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NK T Cell-Induced Protection Against Diabetes in Vα14-Jα281 Transgenic Nonobese Diabetic Mice Is Associated with a Th2 Shift Circumscribed Regionally to the Islets and Functionally to Islet Autoantigen[1]

Véronique Laloux, Lucie Beaudoin, Dirk Jeske,[2] Claude Carnaud, and Agnès Lehuen[3]

The onset of autoimmune diabetes is related to defective immune regulation. Recent studies have shown that NK T cells are deficient in number and function in both diabetic patients and nonobese diabetic (NOD) mice. NK T cells, which are CD1d restricted, express a TCR with an invariant Vα14-Jα281 chain and rapidly produce large amounts of cytokines. Vα14-Jα281 transgenic NOD mice have increased numbers of NK T cells and are protected against diabetes onset. In this study we analyzed where and how NK T cells interfere with the development of the anti-islet autoimmune response. NK T cells, which are usually rare in lymph nodes, are abundant in pancreatic lymph nodes and are also present in islets. IL-4 mRNA levels are increased and IFN-γ mRNA levels decreased in islets from diabetes-free Vα14-Jα281 transgenic NOD mice; the IgG1/IgG2c ratio of autoantibodies against glutamic acid decarboxylase is also increased in these mice. Treatment with IL-12 (a pro-Th1 cytokine) or anti-IL-4 Ab abolishes the diabetes protection in Vα14-Jα281 NOD mice. The protection from diabetes conferred by NK T cells is thus associated with a Th2 shift within islets directed against autoantigen such as glutamic acid decarboxylase. Our findings also demonstrate the key role of IL-4. ***The Journal of Immunology*****, 2001, 166: 3749–3756.**

Nonobese diabetic (NOD)[4] mice are widely used as a model for type 1 diabetes, because they spontaneously develop manifestations very similar to those of the human disease (1). Specific elimination of β cells is preceded by pancreatic islet infiltration by myeloid cells and T and B lymphocytes. Peri-insulitis begins at 3–4 wk of age in NOD mice. The insulitis then becomes invasive and destructive, leading to diabetes from 12 wk of age. Many studies, including transfer experiments with immunoincompetent newborn or *scid* NOD mice have shown that T cells are critical for disease development (2).

However, islet infiltration by T cells does not always lead to overt diabetes. Several protective mechanisms involving T cells have been described in the last decade (reviewed in Ref. 3). Treatments depleting certain T cell subsets, such as thymectomy at weaning, accelerated diabetes development (4). Conversely, transfer of thymocytes or splenocytes from prediabetic mice prevents diabetes onset (5). Several laboratories, including our own, have recently shown that NK T cells, a regulatory subset, can protect against diabetes (6, 7).

NK T cells express $\alpha\beta$ TCR and certain cell surface markers associated with the NK cell lineage (reviewed in Ref. 8). These peculiar T cells have a relatively limited repertoire, as they are biased toward Vβ8 and use an invariant α-chain (Vα14-Jα281) (9). NK T cells, which are either $CD4^+$ or $CD4^-CD8^-$ double negative, are selected on CD1, a nonclassical MHC class I molecule (10), and recognize glycolipids such as glycosylceramides (11, 12) and GPI-anchored Ags (13, 14). NK T cells rapidly produce large amounts of IL-4 and IFN-γ after triggering of their TCR (15–17). They are remarkably conserved through mammalian evolution. They have been implicated in protection against various infections by bacteria such as *Listeria* (18) and by parasites such as *Toxoplasma gondii* (19) and *Plasmodium* (20), and against tumor invasion and metastasis (11, 21, 22). Several reports suggest that the onset of insulin-dependent diabetes mellitus is associated with an NK T cells defect (6, 7, 23–25). Both NOD mice and patients with insulin-dependent diabetes mellitus have subnormal numbers of NK T cells, which are also functionally deficient in IL-4 production.

The NK T cell defect involved in diabetes has been analyzed in transgenic NOD mice overexpressing the invariant α-chain characteristic of these cells. Indeed, Vα14-Jα281 transgenic NOD mice possessing increased numbers of NK T cells were partially protected against diabetes onset. Transfer and cotransfer experiments with transgenic splenocytes showed the ability of NK T cells to regulate the development of pathogenic T cells from normal NOD mice. Splenocytes from Vα14-Jα281 NOD mice released large amounts of IL-4 after anti-CD3 stimulation both in vivo and in vitro, and baseline serum IgE levels were spontaneously elevated. Importantly, the comparison of several Vα14-Jα281 NOD lines revealed a positive correlation among the number of NK T cells, the level of IL-4 secreted, and the efficiency of protection against diabetes (7).

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[4] Abbreviations used in this paper: NOD, nonobese diabetic; GAD, glutamic acid decarboxylase.

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Here, we investigated how NK T cells influence the immune system of Vα14-Jα281 NOD mice, particularly the differentiation of autoreactive cells, by comparing wild-type and transgenic NOD mice. We screened for NK T cells in various organs (including pancreatic lymph nodes and islets); measured IL-4, IFN-γ, and IL-10 transcripts in the spleen, pancreatic lymph nodes, and islets; characterized anti-glutamic acid decarboxylase (anti-GAD) Ab isotypes; analyzed the role of the local Th2 shift in the protection by injecting IL-12 (a pro-Th1 cytokine); and analyzed the role of IL-4 by injecting anti-IL-4 mAb. We found that the diabetes protection conferred by NK T cells was associated with a shift toward Th2 within islets and directed against autoantigens such as GAD. Our findings also demonstrated the key role of IL-4.

Materials and Methods

Mice

The Vα14-Jα281 transgenic NOD line 86 was produced by microinjection of NOD eggs. Vα14-Jα281 line 86, Vα14-Jα281 line 86 $C\alpha^{-/-}$, and congenic NOD.NK1.1 mice have been described previously (7). We used heterozygous transgenic mice, because they provide perfect controls within the same litters. Transgenic and negative littermates on the NOD background were used for functional studies, and NK 1.1 congenic NOD were used for immunofluorescence analysis. All the mice used in this study were raised and housed in strictly controlled specific pathogen-free conditions.

Preparation of pancreatic islets

Mice were killed, and pancreases were dissected free from surrounding lymph nodes. Pancreases were minced with scissors into solution A (PBS, 5% FCS, and 0.06% glucose). Fragments were collected by sedimentation, and 1 ml of PBS containing 15% FCS, 0.06% glucose, and 4 mg/ml collagenase P (Roche, Mannheim, Germany) was added. The tissues were digested at 37°C for 3–4 min with vigorous shaking and were extensively washed with solution A. Islets were hand-picked under an inverted microscope. For immunofluorescence analysis, islet-infiltrating cells were purified mechanically.

Flow cytometry

Islet-infiltrating cells and cell suspensions from the spleen and various lymph nodes were prepared and stained at 4°C in PBS containing 1% BSA and 0.1% azide after blocking Fcγ receptors by incubation with 2.4G2 and aggregated human IgG. Staining was performed with FITC-conjugated anti-TCRαβ (H57 mAb) and PE-conjugated anti-NK1.1 (PK136; PharMingen, San Diego, CA), and islet-infiltrating cells were also stained with biotinylated anti-Thy-1 (30H12 mAb) plus streptavidin-APC (PharMingen) for more accurate gating of T cells. Stained cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) using CellQuest software.

ELISA-PCR assay

Purified islets were lysed in 400 μl of RNABle (Eurobio, Les Ulis, France). As the main source of variability in PCR RNA quantification is not the RT or PCR steps but, rather, the amount of starting material and RNA quality, all the samples were tested in duplicate. Each islet lysate was divided into two equal parts, and 20 μl of chloroform was added to all samples. After 15-min incubation at 4°C, samples were centrifuged, and RNA was precipitated in the presence of 2 μl of Pellet Paint (Novagen, Madison, WI). In parallel, RNA was prepared from 5×10^5 splenocytes and pancreatic lymph node cells. RNA was reverse transcribed (Promega, Madison, WI) into cDNA and then analyzed for cytokine mRNA expression in a kinetic ELISA-PCR method as previously described (26). Briefly, the amplification step is conducted with a pair of primers specific for each cytokines; one primer is biotinylated. The PCR products are captured on avidin-coated microplates and denatured by alkaline treatment. The captured DNA strands were hybridized with FITC-labeled probes. The amount of probe is then measured by an alkaline phosphatase-coupled anti-FITC Ab, and 1,2-dioxetane chemiluminescent substrate (CSPD, Tropix, Perkin-Elmer Applied Biosystems, Foster City, CA) and luminescence enhancer (Sapphire II, Tropix, Perkin-Elmer Applied Biosystems) are added for luminescence detection. mRNA corresponding to a specific T lymphocyte gene, the TCR β-chain gene, was measured as a reference in each sample. Primers and probes were the following: β (5′biotin-AAAAGGCTACCCTCGTGGCTTG-3′; 5′-GAACTGCACTTGGCAGCGGAA-3′ and 5′fitc-TGGCAGGGAAGAAG CCC-3′), IL-4 (5′biotin-CGGCATTTTGAACGAGTCACAGG-3′; 5′-ACTTGGACTCATTCATGGTGCAGC-3′ and 5′fitc-GCTGTGAGGACG TTTGGC-3′), IL-10 (5′biotin-TTGTAGCACCTTGGTCTTGGAGC-3′; 5′-GGTTGCCAAGCCTTATCGAAATG-3′ and 5′fitc-GGCAGTGGAGCAG GTGAA-3′), and IFN-γ (5′biotin-CCTCATGGCTGTTTCTGGCTGTTA-3′; 5′-CATTGAAGCTTGG CGCTGGACC-3′ and 5′fitc-CAATGACTGTGC CGTGGC-3′).

Recombinant mouse GAD65

Recombinant GAD65 protein was produced in SF9 insect cells by a baculovirus expression system and was further purified first by Ni^{2+} affinity and then by preparative SDS-PAGE followed by electroelution.[5] In brief, baculovirus-infected SF9 cells were lysed with Gu/HCl buffer (6 M guanidine/HCl, 100 mM PO_4 buffer, 10 mM Tris-HCl, and Pefabloc (Uptima Interchim, Montlucon, France), pH 8.0), and the lysate was incubated with ProBond Ni^{2+} Resin (Invitrogen, Gronimgen, The Netherlands). The beads were washed with urea buffer (8 M urea, 100 mM PO_4 buffer, and 10 mM Tris-HCl) starting at pH 8.0. The pH was gradually lowered, and GAD65 was eluted at pH 4.5. Fractions were analyzed by 8% SDS-PAGE and GAD65-containing fractions were concentrated in a VivaSpin device (VivaScience, Lincoln, U.K.). For further purification, GAD65 was processed by 8% SDS-PAGE. The band corresponding to GAD65 was visualized by negative zinc/imidazole staining (Bio-Rad, Hercules, CA) and cut out. The gel slices were destained in electroelution buffer (25 mM Tris, 193 mM glycine, and 0.025% SDS), and the protein was eluted from the gel by using the BioTrap electroelution chamber (Schleicher & Schuell, Dassel, Germany). The gel-purified protein was dialyzed (24 h in 25 mM Tris, 193 mM glycine, and 0.1% SDS; then 24 h in 25 mM Tris, 193 mM glycine, and 0.01% SDS; then 24 h in IMDM), then sterilized and quantified by 8% SDS-PAGE with BSA as reference. GAD65 was stored at 4°C.

GAD-specific T cell responses

Cell suspensions were prepared from mesenteric lymph nodes, and 2×10^6 cells were incubated in flat-bottom 96-well plates with IMDM (Glutamax) containing 10% FCS and 25 μg/ml of recombinant GAD65 protein. As a positive control, cells were incubated in wells precoated with anti-CD3 mAb (2 μg/ml). IL-4 and IFN-γ released into the supernatants after 48 h of culture were measured by ELISA methods as previously described (7).

Serum Ig isotype levels

Serum IgG1 was measured with a standard ELISA method. A specific polyclonal Ab against IgG1 (Southern Biotechnology Associates, Birmingham, AL) was used for coating. Sera were diluted from 1/10,000 to 1/80,000, then alkaline phosphatase-conjugated anti-IgG (Sigma, St. Louis, MO) was added for detection. Serum IgG2c was measured with a competitive ELISA method using IgG2c mAb CBPC101 for coating. CBPC101 myeloma cells were generated by M. Potter (National Cancer Institute, Bethesda, MD). For the competitive step, biotinylated anti-IgG2c mAb 5.7.2 (27) was added to wells containing diluted serum (1/5,000 to 1/40,000). After 2 h of incubation, streptavidin-alkaline phosphatase (Amersham International, Les Ulis, France) was added for detection. mAb CBPC101 and mAb 5.7.2 were gifts from Dr. L. Majlessi (Pasteur Institute, Paris, France).

OVA immunization

Thirteen-week-old mice were injected s.c. into hind footpads with 100 μg of OVA in saline emulsified with CFA (Difco, Detroit, MI). Two weeks later, mice were reinjected s.c. in the same footpads with 100 μg of OVA in saline emulsified with CFA. Mice were bled 9 wk after the first injection.

Measurement of GAD- and OVA-specific Abs

Serum IgG1, IgG2c, and IgE specific for GAD or OVA were measured with standard ELISAs. GAD or OVA (Sigma) at a concentration of 10 μg/ml in 0.1 M carbonate buffer (pH 9.6) was used to coat the plates. Diluted sera were incubated overnight at 4°C, and biotinylated polyclonal anti-IgG1 (Southern Biotechnology Associates), biotinylated anti-IgG2c mAb 5.7.2, or biotinylated anti-IgE mAb LO-ME-2 (Biosys, Compiegne, France) was added; streptavidin-alkaline phosphatase (Amersham International, Les Ulis, France) was used for detection.

[5] D. Jeske, K. Jensen, L. Beaudoin, H.-J. Fehling, P. van Endert, R. C. Monteiro, E. E. Sercarz, J.-F. Bach, H. von Boehmer, and A. Lehuen. Expression of GAD65 in NOD B cells does not alter the incidence of diabetes. *Submitted for publication.*

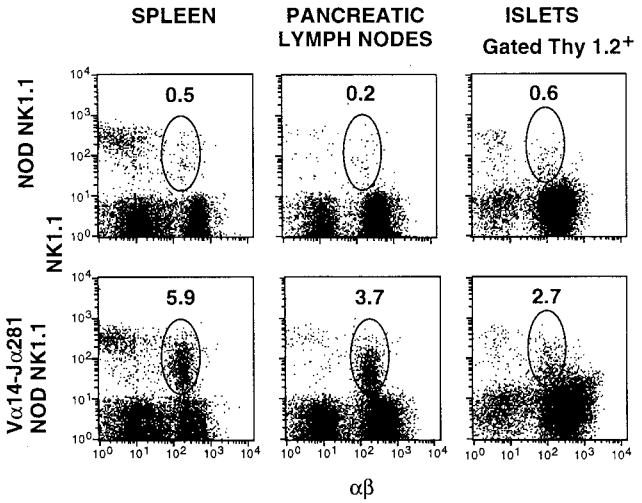

FIGURE 1. Increased NK T cell numbers in spleen, pancreatic lymph nodes, and islets of Vα14-Jα281 transgenic NOD mice. Cells from a 14-wk-old Vα14-Jα281 NOD NK1.1 mouse and a littermate control were either dually stained with anti-TCR-$\alpha\beta$ and anti-NK1.1 or triply stained with anti-Thy-1.2, anti-TCR-$\alpha\beta$, and anti-NK1.1. The numbers represent the percentages of NK T cells among total organ cells or Thy1.2$^+$ islet cells.

In vivo IL-12 treatment

Recombinant mouse IL-12 (gift from Dr. R. O'Hara, Genetics Institute, Cambridge, MA) was diluted in PBS containing 1% syngenic NOD mouse serum. Nine-week-old females were injected daily i.p. with IL-12 at 0.15 μg/20 g (28) or with vehicle alone (PBS containing 1% NOD serum). Mice were tested every day for diabetes. Overt diabetes was defined as three consecutive positive glucosuria tests (Glukotest, Roche, Indianapolis, IN). For flow cytometry and cytokine mRNA measurement by kinetic PCR, females were injected i.p. with 0.15 μg/20 g of IL-12 for 5 and 7 days, respectively.

Cyclophosphamide and Ab treatments

Eight- to 10-wk-old females were injected i.p. with 200 mg/kg cyclophosphamide (day 0). 11B11 (an IgG1 mAb specific for IL-4) or JES5 (an IgG1 mAb specific for IL-10) was injected at a dose of 0. 5 mg/mouse on days -1, 0, 2, and 5. Mice were tested every day for diabetes.

Statistical analysis

Differences in cytokine production were analyzed using Student's *t* test. The incidence of diabetes was studied using the log rank test.

Results

Elevated NK T cell numbers in various lymphoid organs and within islets of Vα14-Jα281 NOD mice

We have previously reported that Vα14-Jα281 NOD mice overexpress NK T cells and are partially protected against spontaneous diabetes (50 vs 90% in normal NOD mice) and totally protected against cyclophosphamide-induced diabetes. Moreover, Vα14-Jα281-expressing NK T cells protect against the disease when coinjected with diabetogenic T cells (7). To determine where NK T cells are present in Vα14-Jα281 NOD and could therefore interfere with the development of autoreactive T cells, we screened for NK T cells by immunofluorescence of pancreatic draining lymph nodes and cells infiltrating pancreatic islets, spleen, and distant lymph nodes. As expected, Vα14-Jα281 mice had increased percentages of NK T cells in all organs analyzed relative to their transgenic negative littermates (Fig. 1 and Table I). NK T cells represented 3.7% of splenocytes of Vα14-Jα281 NOD mice and only 0.46% in control NOD mice; corresponding values for pancreatic lymph node cells were 2.8 and 0.2%. NK T cells represented 1.52% of Thy-1$^+$ islet-infiltrating cells in Vα14-Jα281 NOD and 0.47% in control NOD mice. Interestingly, NK T cells were more abundant in pancreatic and mesenteric lymph nodes than in popliteal, inguinal, and brachial nodes. The presence of large numbers of NK T cells in pancreatic lymph nodes and within the islets means that they could potentially interfere with the development of the autoimmune response in both tissues.

Intraislet cytokine production in Vα14-Jα281 NOD

In a previous study we found that overexpression of NK T cells induced an increase in IL-4 release after anti-CD3 stimulation and an elevated baseline level of serum IgE, suggesting that the immune system is biased toward Th2 responses. To determine whether a similar bias occurred locally in pancreatic lymph nodes and within islets, cytokine mRNAs in these organs were measured by kinetic RT-PCR without in vitro restimulation of T cells. Pancreatic lymph node cells and purified pancreatic islet cells were prepared from individual 13- to 16-wk-old females and compared with those from the spleen of the same individuals (Fig. 2). In the spleen, both IL-4 and IFN-γ transcripts were more abundant in Vα14-Jα281 mice than in negative littermates, whereas IL-10 levels were similar. In pancreatic lymph nodes of Vα14-Jα281 mice, IFN-γ mRNA level were normal, whereas mRNA levels of the Th2 cytokines IL-4 and IL-10 were increased. In islets of Vα14-Jα281 mice, the increase in IL-4 mRNA was associated with a reduction in IFN-γ mRNA, whereas IL-10 mRNA levels were unchanged. The heterogeneity of IL-4 transcript levels in the islets of 13- to 16-wk-old Vα14-Jα281 females could reflect a failure to establish and/or to stabilize a Th2-polarized response inside the islets of Vα14-Jα281 females that would eventually develop diabetes. To test this hypothesis, we analyzed islets from old (>40 wk) protected Vα14-Jα281 NOD females. Interestingly, intraislet IL-4 mRNA levels were high in all protected Vα14-Jα281 mice

Table I. *NK T cell numbers in various organs*[a]

Mice		Spleen	Pancreatic Lymph Nodes	Islets[b]	Mesenteric Lymph Nodes	Popliteal Lymph Nodes	Inguinal Lymph Nodes[c]	Brachial Lymph Nodes[c]
NOD	Cell no.[a]	114 ± 21.3	2.9 ± 1.0	1.0 ± 0.2	17 ± 2.7	2.1 ± 1.4	8.6 ± 5.3	3.2 ± 1.0
	NK T cells[b]	0.46 ± 0.13 (n = 6)[e]	0.20 ± 0.02 (n = 6)	0.47 ± 0.06[c] (n = 6)	0.26 ± 0.12 (n = 6)	0.03 ± 0.01 (n = 6)	0.02[d] (n = 3)	0.04[d] (n = 3)
Vα14-Jα281 transgenic	Cell no.[a]	83 ± 22.2	2.2 ± 0.9	1.1 ± 0.5	12 ± 2.5	0.7 ± 0.6	2.9 ± 0.8	0.7 ± 0.4
	NK T cells[b]	3.70 ± 0.88 (n = 6)	2.85 ± 0.57 (n = 6)	1.52 ± 0.69[c] (n = 6)	2.20 ± 0.46 (n = 6)	0.20 ± 0.14 (n = 6)	0.20[d] (n = 3)	0.40[d] (n = 3)

[a] Measurements of $\times 10^{-6}$ ± SD.
[b] Percentage of the cell population in the whole organs.
[c] Percentage of Thy-1$^+$ cells; the values correspond to three immunofluorescence stainings, each performed with two mice.
[d] Lymph node cells from three mice were pooled for immunofluorescence stainings.
[e] n = number of mice analyzed; mice were analyzed individually except where mentioned.

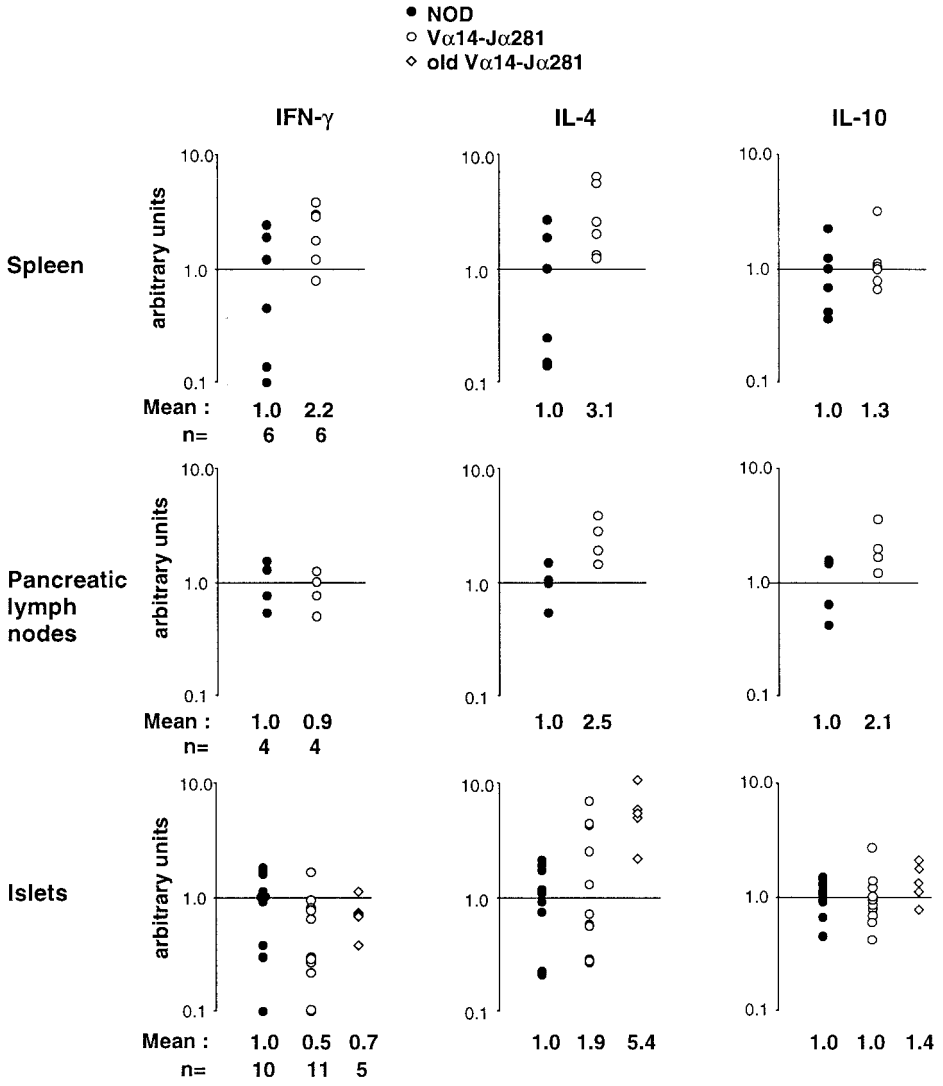

FIGURE 2. Increased IL-4 mRNA and decreased IFN-γ mRNA levels in islets from Vα14-Jα281 NOD mice. IFN-γ, IL-4, and IL-10 mRNA were analyzed in spleens, pancreatic lymph nodes, and islets from 13- to 16-wk-old Vα14-Jα281 NOD mice and negative littermates. Islets from 40-wk-old protected Vα14-Jα281 NOD females were also analyzed. TCR β-chain mRNA was measured as a reference and was used to normalize all the samples. The average level of each cytokine mRNA in control NOD mice is arbitrarily set at 1 U. Each point represents an individual mouse; ●, control NOD mice; ○, Vα14-Jα281 NOD mice; ◇, old Vα14-Jα281 NOD mice. n, number of mice analyzed in each group.

analyzed. IL-2 mRNA levels were reduced in islets from protected Vα14-Jα281 mice, while TGF-β transcript levels were normal (data not shown). Taken together, these results indicated that overexpression of NK T cells in NOD mice tends to reverse the IL-4/IFN-γ ratio at the lesion target site.

Anti-GAD responses in Vα14-Jα281 NOD mice

The Th2 shift in the cytokine profile inside the islets of Vα14-Jα281 NOD mice could be due to cytokine production by infiltrating NK T cells and/or to the response of autoreactive T cells specific for islet Ags. To test the second hypothesis, we analyzed the immune response that has developed in vivo against GAD, a β cell autoantigen involved in diabetes. When stimulated in vitro by recombinant GAD, lymph node cells from Vα14-Jα281 NOD mice produced more IL-4 and IFN-γ than did cells from control females (Fig. 3). Another way to analyze the anti-GAD response in vivo without in vitro restimulation is to determine the levels and the isotypes of anti-GAD Abs. The analysis of sera from >30 Vα14-Jα281 NOD females and 16 negative littermate females revealed a significant reduction in anti-GAD IgG2c in transgenic NOD mice ($p = 0.0003$). This decrease was more pronounced than the reduction in total IgG2c Abs. Arbitrarily attributing control NOD sera with a value of 1 (for both anti-GAD Abs and total IgG), the average values in transgenic NOD serum were 0.4 for anti-GAD IgG2c and 0.9 for total IgG2c (Fig. 4). In old protected Vα14-Jα281 NOD females, the reduction in anti-GAD Abs of the Th1 isotype was associated with an increase in anti-GAD Abs of the Th2 isotype. In contrast, levels of total IgG1 and total IgG2c were very similar in transgenic and control mice and did not change with age. We then sought to determine whether this bias toward a Th2 response was peculiar to the pancreatic autoantigen or whether overexpression of NK T cells could interfere with the response to an exogenous Ag. Interestingly, the Ab response observed in Vα14-Jα281 NOD mice at various times after s.c. immunization with OVA never showed a Th2 bias (Fig. 5).

IL-12 treatment inhibits the protection conferred by NK T cells

If the protection conferred by NK T cells was due to a bias toward Th2 responses, treatment of Vα14-Jα281 NOD females with IL-12, a potent stimulator of Th1 responses characterized by IFN-γ production (29), should abolish it. When 9-wk-old transgenic females were injected daily with 0.15 μg of IL-12, they developed diabetes within 2 wk, like their nontransgenic littermates (Fig. 6). To check that this low dose IL-12 treatment effectively pushed the immune system toward a Th1 response, cytokine mRNAs produced within the islets of Vα14-Jα281 NOD females treated with IL-12 (or by vehicle alone) were measured by quantitative PCR. As shown in Fig. 7, the IL-4 mRNA level was 8-fold lower after 7 days of IL-12 treatment compared with vehicle treatment, whereas the IFN-γ mRNA

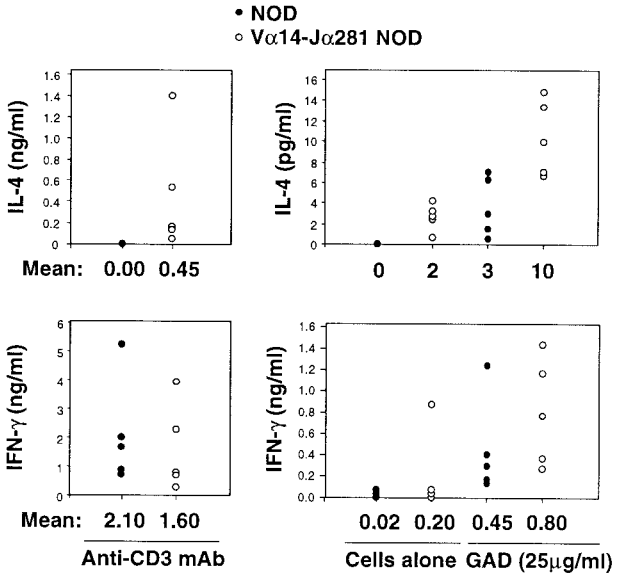

FIGURE 3. Increased cytokine production by lymph node cells from Vα14-Jα281 NOD mice stimulated by GAD65. Two million cells were incubated in flat-bottom 96-well plates with anti-CD3, 25 μg/ml GAD65, or medium alone. IL-4 and IFN-γ production was measured in supernatants harvested after 48-h culture. Vα14-Jα281 females and negative littermate females were analyzed at 14 wk of age. Each point represents an individual mouse. ●, Control NOD mice; ○, Vα14-Jα281 NOD mice.

level was increased 2.8-fold. Thus, the pro-Th1 influence of in vivo IL-12 treatment was detected without in vitro stimulation of islet-infiltrating cells. Interestingly, after 5 days of IL-12 treatment, no NK T cells were detectable by immunofluorescence analysis of the spleen and pancreatic lymph nodes of Vα14-Jα281 NK1.1 congenic NOD mice (Fig. 8). These data show that IL-12 can act on NOD NK T cells and that in vivo IL-12 treatment abolishes the protective action of NK T cells normally observed in Vα14-Jα281 NOD mice.

IL-4, but not IL-10, is required for NK T cell-induced protection against diabetes

Our results suggested that the protection against diabetes conferred by NK T cells was associated with a Th2 shift of the immune response against islet Ags. To determine whether IL-4 and/or IL-10 were required for this protection, specific blocking mAbs were injected in Vα14-Jα281 NOD mice. These treatments were applied to cyclophosphamide-treated mice, as we have previously shown that overexpression of NK T cells totally protects Vα14-Jα281 NOD mice against cyclophosphamide-induced diabetes (7). As shown in Fig. 9, total protection against cyclophosphamide-induced diabetes was confirmed in Vα14-Jα281 NOD mice, whereas nontransgenic NOD mice developed diabetes. Simultaneous treatment with anti-IL-4 mAb and cyclophosphamide abolished the protective effect of NK T cells in Vα14-Jα281 NOD mice, contrary to simultaneous treatment with anti-IL-10 mAb and cyclophosphamide. These results demonstrate that IL-4 is a key mediator of NK T cell-induced protection against diabetes in NOD mice.

Discussion

This study provides several lines of evidence suggesting that the protection against diabetes observed in Vα14-Jα281 NOD mice is associated with a shift toward a Th2 immune response. First, an increase in IL-4 transcripts and a reduction in IFN-γ transcripts

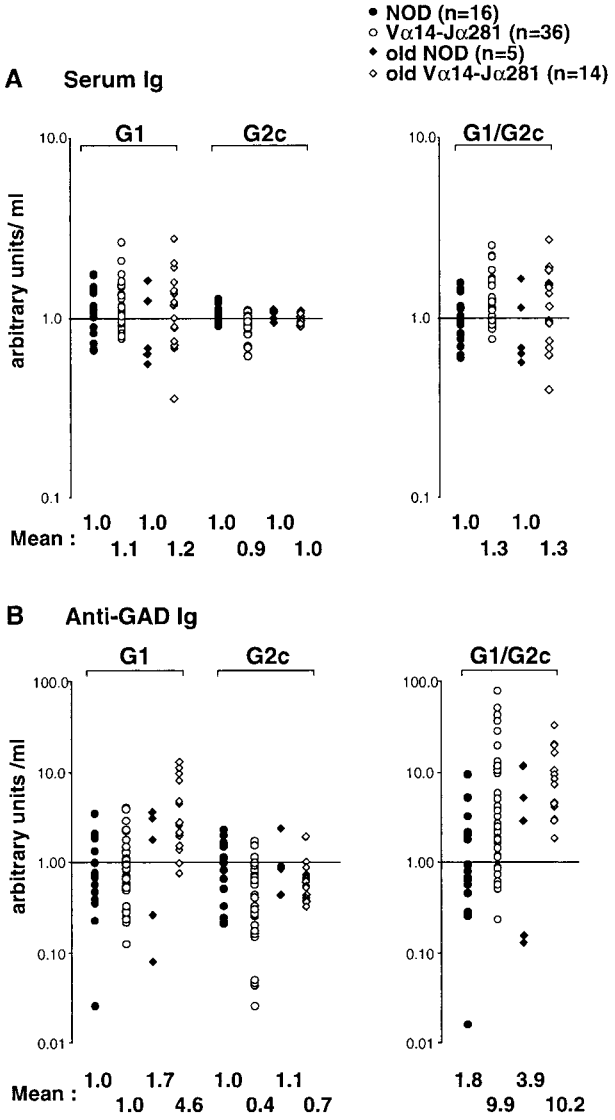

FIGURE 4. Increased IgG1 anti-GAD Ab levels and decreased IgG2c anti-GAD Ab levels in Vα14-Jα281 NOD mice. Sera from 14- to 18-wk-old Vα14-Jα281 females ($n = 36$), negative littermate females ($n = 16$), 40-wk-old protected Vα14-Jα281 females ($n = 14$), and negative littermate females ($n = 5$) were analyzed. The average level of each isotype in young control female NOD mice is arbitrarily set at 1 U/ml. Each point represents an individual mouse. ●, young control NOD mice; ○, young Vα14-Jα281 NOD mice; ♦, old control NOD mice; ◇, old Vα14-Jα281 NOD mice. *A*, Total serum Ig isotypes; *B*, anti-GAD Ig isotypes.

were found within islets. Second, the immune response against the islet autoantigen GAD showed a Th2 bias, as illustrated by the increased IgG1/IgG2c ratio of anti-GAD Abs. Third, IL-12, a pro-Th1 cytokine that favors IFN-γ production and inhibits IL-4 production, abolished the diabetes protection in Vα14-Jα281 NOD mice, with kinetics and incidence similar to those in control mice. Fourth, treatment with anti-IL-4 mAb abrogated the protection normally observed in Vα14-Jα281 NOD mice against cyclophosphamide-induced diabetes. Together, our results identify IL-4 as a key mediator of the immunoregulation induced by NK T cells. Hammond et al. (6) had previously shown that IL-4 and/or IL-10 were required for NK T cell-induced protection against diabetes, but our data suggest a role for IL-4 and not IL-10. Indeed, treatment with anti-IL-10 mAb did not abolish the NK T cell-induced protection against diabetes, and the level of IL-10 transcripts in the

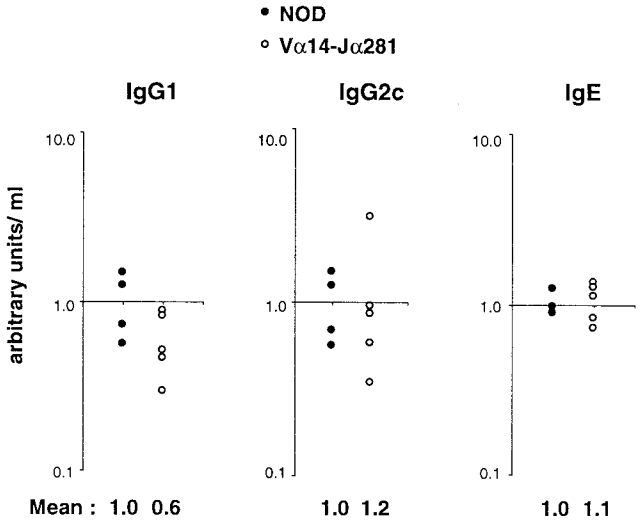

FIGURE 5. The anti-OVA response is not shifted toward Th2 in Vα14-Jα281 NOD mice. Vα14-Jα281 females and negative littermate females were immunized with 100 μg of OVA emulsified in CFA, boosted with OVA 2 wk later, and bled 7 wk later. The average level of anti-OVA Abs of each isotype in serum from negative littermate females is arbitrarily set at 1 U/ml. Each point represents an individual mouse. ●, Negative littermates; ○, Vα14-Jα281 NOD mice.

islets of old protected mice was normal, while the level of IL-4 transcripts was clearly increased. A key role of IL-4 in protection from diabetes has been suggested in previous studies. Indeed, repeated injections of rIL-4 into young NOD mice protect them from

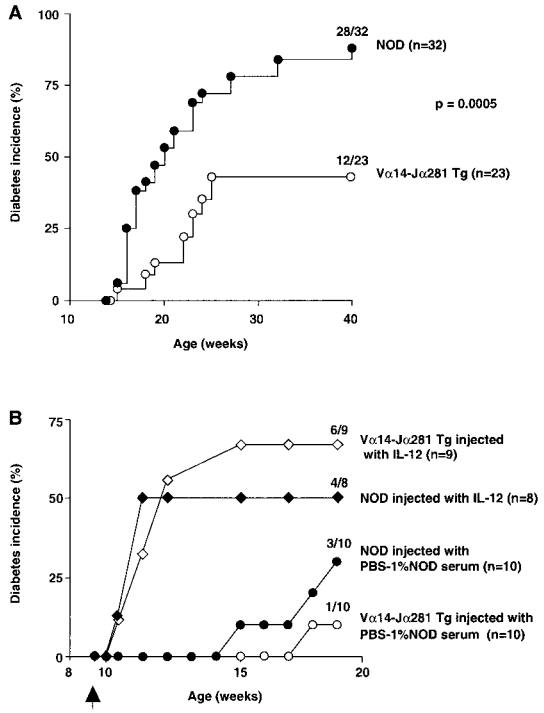

FIGURE 6. In vivo treatment with IL-12 accelerates diabetes onset in Vα14-Jα281 NOD mice, as in negative littermates. *A*, Incidences of spontaneous diabetes in untreated Vα14-Jα281 transgenic NOD females and negative littermates. The difference is statistically significant ($p = 0.0005$). *B*, Prediabetic 9-wk-old females were injected daily i.p. with 0.15 μg of recombinant mouse IL-12 (diamonds) or with vehicle alone (PBS containing 1% NOD serum; circles). Vα14-Jα281 NOD females (open symbols) were compared with negative littermates (filled symbols).

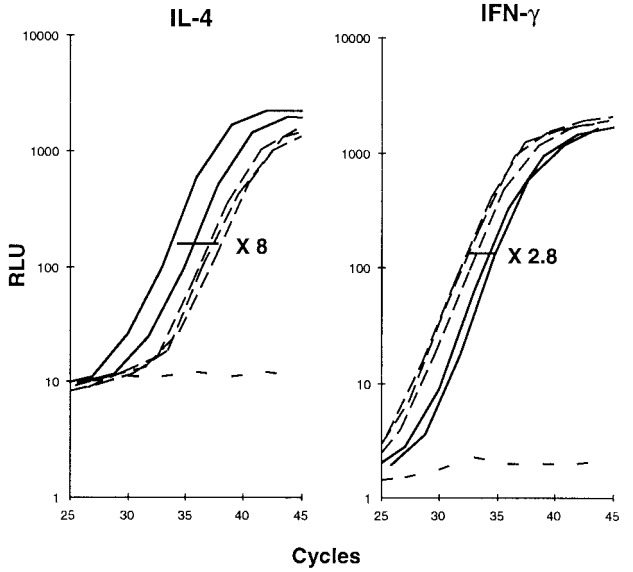

FIGURE 7. In vivo treatment with IL-12 induces a shift toward Th1 in Vα14-Jα281 NOD mice. Prediabetic 9-wk-old Vα14-Jα281 females were injected i.p. for 7 days with 0.15 μg of recombinant mouse IL-12 (dashed line) or with vehicle alone (PBS containing 1% NOD serum; solid line). Islets were purified, mRNA was prepared, and IL-4 and IFN-γ mRNAs were measured by quantitative PCR. The TCR β-chain was also analyzed to normalize the different samples. The negative PCR control (large dashed line) corresponds to PCR without cDNA. The bars connect the mean of the two curves obtained with vehicle treated mice to the mean of the three curves obtained with IL-12-treated mice. The lengths of the bars correspond to the difference in the number of PCR cycles performed to get the same amount of specific cDNA.

disease onset (30, 31), and transgenic NOD mice expressing IL-4 under the control of the insulin promoter do not develop the disease (32).

The induction of Th2 immune responses by NK T cells was first suggested by their ability to rapidly release large amounts of IL-4 after TCR triggering (15, 16). NK T cells have since been shown to help Th1 responses, probably through their production of IFN-γ (33, 34). Similarly, studies using α-galactosylceramide, a specific ligand of Vα14-Jα281 T cells, showed that these cells could promote either Th1 or Th2 responses (35–37). Two of these reports showed that after stimulation, NK T cells could release large amounts of both IFN-γ and IL-4, cytokines that could help Th1 or Th2 responses, but that repeated stimulations of NK T cells would promote IL-4 rather than IFN-γ production (35, 36). The observation that both C57BL/6 (17) and NOD (7) Vα14-Jα281 transgenic mice, which contain large numbers of NK T cells, have increased levels of IgE in the absence of exogenous stimulation further supports the role of these cells in promoting Th2 responses in chronic situations.

Moreover, our data suggest that the location of the immune response is also important. First, the modification of IL-4 and IFN-γ mRNA production in Vα14-Jα281 NOD mice compared with that in control NOD mice is different in spleen, pancreatic lymph nodes, and pancreatic islets. Both IL-4 and IFN-γ transcripts were increased in the spleen, whereas IL-4 transcripts were increased and IFN-γ transcripts decreased in the islets. Second, when Vα14-Jα281 NOD mice were immunized with OVA in the footpad, the immune response never shifted toward Th2, even though the antigenic stimulation was repeated and lasted months. The lymph nodes were huge after these repeated immunizations, containing

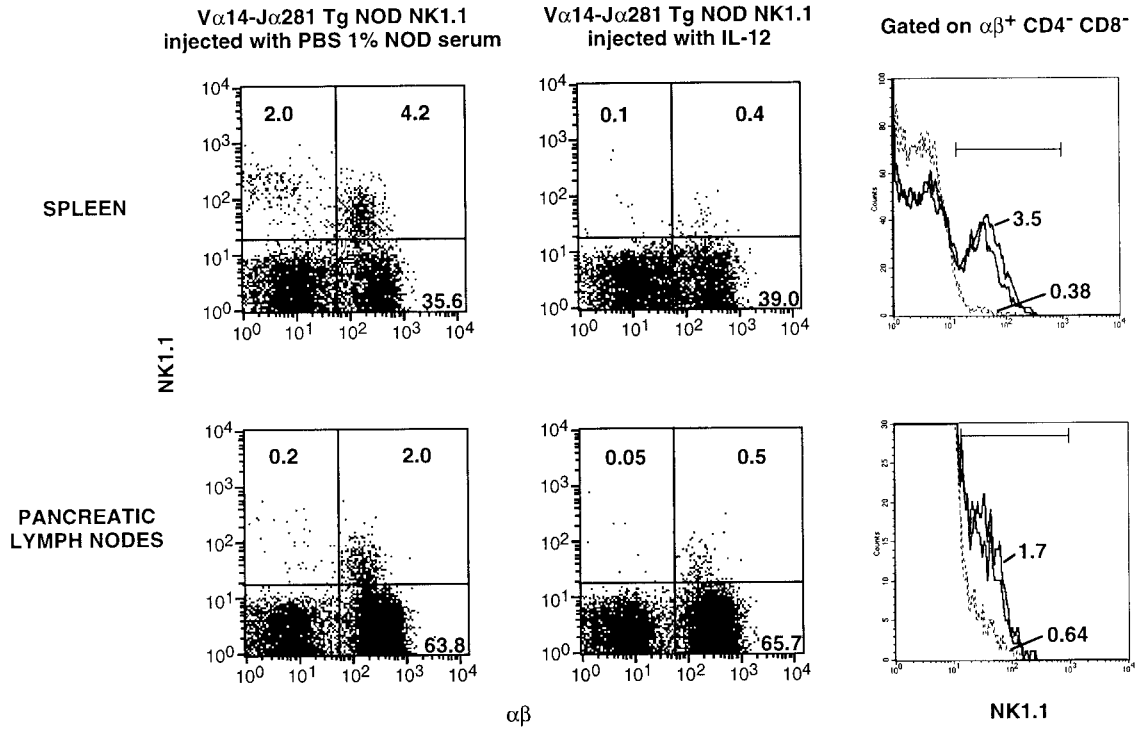

FIGURE 8. NK T cells are not detected in Vα14-Jα281 females treated with IL-12. Prediabetic 9-wk-old Vα14-Jα281 females were injected i.p. for 5 days with 0.15 μg of recombinant mouse IL-12 (dotted line) or with vehicle alone (PBS containing 1% NOD serum; solid line). Spleen and pancreatic lymph node cells were stained with anti-CD4, anti-CD8, anti-TCR-αβ, and anti-NK1.1 mAbs. The numbers represent the percentages of NK T cells in the whole organs.

about 50 million cells, but NK T cells were as rare as in noninflamed popliteal lymph nodes. One hypothesis raised by these data is that autoreactive anti-islet T cells are influenced during their priming in pancreatic lymph nodes by the presence of large numbers of NK T cells. This would fit with several studies showing that anti-islet autoreactive T cells are activated in pancreatic lymph nodes before migrating into the islets (38, 39). Immunofluorescence analysis of NK T cells present in pancreatic lymph nodes showed that they were mainly $CD4^{-}CD8^{-}$, $CD44^{+}$, and $CD122^{+}$ and that approximately 55% of them expressed Vβ8, a phenotype similar to that of splenic NK T cells.

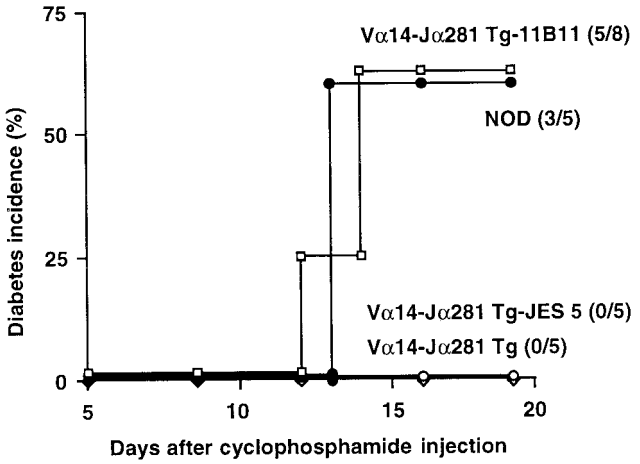

FIGURE 9. IL-4 is required for NK T cell-induced protection against diabetes. Ten-week-old females were injected with cyclophosphamide (200 mg/kg) on day 0. Mice were treated with 0.5 mg of anti-IL-4 mAb (11B11) or anti-IL-10 mAb (JES5) on days −1, 0, 2, and 5.

It is important to emphasize that NK T cells seem to regulate rather than to inhibit the autoimmune responses occurring spontaneously in NOD mice. Indeed, T cell responses against GAD were still present in Vα14-Jα281 NOD mice, and in vitro anti-GAD stimulation led to increased production of both IL-4 and IFN-γ relative to that in control NOD mice. This immunostimulatory role of NK T cells is not surprising, as they are relatively efficient in bolstering various immune responses against certain bacteria and parasites. NK T cells, by shifting the Th1/Th2 balance of the immune response, seem to behave differently from other regulatory T cells such as $CD4^{+}$ $CD25^{+}$ and Tr1 cells, which tend to inhibit T cell activation (40–42).

Several laboratories have shown that NK T cells are deficient in both number and function in NOD mice (7, 23, 24, 43). Recently, it has been proposed that the NK T cell defect, linked to diabetes onset, may mainly be due to the weak response of these cells to IL-12 and their low IFN-γ production (43). However, we found that NK T cells in NOD mice responded to IL-12 injected i.p. even at a low dose (0.15 μg/mouse). After a few days of such treatment NK T cells disappeared from pancreatic lymph nodes as well as spleen of Vα14-Jα281 NOD mice. This was probably due to activation-induced cell death, as NK T cells are known to die by apoptosis after activation by anti-CD3 or IL-12 in vivo (44). Likewise, in vivo IL-12 treatment induced IFN-γ production within islets, and this was associated with vulnerability to diabetes rather than protection. These data confirm results reported by Trembleau et al. (28). Clearly, increased IFN-γ production within islets is not sufficient to protect against diabetes onset. Although, IFN-γ may nonetheless be involved in the protection of Vα14-Jα281 NOD mice, it is important to note that all our data converged to suggest that long term protection is associated with a Th2 shift. Furthermore, old protected Vα14-Jα281 NOD mice showed a reduction in

IFN-γ transcripts within islets and an increase in the IgG1/IgG2c ratio of anti-GAD Abs.

Increased NK T cell numbers in NOD mice are sufficient to protect NOD mice from diabetes. This protection is associated with several modifications of the immune system, locally within islets and in the specific response against GAD. It is puzzling how an increase in NK T cell numbers can push the immune response against a self Ag toward the Th2 pattern, while the same cells efficiently help to induce Th1 responses against infectious agents. Further studies should focus on NK T cells present in pancreatic lymph nodes and the differentiation of anti-islet T cells in this environment.

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