreover, the incidence of diabetes was significantly lower in the insCD1d transgenic NOD mice compared with their negative littermates (37 vs 80%; p < 0.05). Insulitis indexes performed on pancreatic sections confirmed that autoimmune attack to β islets was minimal in insCD1d transgenic mice compared with their negative littermates (Fig. 3B). Interestingly, pancreata of transgenic insCD1d NOD mice showed some degree of peri-insulitis, with lymphocytes surrounding the islets without destroying them (Figs. 2B and 3B). This histological picture
was previously found in other models of NKT cell protection from autoimmune diabetes, suggesting that nonpathogenic mononuclear cells populate pancreatic islets of protected mice (20). Reduced pathogenesis in the T cell compartment of protected insCD1d NOD mice was confirmed by inability of transgenic splenocytes to transfer diabetes into NOD-Scid recipients (Fig. 3C).

**FIGURE 2.** Transgenic insCD1d NOD mice overexpressed CD1d within β cells of pancreatic islets. insCD1d NOD mice were generated by inserting a 1860-bp DNA fragment encompassing murine CD1d gene under the transcriptional control of the human insulin promoter. Transgenic progeny was screened by PCR typing of tail DNA with primers specific for the HBS sequence contained in the transgene, and expression of murine CD1d gene product was confirmed by immunohistochemical analysis. Cryosections of frozen pancreata from 8-wk-old nontransgenic (A) and insCD1d transgenic (B) NOD mice were stained with mAb anti-murine CD1d (clone 1B1). Normal islets did not constitutively express CD1d; however, low levels of expression were found on lymphocytes infiltrating the islets of female NOD mice starting at 8 wk of age (black arrows). Original magnifications, ×20.

**NKT cells were rendered unresponsive by CD1d-induced activation at the target site of autoimmunity**

To test whether protection from autoimmune diabetes in insCD1d transgenic mice was due to activation of immunoregulatory NKT cells within the site of autoimmunity, we analyzed percentages and phenotype of NKT cells from transgenic and negative NOD mice. We performed a FACS analysis on total lymphocytes isolated from pancreata and PLN of the two groups of mice at 6–8 wk of age. In accordance with previous reports (20), we measured extremely low numbers of NKT cells among lymphocyte infiltrates within pancreatic islets of either transgenic or nontransgenic NOD mice (0.05% in transgenic and 0.03% in nontransgenic NOD mice) (data not shown). The percentages of NKT cells were slightly increased in PLN of transgenic mice compared with nontransgenic littermates (p > 0.05) (Fig. 4A). One possible explanation for this finding is that NKT cells are recruited and activated to the target site of autoimmunity by CD1d overexpression and rapidly move toward the draining PLN where they can critically affect generation of the autoimmune T cell repertoire (43, 44). To verify whether selfCD1d recognition activated NKT cells, we analyzed the activation state of NKT cells within draining PLN. Circulating NKT cells normally carry most activation markers of T cells (1). Recent reports indicated that two specific markers of activation, CD40L and CD95, are found exclusively on TCR-activated NKT cells (45, 46). FACS analysis detected comparable levels of those markers on NKT cells from PLN and splenocytes of transgenic and negative female NOD mice (data not shown). In vivo studies suggest that NKT cells behave like classical T cells that rapidly die or become unresponsive following TCR-mediated stimulation to limit excessive clonal expansion (39, 47). We found that NKT cells from transgenic PLN responded poorly in vitro to antigenic stimulation with αGalCer compared with NKT cells from spleens of the same mice (p < 0.001) or from PLN of negative littermates (p < 0.001) (Fig. 4B). IL-15 is a key cytokine for NKT cell growth that prevents activation-induced cell death and favors clonal expansion of NKT cells in vitro (37). NKT cells from PLN of transgenic insCD1d mice failed to expand in response to Ag stimulation with αGalCer in the presence of IL-15, whereas the same cells from negative littermates and from spleen expanded normally (Fig. 4C). Lack of proliferative response and expansion of NKT cells from PLN but not spleen of transgenic mice indicated that unresponsiveness of NKT cells was limited to the site of autoimmunity. Those results suggested that NKT cells from transgenic mice were locally activated by CD1d overexpression within pancreatic tissues and became unresponsive to further stimulation.
CD1d up-regulation affected the generation of the autoimmune T cell repertoire

The mechanisms underlying NKT cell-mediated protection from autoimmune diseases are still unclear. A recent report suggested that NKT cells play their immunoregulatory role by inhibiting the priming of autoimmune T cells rather than the aggressiveness of effector autoreactive T cells (48). To test whether NKT cells triggered by pancreatic CD1d overexpression affected the autoimmune T cell repertoire in transgenic insCD1d NOD mice, we measured primary response of total splenocytes against rGAD65. We used the response to GAD65 autoantigen as a parameter of T cell autoreactivity, because the presence of GAD65-specific T cells has been integrally linked to diabetes pathogenesis in NOD mice (49). Reduced proliferative response against GAD65 in insCD1d NOD mice demonstrated that, in our transgenic model, reduction of diabetes incidence was integrally related to the inhibitory effect that NKT cells play on priming of autoreactive T cells (Fig. 5A). Conversely, humoral autoimmunity measured by IAA was up-regulated in transgenic insCD1d mice compared with normal NOD mice (60% TG+ vs 11% TG−; p < 0.01) (Fig. 5B). This result indicates that, in our transgenic model, a shift from cellular to humoral autoimmunity (lack of anti-GAD65 T cell response and increased IAA positivity) was associated with protection from diabetes.

Recognition of CD1d overexpressed at the site of autoimmunity differentiated NKT cells toward an IL-4-secreting phenotype

Several lines of evidence suggested that NKT cells could mediate their regulatory function through secretion of cytokines such as IL-4 (16, 19, 20). In accordance, in our transgenic model, protection from diabetes correlated with a biased IL-4-secreting phenotype of NKT cells populating PLN of transgenic insCD1d mice (Fig. 6A). This observation reinforces the hypothesis that immunoregulatory NKT cells secrete a larger amount of IL-4 once activated; however, it is still unclear how NKT cells affect T cell autoimmunity. One possibility is that IL-4 released by NKT cells dampened the diabetogenic potential of autoimmune T cells by skewing their cytokine phenotype from an inflammatory Th1 to a protective Th2 type (50). However, when we looked at cytokine profiles of autoreactive T cells, we did not find a shift toward a protective Th2 type in transgenic mice. GAD65-specific T cells from transgenic insCD1d NOD mice did not secrete significant amounts of IL-4. Despite low proliferation in response to GAD65 stimulation, we measured secretion of IFN-γ in short-term cultures of splenocytes from transgenic mice. However, secretion of IFN-γ was lower than that of normal NOD splenocytes (Fig. 6B) in accordance with the reduced number of precursors we found in primary GAD65-stimulated cultures (Fig. 5A) and with their reduced diabetic potential (Fig. 3C). Protection from diabetes in

confirmed by histopathological analysis of pancreata section from insCD1d transgenic and negative NOD mice showing a lower degree of insulitis (■) in transgenic compared with negative NOD mice at 14 wk of age. No difference in the degree of peri-insulitis (□) between the two group of mice was observed. Pancreata from 8- and 14-wk-old mice were formalin-fixed and stained with H&E. The analysis was performed on five to six mice per group. C. Splenocytes of insCD1d transgenic mice contained a lower number of autoreactive T cells than their negative counterpart and were unable to transfer diabetes into immunodeficient NOD recipients. A total of 5 × 10⁶ splenocytes isolated from 12- to 16-wk-old insCD1d transgenic (□) or negative (■) NOD mice were injected i.v. into NOD.Scid recipients (eight to nine mice per group). Diabetes incidence was evaluated by glycemia measurements starting at 2 wk posttransfer.

FIGURE 3. Up-regulation of CD1d expression counterregulated autoimmune diabetes in insCD1d transgenic NOD mice. A, Diabetes incidence was assessed on the progeny of one transgenic insCD1d line. Glycemia was measured weekly starting at 10 wk of age by using Glucometer Elite strips on venous blood. Animals were considered diabetic after two consecutive blood glucose measurements starting at 2 wk posttransfer. Diabetes incidence was integrally related to the inhibitory effect that NKT cells play on priming of autoreactive T cells (Fig. 5A). Conversely, humoral autoimmunity measured by IAA was up-regulated in transgenic insCD1d mice compared with normal NOD mice (60% TG+ vs 11% TG−; p < 0.01) (Fig. 5B). This result indicates that, in our transgenic model, a shift from cellular to humoral autoimmunity (lack of anti-GAD65 T cell response and increased IAA positivity) was associated with protection from diabetes.

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Discussion

NKT cells play a key role in suppression of autoreactive T cells and prevention of autoimmune diseases. This hypothesis was originally suggested by the observation that selective defects in NKT cell number and cytokine phenotype are present in individuals affected by different autoimmune diseases such as insulin-dependent diabetes mellitus (IDDM), multiple sclerosis, systemic lupus erythematosus, systemic sclerosis, rheumatoid arthritis, and inflammatory bowel disease (10–13, 51–53). A recent report by using highly specific CD1d tetramer-based methodology showed that the frequency and cytokine profile of NKT cells are conserved during the course of IDDM (54). In accordance, our data showed that overexpression of CD1d within pancreatic islets of NOD mice protected from autoimmune diabetes through activation of NKT cells and improvement of IL-4 secretion at the site of autoimmunity (i.e., PLN) rather than by increasing frequency of NKT cells. Those findings weakened the hypothesis that a defect of NKT cell number and cytokine phenotype underlies the pathogenesis of autoimmune diabetes. However, there is still strong evidence that CD1d-reactive NKT cells play a suppressor function against autoreactive T cells and are crucial to limit inflammation and prevent autoimmunity. Specific activation of NKT cells by the glycolipid Ag αGalCer protected mice against autoimmune disease (diabetes and encephalomyelitis) (16–20). In addition, lack of NKT cells in CD1d knockout NOD mice led to exacerbation of IDDM (55). Our data, showing that overexpression of CD1d within pancreatic islets prevented autoimmune diabetes in NOD mice, suggest the alternative hypothesis that ineffective NKT cell immunoregulation in autoimmune-prone individuals is not related to an intrinsic NKT cell defect of frequency and cytokine phenotype but rather to defective signals that regulate CD1d expression at the site of tissue damage.

The hypothesis that NKT cells are crucial to secrete IL-4 and down-regulate T cell autoimmunity is apparently contradicted by evidence that they improve T cell immunity against infections and antitumoral immunity through secretion of inflammatory, Th1-type cytokines (21–28). Specifics of CD1d recognition could be important for the NKT cell decision to differentiate toward an...
immunoregulatory rather than inflammatory pathway. The observation that NKT cells isolated from cord blood have an activated/memory phenotype (56) suggests that those cells are stimulated even before birth by recognition of self molecules. In vivo NKT cells interacted with CD1d-expressing cells in the absence of foreign Ags and mediated regulatory mechanisms for suppression of inflammatory responses (14, 32). In our transgenic model, immunoregulatory NKT cells were activated to prevent autoimmune diabetes by recognition of overexpressed CD1d molecules in the absence of foreign Ags. This suggests that the key event for NKT cell activation and differentiation toward an immunoregulatory

**FIGURE 5.** CD1d-reactive NKT cells affected generation of the autoimmune T cell repertoire. A, insCD1d transgenic mice lacked a spontaneous response to GAD65 autoantigen. Splenocytes from 8- to 10-wk-old insCD1 transgenic NOD mice and their transgenic littermates were cultured with or without recombinant murine GAD65 at 20 µg/ml, and proliferation was measured as the mean [3H]thymidine incorporation. Data are expressed as mean cpm ± SEM from two triplicate experiments with two to three animals individually tested in each experiment. B, Transgenic mice showed a shift from cellular to humoral autoimmunity. Sera samples were collected from venous blood of 8- to 12-wk-old transgenic (n = 10) and negative (n = 18) female NOD mice. The average level of IAA among triplicate samples was determined by RIA assay (radioimmunoassay). Each point represents an individual mouse. IAA levels were significantly higher in the insCD1d transgenic group compared with their negative littermates (p < 0.05), and the percentage of IAA-positive mice was increased in the transgenic group (60 vs 11% in the negative group).

**FIGURE 6.** CD1d overexpression induced IL-4 secretion by NKT cells and altered the cytokine microenvironment at the site of autoimmunity. A, NKT cells from PLN of insCD1d transgenic NOD mice showed an increased secretion of IL-4 compared with the same cells from negative NOD mice. Splenocytes and PLN of insCD1d transgenic (TG+) and negative NOD (TG−) were reduced to single-cell suspension and in vitro stimulated with αGalCer (100 ng/ml) and IL-15 (10 ng/ml). Supernatants were collected after 7 days, and cytokine secretion was measured by ELISA. Data are expressed as mean picograms per milliliter ± SEM from two duplicate experiments with four animals used in each experiment. B, Transgenic insCD1d NOD mice expressed down-modulatory cytokines at the site of autoimmunity. Spleens (S), PLN, and pancreata (P) from insCD1d transgenic (S+) and negative littermates (S−) were snap frozen in liquid nitrogen, and RNA was extracted by TRIzol reagent, reverse transcribed, and amplified with specific primers for indicated cytokines. Down-modulatory cytokines, such as IL-4 but also IL-10, were detected in the PLN of insCD1d transgenic mice but not in their negative littermates. One of two representative experiments is shown. C, Cytokine profiles of autoreactive T cells did not shift toward the protective Th2 type. GAD65-specific T cells from transgenic insCD1d NOD mice did not secrete significant amounts of IL-4, although, in accordance with their reduced diabetogenic potential, they released slightly less IFN-γ. Data are expressed as mean picograms per milliliter ± SEM from three duplicate experiments.
pathway could be recognition of CD1d molecules overexpressed by mononuclear cells and complexed with endogenous ligands. Although the nature of those endogenous ligands is unknown, there is some evidence that immunoregulatory NKT cells recognize self-sphingolipids such as ganglioside and ceramide (57). There is evidence that immunoregulatory circuits are triggered by alteration of tissue homeostasis (limited T cell death early on in diabetogenesis protected against autoimmunity; Ref. 58). Hence, it is possible that regulatory NKT cells are activated by recognition of those endogenous ligands released by damaged tissues (dying by necrosis or apoptosis) and presented by overexpressed CD1d molecules. In this view, αGalCer could prevent autoimmune diseases by sharing a structural homology and mimicking the yet-unidentified mammalian glycolipid that serves as natural endogenous ligand for CD1d molecules. In course of infection, foreign Ags could compete with endogenous ligands for CD1d binding and induce inflammatory features upon NKT cells. Alternatively, the NKT cell decision to differentiate toward regulatory or inflammatory cells depends upon the type of CD1d-expressing cells within the target site of autoimmunity. Specifically, NKT cells could lose their inflammatory potential and acquire regulatory properties when they recognize selfCD1d molecules in the absence of costimulatory signals on immature DC or nonprofessional APCs (59) such as pancreatic β cells in our transgenic model.

The mechanisms responsible for NKT cell-mediated counterregulation of autoimmunity are still unclear. Recognition of selfCD1d molecules could drive NKT cells toward a protective pathway either by up-regulating secretion of regulatory cytokines and/or by turning off their inflammatory potential. Evidence so far collected pointed out that down-modulatory cytokines such as IL-4 and IL-10 play a key role in NKT cell-mediated protection against autoimmune diseases (5). In accordance with those reports, we found that regulatory NKT cells from transgenic mice released large amounts of IL-4, and the cytokine microenvironment at the site of priming for the autoreactive T cell repertoire was altered by CD1d overexpression. Protection from autoimmune diabetes in insCD1d NOD mice was related to lack of proliferation together with the inability to transfer diabetes of autoreactive T cells. However, those dysfunctional features of autoreactive T cells were not associated with a biased cytokine profile from an inflammatory Th1 to an IL-4–secreting Th2 type. Moreover, we found up-regulation of regulatory cytokines such as IL-4 and IL-10 also in spleen of transgenic mice where NKT cells did not show a biased IL-4–secreting phenotype. We concluded that alteration of the cytokine microenvironment was not directly linked to NKT cell secretion of IL-4. Previous studies have shown that NKT cells activated by αGalCer administration secreted both IL-4 and IFN-γ and yet counterregulate autoimmune diseases (17–20). In our model, regulatory NKT cells locally activated by selfCD1d recognition secreted a larger amount of cytokines, particularly IL-4 and also IFN-γ, but they did not necessarily function through secretion of IL-4. In fact, NKT cells could play their regulatory function by modulating different cell populations such as DC (60) or other regulatory T cell subsets that are directly responsible for alteration of the cytokine microenvironment. Systemic activation of NKT cells by a model Ag such as αGalCer induced both adjuvant/inflammatory and regulatory features upon NKT cells (18, 41). To design therapies that specifically improve NKT cell immunoregulation in autoimmune-prone individuals, it is necessary to understand the physiological mechanism that drives NKT cells toward a regulatory pathway. In this study, we show that recognition of CD1d molecules overexpressed at the site of autoimmunity generated an NKT cell-mediated immunoregulatory circuit critical for maintenance of T cell homeostasis and prevention of autoimmunity.

Acknowledgments

We thank Enrique Rodriguez, Jae Lee, and Cristina Belloni for technical assistance; Kirin Brewery for providing KRN7000; Mitchell Kronenberg and Stéphane Sidobre for providing murine αGalCer/CD1d tetramers; Massimo Degano and Paolo Dellabona for advice and stimulating discussion; and Massimo Degano for critical reading of the manuscript.

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