α/β–T Cell Receptor (TCR)$^+$CD4$^-$CD8$^-$ (NKT) Thymocytes Prevent Insulin-dependent Diabetes Mellitus in Nonobese Diabetic (NOD)/Lt Mice by the Influence of Interleukin (IL)-4 and/or IL-10

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Summary

We have previously shown that nonobese diabetic (NOD) mice are selectively deficient in α/β-T cell receptor (TCR)$^+$CD4$^-$CD8$^-$ NKT cells, a defect that may contribute to their susceptibility to the spontaneous development of insulin-dependent diabetes mellitus (IDDM). The role of NKT cells in protection from IDDM in NOD mice was studied by the infusion of thymocyte subsets into young female NOD mice. A single intravenous injection of 10^6 CD4$^{-/low}$CD8$^-$ or CD4$^-$CD8$^-$ thymocytes from female (BALB/c × NOD)F1 donors protected intact NOD mice from the spontaneous onset of clinical IDDM. Insulitis was still present in some recipient mice, although the cell infiltrates were principally periductal and periislet, rather than the intraislet pattern characteristic of insulitis in unmanipulated NOD mice. Protection was not associated with the induction of "allogenic tolerance" or systemic autoimmunity. Accelerated IDDM occurs after injection of splenocytes from NOD donors into irradiated adult NOD recipients. When α/β-TCR$^+$ and α/β-TCR$^-$ subsets of CD4$^-$CD8$^-$ thymocytes were transferred with diabetogenic splenocytes and compared for their ability to prevent the development of IDDM in irradiated adult recipients, only the α/β-TCR$^+$ population was protective, confirming that NKT cells were responsible for this activity. The protective effect in the induced model of IDDM was neutralized by anti–IL-4 and anti–IL-10 monoclonal antibodies in vivo, indicating a role for at least one of these cytokines in NKT cell-mediated protection. These results have significant implications for the pathogenesis and potential prevention of IDDM in humans.

NKT cells express cell surface markers of both T cells (such as the α/β-TCR) and NK cells (including NK1.1, CD16, Ly49A, and Ly49C) and appear to play a role in immunoregulation (for review see reference 1). Recent evidence has suggested that they may also play a role in the pathogenesis of insulin-dependent diabetes mellitus (IDDM)[1] in nonobese diabetic (NOD) mice that spontaneously develop IDDM closely resembling the human disease (2).

Zipris et al. (3) found that thymocytes from young NOD mice proliferated poorly after stimulation by concanavalin A or plate-bound anti-CD3 mAb. This peculiarity was subsequently shown to be associated with decreased IL-2 and IL-4 production. Although addition of IL-2 in vitro partially corrected this defect, supplementation with IL-4 not only completely normalized in vitro responses, but also reduced the incidence of spontaneous IDDM of mice treated in vivo (4). They concluded that a failure of NOD thymocytes to produce sufficient IL-4 for the differentiation of regulatory CD4$^+$CD8$^-$ Th2 clones may disrupt the balance between self-tolerance and autoimmunity resulting in IDDM.

A plausible explanation for the decreased levels of IL-4 produced following stimulation of NOD mouse thymocytes was provided when Gombert et al. (5, 6) and our own group (7, 8) independently discovered that these mice are deficient in NKT cells. Gombert et al. (5, 6) reported that NOD mice have a deficit in the number and functional capacity of heat stable antigen (HSA)$^-$, CD8$^-$, α/β-TCR$^+$, Mel-14$^-$, or CD44$^+$ thymocytes, which display the Vβ8-biased TCR repertoire characteristic of NKT cells. The consequence of this deficiency in vivo was demonstrated by the injection of anti-CD3 mAb. Normally, in vivo stimulation of NKT cells by anti-CD3 mAb results in appearance of IL-4 messenger RNA within 30 min of injection followed

[1]*Abbreviations used in this paper:* APC, allophycocyanin; BCG, bacillus Calmette-Guerin; DN, double negative; IDDM, insulin-dependent diabetes mellitus; NOD, nonobese diabetic; RT, room temperature.

D.I. Godfrey and A.G. Baxter contributed equally to this work.

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by a rise in protein production of IL-4 protein peaking at 2 h (9). Gombert et al. (6) found that the amount of IL-4 produced in vitro by cells from young NOD mice after anti-CD3 challenge was much lower than that produced by cells from C57BL/6 mice and similar to the amount produced by cells from NKT cell–deficient β2 microglobulin$^{-/-}$ targeted mutant mice (10). Although there was some increase in IL-4 production by cells from older NOD mice, the levels remained fourfold lower than controls (6).

A comparable deficiency in thymic NKT cells in NOD mice was identified by our group (7, 8) during a multiparameter flow cytometric study of thymic T cell development in nine different mouse strains. The results revealed a three- to sevenfold reduction in the numbers of α/β-TCR$^+$CD4$^-$CD8$^-$ (double negative; DN) thymocytes from NOD mice compared with other nondiabetes-prone strains. This DN cell population not only shows the Vβ8-biased TCR repertoire described above (9, 11), but is known to secrete large amounts of IL-4 rapidly after activation in vitro (12). In addition to the thymic defect, spleen and lymph nodes of NOD mice also had reduced numbers of α/β-TCR$^+$DN cells (13). Evidence for the potential relevance of this defect to the pathogenesis of IDDM was obtained by demonstrating that transfer of DN thymocytes, but not unfractionated thymocytes, from either semiallogeneic (F1) donors or 35-wk-old syngeneic NOD mice prevented the spontaneous onset of IDDM (13).

These studies prompted two main lines of inquiry. The first was to determine whether α/β-TCR$^+$CD4$^-$CD8$^-$ (NKT) cells were responsible for the protective activity within the population of DN thymocytes, which included both α/β-TCR$^+$ and α/β-TCR$^-$ cells. The second goal was to define the mechanism of action by which these cells prevented IDDM.

Materials and Methods

Mice. Female NOD/Lt//Arc, BALB/c//Arc, and (NOD × BALB)F1 mice were obtained from the Animal Resources Centre (Canning Vale, Perth, Australia) and maintained in clean conditions in the Centenary Institute Animal House (Sydney, Australia). Sentinel mice were tested by serology at four monthly intervals for the following pathogens: mouse hepatitis virus, rotavirus, ectomelia, mouse cytomegalovirus, polyoma virus, murine adenovirus, lymphocytic choriomeningitis virus, mouse pneumonia virus, reovirus, Sendai virus, Theiler's murine encephalitis virus, *Bacillus piliformis*, *Mycoplasma pulmonis*, *Bordtella bronchiseptica*, *Corynebacterium kutscheri*, *klebsiella* species, *Pasturella multocide*, *Pasturella pneumotropica*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Citrobacter freundii*, and *salmonella* species. No mice tested positive for any of these pathogens. Mice were housed at 21°C and 40% humidity and were fed mouse chow (Barastock, Melbourne, Australia) and acidified water ad libitum.

Isolation of DN Thymocytes. CD4$^+$ and CD8$^+$ cells were depleted by a 30-min incubation with anti-CD4 (clone RL-172.4) and anti-CD8 (clone 3.155) supernatants at 4°C followed by a 30-min incubation with rabbit complement (C-six Diagnostics, Mecquon, WI) at 37°C. Viable cells were recovered over Histopaque 1083 (Sigma Chemical Co., Castle Hill, Australia), washed and labeled with anti-CD4-PE (clone RM4-5; PharMingen, San Diego, CA), anti-CD8α-allophycocyanin (APC) (clone 53-6.7, PharMingen), and anti–α/β-TCR–FITC (clone H57-597; PharMingen). In some experiments, DN cells were further purified from the depleted population using a FACStarPLUS® (Becton Dickinson, San Jose, CA) with or without further separation of DN cells into α/β-TCR$^-$ and α/β-TCR$^+$ subsets. Sorted populations were 98–99.9% pure.

Flow Cytometric Analysis. For multiparameter labeling of lymphocytes, cells were stained with anti–α/β-TCR–FITC (clone H57-597; PharMingen), anti-CD4-PE (clone RM4-5; PharMingen), or anti-CD4-biotin (clone CT-CD4; Caltag Laboratories, San Francisco, CA) and anti-CD8α-APC (clone 53-6.7; PharMingen). Biotinylated mAbs were detected with streptavidin-conjugated Texas red (Molecular Probes, Eugene, OR). For intracellular cytokine staining, cells were incubated overnight on 24-well plates that had been previously coated with 10 μg/ml anti-CD3 mAb (KT3) with the addition of 10 μg/μl Brefeldin A (Sigma Chemical Co.) 3 h after initiation of the culture. The next day, the cells were surface stained for CD4 and CD8 expression. The cells were then fixed with 4% paraformaldehyde for 20 min and stained for cytokines using anti–IL-4–PE (clone 11B11; PharMingen) and anti–IFN-γ–FITC (clone XMG1.2; PharMingen) in 0.5% saponin (Sigma Chemical Co.). Analysis was performed on a FACStarPLUS® (Becton Dickinson, San Jose, CA).

Adoptive Transfer of Thymocytes. DN thymocyte subpopulations were tested for efficacy in two models of IDDM. In the first model, prediabetic 3–4-wk-old female unmanipulated NOD/Lt mice were injected intravenously with 1.0×10^6–2.0×10^6 thymocytes (either unfractionated or fractions prepared as described above) in 200 μl of PBS or with 200 μl of PBS alone. Recipients were then bled from 12–35 wk of age at two weekly intervals for blood glucose estimations. Mice were declared diabetic if two blood glucose readings >11.1 mM were obtained or if death occurred immediately after a single reading >11.1 mM.

In the second model, nondiabetic 10–12-wk-old female gamma-irradiated (700 R) NOD/Lt mice were injected intravenously with 3.8×10^5–2.0×10^6 thymocytes (prepared as described above) in 200 μl of PBS, or with 200 μl of PBS alone immediately followed by 2.0×10^7 splenocytes from female diabetic NOD mice in 200 μl of PBS. Mice were bled for blood glucose estimations 3 and 4 wk later and were declared diabetic after a single reading >11.1 mM. The end point was determined by either the onset of IDDM or at 4 wk.

As lymphoid tissue from young NOD mice contained insufficient numbers of α/β-TCR$^+$DN cells, cells for transfer were derived from (NOD × BALB/c)F1 mice, which have much greater numbers and are partially isogenic with NOD mice (13).

Blood glucose estimations were performed on retroorbital venous blood samples by the glucose oxidase technique using Glucofilm test strips (Bayer Australia, Pymble, Australia) assayed on a Glucometer M+ meter (Bayer Diagnostics, Pymble, Australia).

Neutralization of IL-4 and IL-10 by mAb Treatment In Vivo. mAbs 11B11 rat anti–mouse IL-4 (IgG1) and JES5-2A5 rat anti–mouse IL-10 (IgG1; gifts of John Abrams, DNAX, Palo Alto, CA) were grown as ascites in pristane-primed (1.0 ml/mouse intraperitoneally, 2, 6, 10, 14 tetramethylpentadecane 98%; Aldrich Chemical Company, Milwaukee, WI) BALB/c-*nu/nu* mice. Ascites supernatants and rat IgG (Sigma Chemical Co.) were purified on a protein G–Sepharose column (Pharmacia Biotech, Uppsala, Sweden), dialyzed against PBS, and concentrated by osmosis using Aquacide (Calbiochem, La Jolla, CA). Mice were injected intraperitoneally on the day of transfer (day 0), and days 1,

3, and 6 after transfer with either 0.5 mg 11B11 together with 0.5 mg JES5-2A5, or 1.0 mg rat IgG or PBS.

Skin Grafts. Donor BALB/c mice were killed by cervical dislocation and the tails removed. The tail skin was incised longitudinally, stripped from the underlying tissues, and placed in a sterile petri dish, dermis down, on a bed of filter paper moistened in PBS and then cut into segments ~1 cm^2. Recipient NOD mice were anesthetized with 1.25–1.75 mg Ketamine (Apex Laboratories, St. Mary's, Australia) and 0.25–0.35 mg Xylazine (Bayer Australia) in PBS subcutaneously. The superficial layers of the skin over the graft site on the flanks of the recipients were removed with surgical scissors to prepare a bloodless vascular field for the graft. The graft was held in place with petroleum jelly impregnated gauze and a bandage of adhesive surgical tape. Postoperative analgesia was achieved with 0.02 mg buprenorphine (Reckitt and Coleman, Bristol, UK) injected subcutaneously immediately before surgery. 8 d after grafting, the dressing was removed and grafted mice were placed in individual cages for monitoring of graft survival.

Insulitis Scoring. Pancreata were either fixed in 10% formalin in saline and paraffin embedded or embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN) and frozen on a mixture of ethanol and dry ice. Three serial 6-μm sections were cut at each of three levels 100 μm apart and stained with hematoxylin and eosin. Each islet was examined at ×100 and given a score from 0 to 4 in which 0 represented a noninfiltrated islet and 4 represented complete infiltration or a "burned-out" islet. The insulitis score for each mouse was calculated by expressing the total of the scores as a percentage of the sum of the maximum possible scores.

Hematocrit Measurement and Direct Coombs' Test. 75 μl of blood were drawn up into a heparinized capillary tube (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 1,600 *g* for 30 min. The height of the column of packed red cells was divided by the total height of the column of blood and expressed as a percentage.

10 μl of packed cells were resuspended in 5 ml PBS with 0.3% bovine serum albumin, washed, and resuspended in 1 ml of the same solution. For two aliquots, 100 μl of each sample were placed in 96-well round-bottomed plates (Nunc, Copenhagen, Denmark) with three serial one-half dilutions. 10 μl of 10 μg/ml polyclonal goat anti–mouse IgG-Fc (Jackson ImmunoResearch Laboratories, Austin, TX) were added to one of the sets of dilutions and the plates were vortexed gently and incubated at 37°C for 3 h. Wells in which the cells collected in a button at the bottom were recorded as negative, whereas those in which the cells remained spread over the surface of the well were recorded as positive.

Assessment of Antinuclear Antibodies. Sera were assessed for binding to HEp-2 slides (Sanofi Diagnostics Pasteur Inc., Chaska, MN). Slides were incubated in PBS for 10 min. Sera diluted in PBS (starting concentration 1:100) were incubated on the slides at room temperature (RT) for 30 min in a moist chamber. Slides were then washed three times for 5 min in PBS and incubated for 30 min at RT with 50 μg/ml FITC-conjugated rat anti–mouse IgG gamma chain–specific antisera (Southern Biotechnology Associates, Birmingham, AL). Slides were again washed three times for 5 min with PBS, mounted with polyvinyl alcohol mounting media (Sanofi Diagnostics Pasteur Inc.), and examined on a fluorescence microscope (Leica Mikoskopie, Postfach, Germany). Control sera were obtained from bacillus Calmette-Guerin (BCG)-treated NOD mice previously demonstrated to produce antinuclear antibodies and BALB/c-*nu/nu* mice.

Detection of Glomerular Immune Complex Deposits. Kidneys were embedded in Tissue-Tek OCT compound (Miles) and frozen for histological analysis. 6-μm sections were cut on a cryostat (Microm, Waldorf, Germany), mounted on microscope sides, air dried, acetone fixed for 10 min, and stored at −80°C in an airtight bag containing silica desiccant. When slides were stained, they were thawed to RT, fixed in acetone for a further 5 min, and blocked with 4% FCS (CSL, Melbourne, Australia). Sections were stained with goat anti–mouse C3c polyclonal IgG (Nordic Immunological Laboratories, Tilburg, Holland) at a 1:10 dilution in PBS for 45 min. Slides were then washed three times in PBS, cover slipped with polyvinyl alcohol mounting media (Sanofi Diagnostics Pasteur Inc.), and examined on a fluorescence microscope (Leica Mikoskopie, Postfach, Germany).

ELISA for IL-4 and IFN-γ. IL-4 and IFN-γ were detected by sandwich ELISA. The capture antibody 11B11 (anti–IL-4) or AN18 (anti–IFN-γ) was bound to round-bottomed microtiter plates that were then blocked with 2% FCS in PBS at 37°C for 1 h. Sample supernatants and cytokine standards were titrated and incubated for 2 h on the plates that were then washed and the detection layer (BVD6-biotin for IL-4 or XMG-biotin for IFN-γ) was added. Bound detection antibody was detected with streptavidin-horseradish peroxidase (Jackson ImmunoResearch, West Baltimore, PA). The substrate used was 2,2′ azino-bis(3-ethylbenzthiazoline)-6 sulfonic acid (Sigma Chemical Co.) and results were determined by optical density scanning on a Labsystems Multiscan Multisoft (Helsinki, Finland).

Statistical Analysis. Qualitative differences between samples were examined using the fourfold table (χ^2) test unless the expected value in any cell was 5 or less, in which case the Fisher's exact test was used. Quantitative differences between samples were compared with the Mann Whitney U (rank sum) test.

Results

DN Thymocytes Prevent Spontaneous IDDM in NOD Mice. Intracellular cytokine staining of NK1.1-expressing C57BL/6 mice revealed that about half of the IL-4–producing α/β-$TCR^+NK1.1^+$ thymocytes are DN and half are $CD4^+CD8^-$ (data not shown). Since NOD mice do not express the NK1.1 allelic marker and α/βTCR^+ $NK1.1^+$ cells comprise a much larger proportion of DN thymocytes than of $CD4^+CD8^-$ thymocytes in C57BL/6 mice, the α/β-TCR^+DN phenotype was used as a surrogate marker of NKT cells in NOD mice. In addition to confirming that young female NOD mice are deficient in α/β-TCR^+DN thymocytes (7, 8, 13), it was found that very few IL-4–producing cells could be identified in anti-CD3–stimulated short-term cultures of NOD thymocytes (Fig. 1). The role of NKT cells in protection from IDDM was therefore investigated by using DN thymocytes from (BALB × NOD)F1 donors because we had previously demonstrated that these mice contained normal numbers of α/β-TCR^+DN thymocytes (13) and subsequently found that a large proportion of their thymocytes secrete IL-4 after short-term anti-CD3 stimulation (Fig. 1).

In a series of experiments, different numbers (1–2 × 10^6/mouse) of DN thymocytes from (NOD × BALB)F1 donors were injected intravenously into 3–4-wk-old female NOD recipients (Fig. 2). Administration of DN thymocytes conferred substantial resistance to IDDM with only 2/19 of the recipients developing diabetes, compared

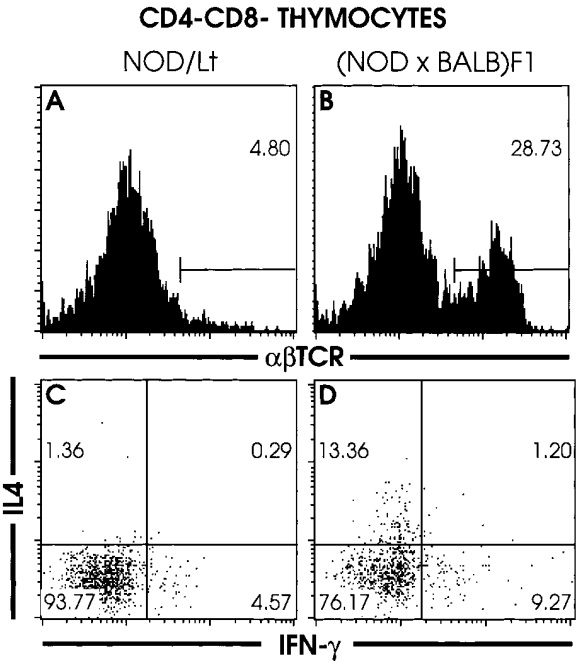

Figure 1. Expression of the αβ-TCR on (*A* and *B*), and IL-4 production by (*C* and *D*) DN thymocytes from NOD (*A* and *C*) and (NOD × BALB)F1 (*B* and *D*) mice.

with controls that either received whole thymocytes (13/19, $P < 0.0001$, χ^2 fourfold table test) or PBS (12/21, $P < 0.001$, χ^2 fourfold table test). For these experiments, two methods of enrichment of DN thymocytes were used. In the first, complement-mediated depletion of anti-CD4 and anti-CD8 antibody-binding cells served to produce a population consisting of $CD4^{-/low}CD8^-$ thymocytes with an overall purity >97%. The second method involved complement-mediated depletion followed by FACS® to exclude the $CD4^{low}$ population and purify only the DN population with >99% purity. Both sources of thymocytes protected recipients from the development of diabetes, indicating that protective cells were contained within the DN population. The numbers of cells transferred, as well as the relative proportion of $\alpha/\beta\text{-}TCR^+$ cells within each inoculum, varied between experiments; consequently, the total numbers of $\alpha/\beta\text{-}TCR^+CD4^{-/low}CD8^-$ or $\alpha/\beta\text{-}TCR^+$DN thymocytes injected varied from $\sim 10^5$–10^6/mouse. The only two treated mice to develop IDDM were injected with the lowest number (1.12×10^5) of $\alpha/\beta\text{-}TCR^+$DN thymocytes, consistent with a minimum of $\sim 1.5 \times 10^5$ $\alpha/\beta\text{-}TCR^+$DN thymocytes being required for protection.

Histological analysis of the pancreata of mice surviving to the termination of the experiment was performed. Although there was no significant difference between the insulitis scores of treated mice and nondiabetic control mice, the pattern of insulitis in the treated mice was qualitatively different and could be subdivided into three distinct patterns. 7/15 mice had florid periductal and/or periislet lymphoid collections with intact islets. One third of the mice had no signs of insulitis or other infiltrates at all, whereas

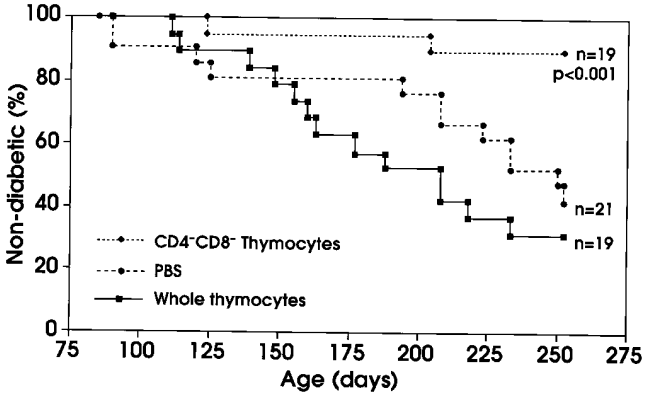

Figure 2. Diabetes-free survival of female NOD mice after injection at 3–4 wk of age with DN thymocytes (*dotted line*), unfractionated thymocytes (*solid line*), or PBS (*dashed line*).

the remainder had intraislet infiltrates qualitatively similar to those normally found in nondiabetic untreated mice (Fig. 3).

IDDM Protection by DN Thymocytes Is Not Associated with Tolerance to Alloantigen. The preceding experiments indicated that protective cells reside in the DN population of thymocytes, but did not tell us whether or not $\alpha/\beta\text{-}TCR^+$ cells mediate this effect as γ/δ T cells, NK cells, B cells, and macrophages are also found within this population. Consequently, the expression of semiallogeneic major histocompatibility products by antigen-presenting cells could have led to protection by inducing "allogenic tolerance", as Bendelac et al. (14) reported that the induction of neonatal tolerance to F1 splenocytes resulted in significant protection from both insulitis and diabetes. To examine the outcome of treatment with DN thymocytes on the allogeneic responses of young NOD recipients, 4-wk-old female NOD mice were injected with 3.0×10^6 complement-depleted (BALB × NOD)F1 thymocytes (>96% $CD4^{-/low}CD8^-$; 39.9% $\alpha/\beta\text{-}TCR^+$; equates to 10^6 $\alpha/\beta\text{-}TCR^+CD4^{-/low}CD8^-$ thymocytes) and were grafted 2 mo later with BALB/c tail skin grafts. The tail skin grafts on all six recipients of DN thymocytes were rejected by day 8. Five of seven grafts on PBS-treated control recipients rejected by day 8, and the remaining two by day 14. Although these results failed to indicate a role for allogenic tolerance in protection against IDDM in this model, they did not exclude the possibility that protection was mediated by the transfer of antigen-presenting cells, which could exert a mild immunostimulatory action (15).

Subfractionation of DN Thymocytes in Induced IDDM. To dissect the mechanism of protection in more detail, a more rapid assay was adopted involving the induction of diabetes within 3–4 wk by injection of 2.0×10^7 splenocytes from spontaneously diabetic NOD donors into irradiated nondiabetic adult NOD recipients (16). This model is relatively robust since it is refractory to many treatments reported to protect intact NOD mice from the onset of spontaneous IDDM, such as the administration of CFA or BCG (data not shown).

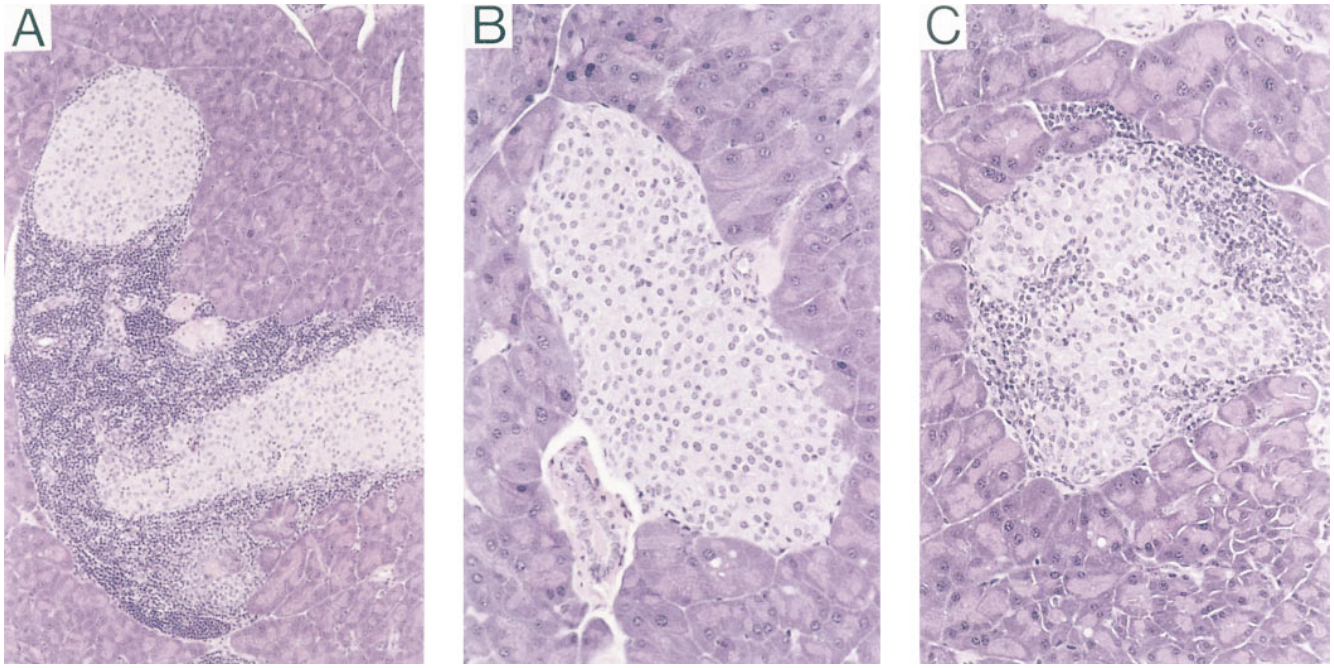

Figure 3. Typical histological appearance at 35 wk of hematoxylin and eosin–stained islets from nondiabetic female NOD mice injected at 3–4 wk of age with DN thymocytes (*A*, ×400; *B*, ×200) or unfractionated thymocytes (*C*, ×400).

Varying numbers (5.0×10^5–2.0×10^6) of DN thymocytes from nondiabetes prone (NOD × BALB)F1 donors were injected together with diabetogenic splenocytes intravenously into 10–12-wk-old irradiated nondiabetic female NOD recipients. 3/14 (21%) recipients of DN thymocytes subsequently developed IDDM, compared with 7/12 and 7/10 recipients given PBS or unfractionated thymocytes, respectively (64%, $P < 0.05$, χ^2 fourfold table test; Table 1). The numbers of cells transferred, as well as the relative proportion of α/β-TCR$^+$ cells within each inoculum, varied between experiments so that the total numbers of α/β-TCR$^+$CD4$^{-/low}$CD8$^-$ thymocytes injected ranged from $\sim 3 \times 10^4$ to 9×10^5/mouse. The only treated mice to develop IDDM received the lowest numbers of α/β-TCR$^+$CD4$^{-/low}$CD8$^-$ thymocytes, which were below the threshold of 1.5×10^5 identified previously. Two additional treatment groups were included in this series of experiments. In the first (Table 1, group *2*) recipients were given 5×10^5–1×10^6 FACS® sorted α/β-TCR$^-$DN thymocytes resulting in development of IDDM in 10/15 of them ($P < 0.05$, c.f., DN thymocyte–treated group, Fisher's exact test). Members of the other group (Table 1, group *3*) received 4×10^5–5×10^5 α/β-TCR$^+$DN thymocytes and 1/6 developed diabetes (NS, c.f., DN thymocyte–treated group, Fisher's exact test). Thus, protection mediated by DN thymocytes resides in the α/β-TCR$^+$DN population, excluding a role for γ/δ T cells, NK cells, and professional antigen-presenting cells in the process.

The Role of Cytokine Production in NKT Cell–mediated Protection. Pancreatic lymph nodes were removed at the termination of some of the experiments involving use of the induced model of IDDM described above (Table 1, experiments *2* and *3*). Lymphocytes obtained from this source were stimulated for 2 d with immobilized anti-CD3 mAb and ELISAs for IL-4 and IFN-γ were performed on the supernatants. There were no consistent differences in the elicited cytokine responses between cells from the various groups of mice (data not shown). As NKT cells might have exerted their effect at a site other than the draining lymph node, a combination of anti–IL-4 and anti–IL-10 mAb was administered systemically to irradiated recipients that had been given a protective inoculum of these cells. Three of five recipients of diabetogenic splenocytes alone developed IDDM. 15 mice divided into three groups of five were treated with 10^6 DN thymocytes (99.9% pure, 15.3% α/β-TCR$^+$) in addition to the splenocytes. One of five mice in the group given PBS and none of five mice treated with control rat IgG developed IDDM, whereas four of five mice treated with anti–IL-4 and anti–IL-10 mAb did so ($P < 0.05$, c.f., combined control groups, Fisher's exact test; Table 2), indicating that IL-4 and/or IL-10 are important for NKT cell–mediated protection in this system.

Effect of DN Thymocyte Transfer on Systemic Autoimmunity. Some therapies for IDDM, which are associated with response class switching, have been shown to induce a lupus-like syndrome in treated NOD mice, despite exerting a protective effect against diabetes (e.g., BCG; reference 17). Consequently, all of the protected NOD mice treated with DN thymocytes at 3–4 wk were examined at 35 wk for signs of systemic autoimmunity. None of them had a hematocrit below 50%, nor were any positive for Coombs' autoantibodies. Moreover, there was no significant differ-

Table 1. *Protection from Induced IDDM by DN Thymocytes*

Experiment	DN No.	Sorted	Purity	α/β Percent	α/β DN No.	Diabetic (Percent)
Group 1						
1	2.0×10^6	No	81.0	55	8.9×10^5	0/5 (0)
2	5.0×10^5	Yes	99.8	19	9.4×10^4	0/3 (0)
3	5.0×10^5	Yes	99.9	15	7.4×10^4	1/3 (33)
4	5.0×10^5	Yes	99.9	8.9	3.4×10^4	2/3 (67)
Group total						3/14 (21)
Group 2						
2	1.0×10^6	Yes	99.8	–	–	2/3 (67)
3	1.0×10^6	Yes	99.9	–	–	2/3 (67)
4	1.0×10^6	Yes	99.6	–	–	2/3 (67)
2	5.0×10^5	Yes	99.8	–	–	1/2 (50)
3	5.0×10^5	Yes	99.9	–	–	2/2 (100)
4	5.0×10^5	Yes	99.6	–	–	1/2 (50)
Group total						10/15 (67)
Group 3						
2	5.0×10^5	Yes	99.2	–	4.9×10^5	0/2 (0)
3	5.0×10^5	Yes	97.1	–	5.0×10^5	0/2 (0)
4	3.8×10^5	Yes	98.8	–	3.8×10^5	1/2 (50)
Group total						1/6 (17)
Group 4						
1	2.0×10^6	No	4.2*	53	4.5×10^4	2/3 (67)
2	1.0×10^6	No	2.3*	19	4.3×10^3	1/2 (50)
3	1.0×10^6	No	3.4*	25	8.6×10^3	3/3 (100)
4	1.0×10^6	No	2.8*	16	4.4×10^3	1/2 (50)
Group total						7/10 (70)
Group 5						
Group total (experiments 1–4)						7/12 (58)

Group 1, DN thymocyte recipients; group 2, α/β-TCR$^-$ DN thymocyte recipients; group 3, α/β-TCR$^+$ DN thymocyte recipients; group 4, unfractionated thymocytes; group 5, PBS recipients.
*Percent DN of unfractionated thymocytes.

ence in the incidence or level of antinuclear autoantibodies between the groups treated with DN thymocytes, unfractionated thymocytes, or PBS. Finally, only 1/17 DN thymocyte–treated mice had any evidence of C3c deposition in the renal glomeruli, and in this case it was minimal, being detected at a similar level to that seen in 4/25 control NOD mice. In other words, delayed induction of systemic autoimmunity did not occur in mice treated with DN thymocytes.

Discussion

The work described here has led to identification of a population of thymocytes that can protect prediabetic NOD mice from developing IDDM. This effect is likely to be due to NKT cells for several reasons. First, NKT cells were contained within the protective population. Although NKT cells are often defined by the coexpression of cell surface markers characteristic of both T cells (e.g., α/β-TCR) and NK cells (e.g., NK1.1), NK1.1 can not be used for this purpose in strains that do not express this allelic marker, including NOD mice. Moreover none of the other proposed markers such as CD44, CD22, and CD122 adequately define NKT cells in C57BL/6 mice, thereby making it difficult to distinguish and separate NKT cells from a population of whole thymocytes with any degree of certainty. For this reason, we have focussed on α/β-TCR$^+$DN thymocytes, which are known to be highly enriched for NKT cells and can be readily identified in most strains of mice. After transfer of subpopulations of thymocytes into young NOD mice, the spontaneous onset of IDDM was prevented both by CD4$^{-/low}$CD8$^-$ thymocytes, as well as by FACS®-sorted DN thymocytes in which the CD4^{low} thymocytes had been excluded (Fig. 2).

Table 2. *Role of IL-4 and IL-10 in NKT Cell–mediated Protection from Induced IDDM*

Group	Cells transferred		Treatment	Diabetic
	Splenocytes	NKT		
1	2.0×10^7	0	PBS	3/5 (60)
2	2.0×10^7	1.0×10^6	Rat IgG	0/5 (0)
3	2.0×10^7	1.0×10^6	PBS	1/5 (20)
4	2.0×10^7	1.0×10^6	αIL-4/αIL-10	4/5 (80)*

*$P < 0.05$, group 4 versus groups 2 and 3, Fisher's exact test.

The second reason why the protective activity of the thymocyte subsets used here is likely to be due to NKT cells is related to the fact that protection observed in the induced model of IDDM resided within the α/β-TCR$^+$ population and not the α/β-TCR$^-$ (non–T cell) population of DN thymocytes (Table 1). Therefore, this finding not only excluded involvement of NK or γ/δ cells in protection mediated by the DN thymocyte fraction, but also ruled out a protective role for professional APCs that could have exerted an immunostimulatory effect similar to that of treatment with BCG, CFA, IL-2, or Poly(I:C) (15). Moreover, many preventative therapies for IDDM in intact NOD mice, including immunostimulatory agents such as CFA and BCG, are ineffective in the induced model used here, whereas DN thymocytes remained effective, highlighting the potency of the α/β-TCR$^+$ DN cells in protection from IDDM.

The third reason why the protective activity of DN thymocytes is likely to be due to NKT cells is related to the known functional properties of these cells. Depletion of NKT cells with either anti-NK1.1 or anti-Vα14 mAb in C57BL/6.*lpr* mice, C3H.*gld* mice, or (NZB × NZW)F1 mice exacerbated autoimmune disease (18, 19), whereas disease onset was delayed by adoptive transfer of NKT cells into preautoimmune C57BL/6.*lpr* recipients (19). Furthermore, clones expressing the invariant Vα14Jα281 TCR characteristic of NKT cells have been reported that suppress delayed-type hypersensitivity after skin painting with 2,4-dinitrobenzene sulfonate (20) and responses to tumors in vivo and in vitro (21). NKT cells also appear to be responsible for mediating anterior chamber-associated immune deviation after intraocular administration of low doses of foreign antigen (22).

The protective activity of transferred NKT cells in NOD mice appears to be mediated by the Th2-associated cytokines IL-4 and/or IL-10 since neutralization of IL-4 and IL-10 by mAb treatment during the first week after NKT cell transfer inhibited protection by these cells (Table 2). Significantly, the effect of NKT cells described here is consistent with the action of IL-4 and/or IL-10 in other experimental systems involving NOD mice. For example, administration of IL-4 to NOD mice reduced the incidence of spontaneous IDDM (4), whereas NOD mice expressing IL-4 under an insulin promoter were also completely protected from insulitis and diabetes (23). Rabinovich et al. (24) reported that IL-10 together with IL-4 reduced islet cell graft destruction in diabetic NOD mice, whereas according to Pennline et al. (25), administration of IL-10 alone to NOD mice reduced the incidence of spontaneous IDDM. Paradoxically, an accelerated form of diabetes occurred in NOD mice expressing very high levels of IL-10 under an insulin promoter (26, 27), although in non-NOD strains, islet expression of IL-10 did not cause diabetes but was associated with extensive periislet infiltrates similar to those seen in some NKT cell–treated NOD mice described here (28; Fig. 3).

The results reported here, when taken in conjunction with previous data, shed new light on the pathogenesis of IDDM in NOD mice. Although an unopposed Th2 antiislet response can, under some circumstances, lead to IDDM in NOD mice (26, 27), a more typical scenario of spontaneous disease in this strain appears to be a period of mixed intraislet cytokine responses (29, 30) that become polarized over time towards either a Th2 or Th1 phenotype (31, 32). Polarization to a Th1 response probably results in more rapid progression to IDDM because the "destructive" insulitis, which occurs in NOD mice at high risk of IDDM, is associated with a higher frequency of cells producing IFN-γ and a lower frequency of cells producing IL-4 than the "nondestructive" insulitis, which occurs in CFA-treated female NOD mice and male NOD mice (33). Moreover, the destructive antiislet response in diabetic NOD mice is usually accompanied by the production of, and is dependent on, Th1-associated cytokines because administration of anti–IFN-γ mAb reduces both the severity of insulitis and the incidence of diabetes in cyclophosphamide accelerated (34), adoptively transferred (35), and spontaneous IDDM (25).

These findings lead to the concept of "plasticity" of the early, mixed-cytokine phase of insulitis. On the one hand, IL-12 can precipitate IDDM in young NOD mice (36) and cyclophosphamide treatment, which also leads to accelerated onset of IDDM in these mice, is associated with the rapid induction of IL-12 gene expression in vivo (37). On the other hand, NKT cell therapy may prevent onset of IDDM by exerting an opposite effect on the pattern of cytokine production within the intraislet infiltrate. Thus, the directed production of IL-4 and/or IL-10 by NKT cells may redress the emerging polarization of the pattern of intraislet cytokine production resulting in a more balanced cytokine profile associated with a slower rate of islet destruction.

We therefore propose that the deficiency of NKT cells in NOD mice contributes to the pathogenesis of IDDM by permitting a disproportionate Th1 response to emerge. The prevention of IDDM, which occurs when approximately normal numbers of these cells are replaced or their function is simulated by the administration of IL-4 (4), represents one of very few examples of prevention of IDDM by correcting an underlying immunological defect in NOD mice, the only other well-documented instance being correction of the unusual MHC class II haplotype of NOD mice by

transgenesis (38–42). It is important to note that the immunomodulatory activity of NKT cells reported here can interfere with an established effector response as illustrated by their efficacy in the induced model of IDDM, whereas a similar approach based on the use of islet-specific Th2 T cell clones did not appear to be as effective (43, 44).

The ultimate aim of studying models such as the NOD mouse is to translate the findings into clinical practice. NKT cells have been identified in humans and, like their counterpart in the mouse, they express a limited diversity of TCRs. Thus, they express the Vα24, JαQ, and Vβ11 chains, which are homologous to the Vα14, Jα281, and Vβ8.2 chains used by mouse NKT cells (45–49) and produce high levels of IL-4 after stimulation through the TCR (50, 51). It will therefore be important to study NKT cell numbers and function in patients with IDDM and their families to determine whether a defect similar to that found in NOD mice occurs. If it does, such a defect would have profound implications, both for our understanding of the pathogenesis of IDDM, as well for prospective preventative therapy.

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