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 10⁶ CD4⁻⁺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 perilislet, 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) 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

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/J Arc, BALB/c/J 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, echovirus, enterovirus, mouse cytomegalovirus, polyoma virus, murine adenovirus, lymphocytic choriomeningitis virus, mouse pneumonia virus, and reovirus. Sendai virus, T helier’s murine encephalitis virus, B aillus pilliformis, M ycoplasma pulmonis, B ordetella bronchiseptica, C orynebacterium kutscheri, k ldesiella species, P asturella multoidea, P asturella pneumotropia, Pseudomonas aeruginosa, S taphylococcus aureus, S treptococcus pneumoniae, C itrobacter freundii, and S almonella species. N o mice tested positive for any of these pathogens. Mice were housed at 21°C and 40% humidity and were fed mouse chow (Bara stock, M elbourne, 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 R-L72.4) and anti-CD8 (clone 3.155) supernatants at 4°C followed by a 30-min incubation with rabbit complement (C-6x Diagnostics, M ecquon, W I) at 37°C. Viable cells were recovered over Histopaque 1083 (Sigma Chemical Co., C astle Hill, Australia), washed and labeled with anti-CD4-PE (clone R M 4-5; P harmingen, S an Diego, C A), anti-CD8α-allophycocyanin (APC) (clone 53-6.7, P harmingen), and anti-α/β-TCR-FITC (clone H 57-597; P harmingen). In some experiments, DN cells were further purified from the depleted population using a FACStarPLUS® (Becton Dickinson, San Jose, C A) 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 R M 4-5; P harmingen), or anti-CD4-biotin (clone CT-CD4; C altag Laboratories, S an Francisco, C A) and anti-CD8α-APC (clone 53-6.7; P harmingen). Biotinylated mAbs were detected with streptavidin conjugated Texas red (M olecular Probes, E ugene, O R). 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/ml 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, C A).

Adoptive Transfer of Thymocytes

DN thymocyte subpopulations were tested for efficacy in two models of IDDM. In the first model, prediabetic 3- to 4-wk-old female unmanipulated NOD/Lt mice were injected intravenously with 1.0 × 106 to 107 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 × 105 to 106 thymocytes (prepared as described above) in 200 μl of PBS, or with 200 μl of PBS alone immediately followed by 2.0 × 106 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 G lucofim test strips (Bayer Australia, P ymble, Australia) assayed on a Glucometer M + meter (Bayer D iagnostics, P ymble, Australia). Neutrophils of IL-4 and IL-10 by mAb Treatment In V i vo.

mAbs 11B11 rat anti–mouse IL-4 (IgG1) and J E5-2A5 rat anti–mouse IL-10 (IgG1; gifts of J ohn A brams, D NAX, P ao Alto, C A) were grown as ascites in pristane-primed (1 ml/mouse intraperitoneally, 2, 6, 10, 14 tetramethylpentadecane 98%; A ldrich C hemical Company, M ilwaukee, W I) B ALB/c-nu/nu mice. As cites 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 A quacide (C albiochem, L a Jolla, C A). 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 cm². 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 analysis was achieved with 0.02 mg buprenorphine (Reckitt and Coleman, Bristol, U.K.) injected subcutaneously immediately before surgery. 8 days 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 Inc., Elkhart, IN) and frozen on a mixture of OCT compound (Miles, Elkhart, IN) and frozen on a mixture of OCT compound (Miles) and frozen on a mixture of OCT compound (Miles) and frozen on a mixture of OCT compound (Miles) and frozen. 30 min at RT with 50% PBS were then washed three times for 5 min in PBS and incubated for 30 min at RT in a moist chamber. Slides were incubated in PBS for 10 min. Sera diluted in PBS and then cut into segments ~1 cm².
with controls that either received whole thymocytes (13/19, \( P < 0.0001 \), \( \chi^2 \) fourfold table test) or PBS (12/21, \( P < 0.001 \), \( \chi^2 \) 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\(^{\circ}\) 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 \( \alpha/\beta \)-TCR\(^{+}\) cells within each inoculum, varied between experiments; consequently, the total numbers of \( \alpha/\beta \)-TCR\(^{+}\)CD4\(^{low}\)/CD8\(^{-}\) or \( \alpha/\beta \)-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 \( \times 10^5 \)) of \( \alpha/\beta \)-TCR\(^{+}\)/DN thymocytes, consistent with a minimum of \( \sim 1.5 \times 10^5 \) \( \alpha/\beta \)-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 perislet lymphoid collections with intact islets. One third of the mice had no signs of insulitis or other infiltrates at all, whereas the remainder had intraislet infiltrates qualitatively similar to those normally found in nondiabetic untreated mice (Fig. 3).

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

**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).

IDDM Protection by DN T lymphocytes is N or associated with tolerance to A alloantigen. The preceding experiments indicated that protective cells reside in the DN population of thymocytes, but did not tell us whether or not \( \alpha/\beta \)-TCR\(^{+}\) cells mediate this effect as \( \gamma/\delta \) 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 alloimmune responses of young NOD recipients, 4-wk-old female NOD mice were injected with 3.0 \( \times 10^6 \) complement-depleted BALB \( \times \) NOD/F1 thymocytes (>96% CD4\(^{low}\)/CD8\(^{-}\); 39.9% \( \alpha/\beta \)-TCR\(^{+}\); equates to \( 10^6 \) \( \alpha/\beta \)-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 lymphocytes 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 \( \times 10^6 \) 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).
Varying numbers \((5.0 \times 10^5-2.0 \times 10^6)\) of DN thymocytes from nondiabetes prone \((N\text{OD} \times \text{BALB})\)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%, \(P<0.05\), \(x^2\) fourfold table test; Table 1). The numbers of cells transferred, as well as the relative proportion of \(\alpha/\beta\)-TCR\(^+\) cells within each inoculum, varied between experiments so that the total numbers of \(\alpha/\beta\)-TCR\(^+\)CD4\(^{low}\)CD8\(^+\) thymocytes injected ranged from \(\sim 3 \times 10^4\) to \(9 \times 10^6\)/mouse. The only treated mice to develop IDDM received the lowest numbers of \(\alpha/\beta\)-TCR\(^+\)CD4\(^{low}\)CD8\(^+\) thymocytes, which were below the threshold of \(1.5 \times 10^5\) identified previously. Two additional treatment groups were included in this series of experiments. In the first (Table 1, group 2) recipients were given \(5 \times 10^5-1 \times 10^6\) FACS\(^\circledast\)-sorted \(\alpha/\beta\)-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 \(4 \times 10^5-5 \times 10^6\) \(\alpha/\beta\)-TCR\(^+\)DN thymocytes and 1/6 developed diabetes (N.S., c.f., DN thymocyte-treated group, Fisher’s exact test). Thus, protection mediated by DN thymocytes resides in the \(\alpha/\beta\)-TCR\(^+\)DN population, excluding a role for \(\gamma/\delta\) 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-\(\gamma\) 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% \(\alpha/\beta\)-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-

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, \(\times 400\); B, \(\times 200\)) or unfractionated thymocytes (C, \(\times 400\)).
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.

**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 × 10^3 | Yes    | 99.8   | 19          | 9.4 × 10^4 | 0/3 (0)          |
| 3          | 5.0 × 10^3 | Yes    | 99.9   | 15          | 7.4 × 10^4 | 1/3 (33)         |
| 4          | 5.0 × 10^3 | 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    |        |        |        |             |            | 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.
of spontaneous IDDM (4), whereas NOD mice expressing
ministration of IL-4 to NOD mice reduced the incidence
imental systems involving NOD mice. For example, ad-
consistent with the action of IL-4 and/or IL-10 in other exper-
IL-10 by mAb treatment during the first week after NKT
mice appears to be mediated by the Th2-associated cyto-
doses of foreign antigen (22).

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

| Group | Splenocytes | NKT Cells | Treatment | Diabetic |
|-------|-------------|-----------|-----------|---------|
| 1     | 2.0 x 10^7  | 0         | PBS       | 3/5 (60) |
| 2     | 2.0 x 10^7  | 1.0 x 10^6| R at IgG  | 0/5 (0)  |
| 3     | 2.0 x 10^7  | 1.0 x 10^6| PBS       | 1/5 (20) |
| 4     | 2.0 x 10^7  | 1.0 x 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+ popula-
tion and not the α/β-TCR− (non-T cell) population of DN
thymocytes (Table 1). Therefore, this finding not only ex-
cluded involvement of NK or γ/δ cells in protection medi-
ated by the DN thymocyte fraction, but also ruled out a pro-
tective 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 preven-
tative therapies for IDDM in intact NOD mice, including
immunostimulatory agents such as CFA and BCG, are ine-
effective in the induced model used here, whereas DN
thymocytes remained effective, highlight-
ing the potency of the α/β-TCR+ DN cells in protection
from IDDM.

The third reason why the protective activity of DN thym-
cytes 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-α/β mAb in
C57BL/6 mice, C3Hlpr mice, or NZB × NZWF1 mice exacer-
band autoimmunity disease (18, 19), whereas disease
onset was delayed by adoptive transfer of NKT cells
into preautoimmune C57BL/6 mice (19). Fur-
thermore, many clones expressing the invariant Vα14/α281 TCR
characteristic of NKT cells have been reported that could
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 cyto-
kines 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 con-
sistent with the action of IL-4 and/or IL-10 in other exper-
imental systems involving NOD mice. For example, ad-
ministration 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 pro-
tected 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 ac-
according to Pennline et al. (25), administration of IL-10
alone to NOD mice reduced the incidence of spontane-
ous IDDM. Paradoxically, an accelerated form of diabetes oc-
curred 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 perilislet 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 anti-
set response can, under some circumstances, lead to IDDM
in NOD mice (26, 27), a more typical scenario of sponta-
neous 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” insu-
litis, 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 fe-
male NOD mice and male NOD mice (33). Moreover, the
destructive antiset response in diabetic NOD mice is usu-
ally 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 acceler-
ated 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 cy-
tokine production within the islet infiltrate. Thus, the
directed production of IL-4 and/or IL-10 by NKT cells
may redress the emerging polarization of the pattern of in-
traislet cytokine production resulting in a more balanced
cytokine profile associated with a slower rate of islet de-
struction.

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 cor-
recting 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 V\textsubscript{a}24, J\textsubscript{a}Q, and V\textsubscript{b}11 chains, which are homologous to the V\textsubscript{a}14, J\textsubscript{a}281, and V\textsubscript{b}8.2 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 as for prospective preventative therapy.

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