Overexpression of Natural Killer T Cells Protects Vα14-Jα281 Transgenic Nonobese Diabetic Mice against Diabetes

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Summary

Progression to destructive insulitis in nonobese diabetic (NOD) mice is linked to the failure of regulatory cells, possibly involving T helper type 2 (Th2) cells. Natural killer (NK) T cells might be involved in diabetes, given their deficiency in NOD mice and the prevention of diabetes by adoptive transfer of α/β double-negative thymocytes. Here, we evaluated the role of NK T cells in diabetes by using transgenic NOD mice expressing the T cell antigen receptor (TCR) α chain Vα14-Jα281 characteristic of NK T cells. Precise identification of NK1.1$^+$ T cells was based on out-cross with congenic NK1.1 NOD mice. All six transgenic lines showed, to various degrees, elevated numbers of NK1.1$^+$ T cells, enhanced production of interleukin (IL)-4, and increased levels of serum immunoglobulin E. Only the transgenic lines with the largest numbers of NK T cells and the most vigorous burst of IL-4 production were protected from diabetes. Transfer and cotransfer experiments with transgenic splenocytes demonstrated that Vα14-Jα281 transgenic NOD mice, although protected from overt diabetes, developed a diabetogenic T cell repertoire, and that NK T cells actively inhibited the pathogenic action of T cells. These results indicate that the number of NK T cells strongly influences the development of diabetes.

Key words: nonobese diabetic • type 1 diabetes • natural killer T cells • transgenic mice • interleukin 4

Nonobese diabetic (NOD)[1] mice develop a spontaneous form of type 1 diabetes very similar to the human disease (1). Autoimmune destruction of β cells is preceded by infiltration of pancreatic islets by macrophages and B and T lymphocytes. The key role of T cells in diabetes development is demonstrated by the capacity of purified T cells or T cell clones to transfer the disease to immunoincompetent NOD mice (2). The disease evolves in two stages. Noninvasive insulitis begins at 3 wk of age but does not result in destructive insulitis (at the origin of clinical diabetes) before 3–4 mo of age. This long period of clinically silent insulitis and its progression to diabetes are best explained by the involvement of immunoregulatory T cells (1). Converging data suggest that cytokine imbalance plays a key role in the pathogenesis of diabetes, with overexpression of Th1 diabetogenic T cells relative to defective regulatory Th2 cells. Systemic administration of IL-4 or anti–IFN-γ mAb prevents diabetes onset in NOD mice (3–5). Conversely, IL-12, which promotes the development of Th1 cells, accelerates disease onset in NOD mice (6). Furthermore, diabetogenic T cell clones derived from NOD mice exhibit a Th1 phenotype (7–9), and cytokine production in the islets of NOD mice correlates with the severity of histological lesions: IL-4–producing cells are associated with nondestructive insulitis and IFN-γ–producing cells are associated with destructive insulitis (10, 11).

Recently, it has been shown that NOD mice and patients with insulin-dependent diabetes mellitus (IDDM) have decreased numbers of NK T cells, a subset of α/β T cells which express surface receptors that are normally associated with the NK lineage (12, 13). These cells, which are either CD4$^+$ or CD4$^-$CD8$^-$ double-negative (DN), are positively selected by the conserved MHC class I–like CD1 molecule and have a highly restricted TCR repertoire (14–19). In both mice and humans, they express an invariant TCR α chain composed of Vα14 and Jα281 segments in mice, or Vα24 and JαQ segments in humans (14, 17). In a

[1]*Abbreviations used in this paper:* DN, double negative; IDDM, insulin-dependent diabetes mellitus; NOD, nonobese diabetic; SA-APC, streptavidin-allophycocyanin.

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study of twins discordant for diabetes, a correlation was observed between the occurrence of IDDM and the loss of the capacity of Vα24-JαQ$^+$ T cells to secrete IL-4 (13). Similarly, in 3–6-wk-old NOD mice, IL-4 production is markedly reduced both in vitro (thymocytes stimulated by anti-CD3 antibody) and in vivo (spleen cells after CD3 antibody injection) (12). The NK T cell defect in NOD mice might account for the deficient IL-4 production by thymocytes and splenocytes from this strain (3). In nonpathological strains of mice, large amounts of IL-4 released by NK T cells appear to favor Th2 over Th1 responses (20–22). Therefore, this defect in NOD mice could be responsible, in part, for the emergence of antiislet Th1 cells, leading to the onset of destructive insulitis and diabetes. Baxter and colleagues have recently reported that the transfer into 3–4-wk-old NOD mice of α/β$^+$ CD4$^-$CD8$^-$ double-negative (α/β DN) thymocytes prevented diabetes development (23). However, because NK T cells are not the only subset present in α/β DN thymocytes, it could not be concluded that protection had been specifically mediated by those lymphocytes. Furthermore, the fact that for technical reasons, semiallogeneic instead of syngeneic thymocytes had to be inoculated raised the concern of a possible allogeneic effect being responsible for reduced disease incidence (24).

To obtain more direct evidence in favor of a control exerted by NK T cells upon diabetes, we adopted a different strategy, consisting in generating a panel of transgenic lines of NOD mice overexpressing the invariant TCR α chain, Vα14-Jα281, of NK T cells. We observed a heterogeneous increase in NK T cell numbers, IL-4 production, and serum IgE level that varied according to the line of Vα14-Jα281 transgenic mice considered. Most interestingly, the three transgenic lines that had the largest numbers of NK T cells were also those that manifested resistance against the development of diabetes.

Materials and Methods

Constructs, Transgenesis, and Mice. A construct coding for the TCR α chain Vα14-Jα281 was used as previously described for transgenesis of C57BL/6 mice (22). This TCR α chain was cloned from a CD1-reactive T cell hybridoma, DN32D3 (14). Electroeluted DNA was microinjected into fertilized NOD eggs, and transgenic mice were identified by PCR of tail DNA, using transgene-specific primers. We used heterozygous mice, as they provide perfect controls within the same litters. The Cα$^{-/-}$ mutation was introduced into Vα14-Jα281 transgenic mice (line 86) by two consecutive crosses with NOD Cα$^{-/-}$ mice (a gift from Christophe Benoist and Diane Mathis, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France). Transgenic mice homozygous for the Cα null mutation were first selected by immunofluorescence of blood lymphocytes and then confirmed by PCR typing of their progeny. Congenic NOD.NK1.1 mice were established as a homozygous line at backcross 10. The chromosome 6 segment containing the NK receptor complex and integrated into the NOD genome originally derived from the C57BL/6 strain (C. Carnaud, unpublished data). NOD scid/scid mice were originally obtained from Leonard Schultz, The Jackson Laboratory (Bar Harbor, ME). All of the strains of mice used throughout this study, including BALB/c, C57BL/6, and NOD, were raised and housed in strictly controlled specific pathogen–free conditions.

Flow Cytometry. Cell suspensions from thymus and spleen 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. For thymocytes, four-color staining was performed with PE-conjugated anti-CD8 (Caltag Laboratories, Inc., San Francisco, CA), Red 613–conjugated anti-CD4 (GIBCO BRL, Gaithersburg, MD), FITC-conjugated anti–TCR-α/β (H57 mAb), biotinylated anti-NK1.1 (PK 136; PharMingen, San Diego, CA), and streptavidin-allophycocyanin (SA-APC; PharMingen). For splenocytes, two-color staining was performed using PE-conjugated anti-CD4 (Caltag Laboratories, Inc.) and Red 613–conjugated anti-CD8. For three-color staining, biotinylated anti-NK1.1, FITC-conjugated anti-α/β, PE-conjugated anti–Thy-1 (30H12 mAb), and SA-APC were used. Cells were analyzed on a FACSCalibur® (Becton Dickinson, Mountain View, CA) using CellQuest software.

Cytokine Release after CD3 Stimulation. For in vivo CD3 stimulation, 4 μg of anti-CD3 mAb 2C11 was injected intravenously. Spleens were removed 90 min later, reduced to single-cell suspensions, and cultured, without further stimulation, at a density of 10^7 cells in 1 ml of complete medium (RPMI containing 10% FCS, glutamine, 2-ME, penicillin-streptomycin) for 2 h to measure IL-4 release in the culture supernatants. For other cytokines (IFN-γ, IL-2, and IL-10), splenocytes were incubated at the same density for 24 h in anti-CD3–coated plates. IL-4 was quantitated in a bioassay using the IL-4–dependent CT-4S cell line (25) in the presence of anti–IL-2 mAb S4B6 at 10 μg/ml, or in an ELISA using mAbs 11B11 (26) and BVD6 (DNAX Research Institute, Palo Alto, CA). IFN-γ, IL-2, and IL-10 were measured by ELISAs using mAbs AN18, R46A2, 1A12, 5H4, JES5, and SXC1 (the last five mAbs were a gift from DNAX Research Institute). Recombinant mouse IL-4, IL-10, and IFN-γ were from R & D Systems (Abingdon, Oxfordshire, UK), and IL-2 was from Genzyme Corp. (Cambridge, MA).

Serum Ig Isotype Levels. Serum levels of Ig isotypes were measured by using standard sandwich ELISAs. Specific polyclonal antibodies against IgG1, IgG2a, IgG2b, and IgG3 (Southern Biotechnology Associates, Inc., Birmingham, AL) and the anti-IgE mAb LOME (Caltag Laboratories, Inc.) were used for coating, and alkaline phosphatase–conjugated anti-IgG (Sigma Chemical Co., St. Louis, MO) or biotinylated anti-IgE LO-ME-2 (Biosys, Compiègne, France) plus SA-AP (Amersham International, Les Ulis, France) was used for detection.

Diabetes Incidence and Histology. Females and males were tested every week for diabetes. Overt diabetes was defined as two consecutive positive glucosuria tests and glycemia >200 mg/dl. Glukotest and Haemoglukotest were purchased from Boehringer Mannheim (Mannheim, Germany). For cyclophosphamide-induced diabetes, 10-wk-old males were injected once intraperitoneally with 300 mg/kg cyclophosphamide (Sigma Chemical Co.) diluted to 30 mg/ml in PBS immediately before injection. Insulitis was evaluated on noncontiguous 4-μm-thick sections of pancreas from 11–12-wk-old females. Sections were stained with hematoxylin and eosin. Insulitis was scored according to the degree of infiltration.

Transfer Experiments. Recipients were 4–5-wk-old scid NOD mice. According to the type of experiment, diabetogenic splenocytes were obtained from overtly diabetic mice or from old asymptomatic donors. After red cell lysis in NH_4Cl, the percentage of T cells was determined by immunofluorescence staining, and

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the equivalent of 1 or 2 × 10^6 T cells were injected intravenously, alone or with other lymphocyte populations. Splenocytes from transgenic mice of line 86 $C\alpha^{-/-}$ were further depleted of B cells by panning on anti-IgM–coated plates. Immunofluorescence staining was performed, and the equivalent of 10^7 T cells (α/β^+) were coinjected with diabetogenic T cells (200 μl/mouse). In one experiment, the same number of control cells (splenocytes from $C\alpha^{-/-}$ mice) were injected. In transfer experiments, splenocytes were prepared from three groups of female NOD mice: nondiabetic transgenic, control littermates of >52 wk of age, or overtly diabetic mice. Spleens from mice of each group were pooled, red cells were lysed, and B cells were partially removed by panning on anti-IgM–coated plates. The percentage of T cells in each population was determined by immunofluorescence staining and varied between 53 and 75%. The equivalent of 2 × 10^6 T cells were injected intravenously into scid NOD females. The same experiment was performed with splenocytes from old transgenic nondiabetic males, using diabetic males as positive controls.

Statistical Analysis. Differences in cytokine production were analyzed using Student's *t* test. Diabetes incidence was studied using the log rank test.

Results

Vα14-Jα281 Transgenic NOD Mice Have an Increased Number of NK1.1⁺ T Cells. Although α/β DN thymocytes have often been used as a reliable source of NK T cells (23), they are heterogenous in their composition and contain both $NK1.1^+$ and $NK1.1^-$ lymphocytes. As illustrated in Fig. 1, $NK1.1^+$ T cells represent roughly 1/3 (27%) of the α/β DN compartment. The remaining 2/3 include Vα14-positive and -negative cells as well as CD1 nonrestricted lymphocytes, clearly identified in CD1 knockout mice (Park, S.-H., and A. Bendelac, unpublished results). Thus, in order to evaluate precisely the role of NK T cells in the pathogenesis of diabetes, we generated a panel of NOD founders expressing the invariant TCR α chain of NK T cells, using the Vα14-Jα281 construct validated in C57BL/6 mice (22). As NOD mice do not express the NK1.1 allele, founder lines were first screened by immunofluorescence analysis of lymphoid organs for an increased percentage of DN thymocytes expressing a Vβ8-biased repertoire (data not shown). By crossing with NK1.1 congenic NOD mice, we confirmed that the six selected transgenic lines had an increased frequency of $NK1.1^+$ T cells in the thymus and spleen (Fig. 2, and Table 1). In the 6 lines, the increase in $NK1.1^+$ α/β T cell numbers varied from 2.5- to 6.2-fold in the spleen. Interestingly, $NK1.1^+$ T cells were mainly of the DN phenotype, whereas the remainder expressed CD4 (Fig. 2). The transgenic line 86 (with the highest frequency of $NK1.1^+$ T cells) was further crossed with a $C\alpha^{-/-}$ NOD mouse to eliminate endogenous α chain rearrangements. Surprisingly, the percentage of $NK1.1^+$ T cells was not significantly modified, suggesting that the pairing of the transgenic α chain with various β chains allowed the development of a large number of T cells without the NK1.1 marker. As described previously, $NK1.1^+$ T cells in all six lines expressed intermediate levels of TCR and were positive for the two activation markers CD44 and CD122 (data not shown). These results showed that enforced expression of the Vα14-Jα281 chain in

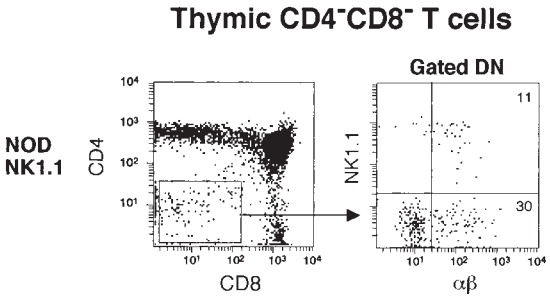

Figure 1. NK T cells represent only a fraction of α/β $CD4^-CD8^-$ DN thymocytes. Thymocytes from an 8-wk-old NOD NK1.1 mouse were quadruply stained with mAbs anti-CD4, anti-CD8, anti–TCR-α/β, and anti-NK1.1. Percentage values are relative to the gated DN population.

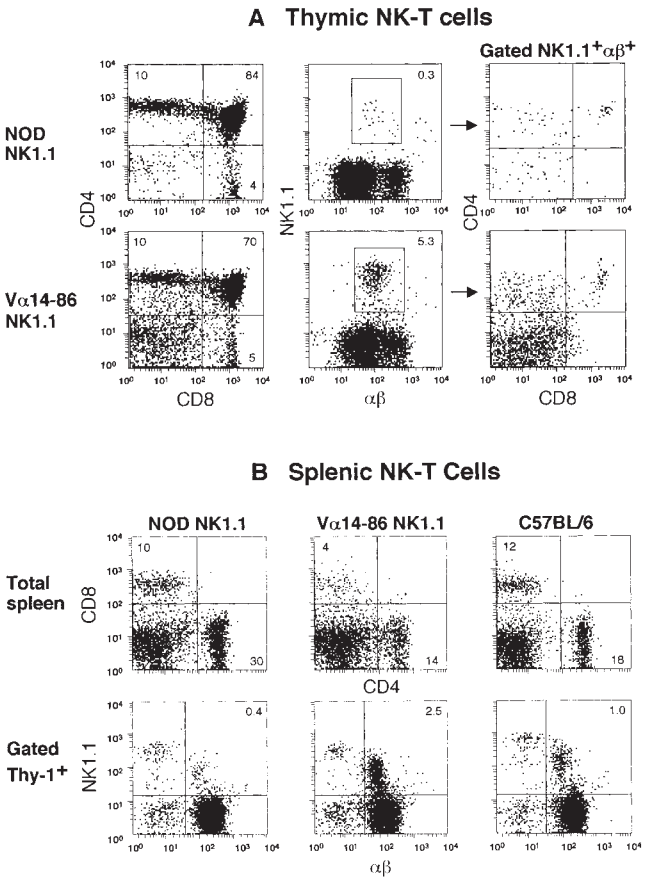

Figure 2. Increased expression of $NK1.1^+$ T cells in Vα14-Jα281 transgenic mice. (*A*) Thymocytes from an 8-wk-old Vα14-Jα281 transgenic (line 86) NOD NK1.1 mouse and a littermate control were quadruply stained with mAbs anti-CD4, anti-CD8, anti–TCR-α/β, and anti-NK1.1. Percentage values are relative to the whole thymocyte population. (*B*) Splenocytes from a 12-wk-old Vα14-Jα281 transgenic (line 86) NOD NK1.1 mouse, a littermate control, and a C57BL/6 mouse were either dually stained with anti-CD4 and anti-CD8, or triply stained with anti–Thy-1, anti–TCR-α/β, and anti-NK1.1. Percentages are relative to the whole splenocyte population.

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Table 1. *T Cell Subsets in the Spleen of the Various Transgenic Lines (Females ≥6 wk of age)*

Mice	Cell no.	α/β^{+}*‡	α/β^{+} $CD4^{+}$*‡	α/β^{+} $CD8^{+}$*‡	α/β^{+} DN*‡	α/β^{+} $NK1.1^{+}$‡	α/β^{+} $NK1.1^{+}/\alpha/\beta^{+}$§
	$\times 10^{-6} \pm SD$	%	%	%	%	%	%
NOD	113 ± 34 (n = 64)	38.3 ± 6.1	25.5 ± 4.4	8.4 ± 1.5	4.4 ± 1.9	0.4 ± 0.2 (n = 11)	1.0
Tg A14-14	95 ± 33 (n = 7)	33.1 ± 7.0	16.1 ± 2.2	5.2 ± 1.6	11.8 ± 3.4	1.0 ± 0.1 (n = 3)	3.0
Tg A14-36	101 ± 34 (n = 7)	36.0 ± 2.4	18.0 ± 1.4	6.0 ± 1.1	12.0 ± 2.5	1.2 ± 0.1 (n = 4)	3.3
Tg A14-10	87 ± 23 (n = 5)	36.3 ± 7.8	15.8 ± 2.7	5.7 ± 1.6	14.8 ± 4.9	1.4 ± 0.3 (n = 2)	3.9
Tg A14-95	89 ± 31 (n = 19)	31.1 ± 0.6	14.7 ± 1.5	4.2 ± 0.3	12.2 ± 1.7	1.9 ± 0.7 (n = 2)	6.1
Tg A14-78	101 ± 43 (n = 21)	32.2 ± 1.9	14.1 ± 1.0	3.5 ± 0.4	14.6 ± 1.5	2.1 ± 0.2 (n = 3)	6.5
Tg A14-86	74 ± 28 (n = 26)	31.8 ± 1.0	13.3 ± 0.3	3.3 ± 0.6	15.2 ± 1.1	2.5 ± 1.4 (n = 14)	7.9
Tg A14-86 CαKO	89 ± 43 (n = 5)	24.4 ± 3.7	5.7 ± 1.0	1.9 ± 0.4	16.8 ± 3.0	1.9 ± 0.5 (n = 8)	7.8
C57BL/6	73 ± 8 (n = 8)	32.4 ± 2.7	18.4 ± 1.2	12.3 ± 0.7	1.7 ± 0.8	0.9 ± 0.2 (n = 9)	2.8

Tg, transgenic. *KO*, knockout.
*n = 3.
‡Percent of the cell population in the whole spleen.
§Percent of the ratio.

NOD mice leads to a substantial increase in $NK1.1^{+}$ T cells in both the thymus and periphery.

Increased Cytokine Production by Splenocytes after CD3 Stimulation. We then measured IL-4 release after in vivo stimulation by anti-CD3 mAb in 10-wk-old transgenic and wild-type female littermates. This assay rigorously reflects NK T cell activity (27). As shown in Fig. 3, splenocytes from the six transgenic lines released higher amounts of IL-4 than those from control NOD mice. In three of the lines, levels were comparable to those obtained with BALB/c and C57BL/6 mice, i.e., four to six times the amount released by control NOD mice. In the other three lines, the increase was 13–25-fold relative to the amount released by control NOD mice. Interestingly, the increase in IL-4 production ran parallel to the increase in $NK1.1^{+}$ T cell numbers in the six transgenic lines. As NK T cells can produce cytokines other than IL-4 and influence the production of various cytokines by other T cells (16), we also measured the production of IFN-γ, IL-2, and IL-10 after in vitro culture on anti-CD3–coated plates. Splenocytes from lines 95, 78, and 86 released slightly more (1.4–1.8-fold) IFN-γ than controls, but the differences were not statistically significant. IL-2 production by splenocytes from the six lines was decreased relative to controls, whereas IL-10 production was slightly increased. These data clearly showed that NK T cells in the transgenic mice were functional.

In all six lines, the enhancement of IL-4 production after in vivo triggering by anti-CD3 antibody was already observable at 3 wk of age (data not shown), indicating that functional NK T cells are present at a higher frequency in the spleen of transgenic mice before islet infiltration begins.

Selective Increase in Th2-associated Ig Isotypes. IgE and IgG1 production is a characteristic feature of Th2 responses. If overexpression of NK T cells indeed results in higher production of IL-4, which in turn can shift the immune system towards a Th2 phenotype, increased Th2-controlled Ig isotype serum levels should be found. As shown in Fig. 4 *A*, transgenic NOD mice from line 86 had elevated levels of serum IgE (4-fold) and IgG1 (1.3-fold) compared with control littermates, whereas the levels of the other isotypes were unchanged. All transgenic lines had increased levels of serum IgE compared with control NOD mice, and the increase in serum IgE levels was strongest in line 86, which expressed the largest number of NK T cells (Fig. 4 *B*). Interestingly, line 86 $C\alpha^{-/-}$ exhibited even higher serum IgE levels. Generally, IL-4 and IgE levels were lower in male transgenics than in their female counterparts (data not shown).

Incidence of Spontaneous Diabetes in Females and Males. The incidence of diabetes in females was decreased in three lines, corresponding to those with the highest NK T cell numbers (Fig. 5 *A*). The level of protection in each line matched the increase in NK T cell numbers and in the IL-4

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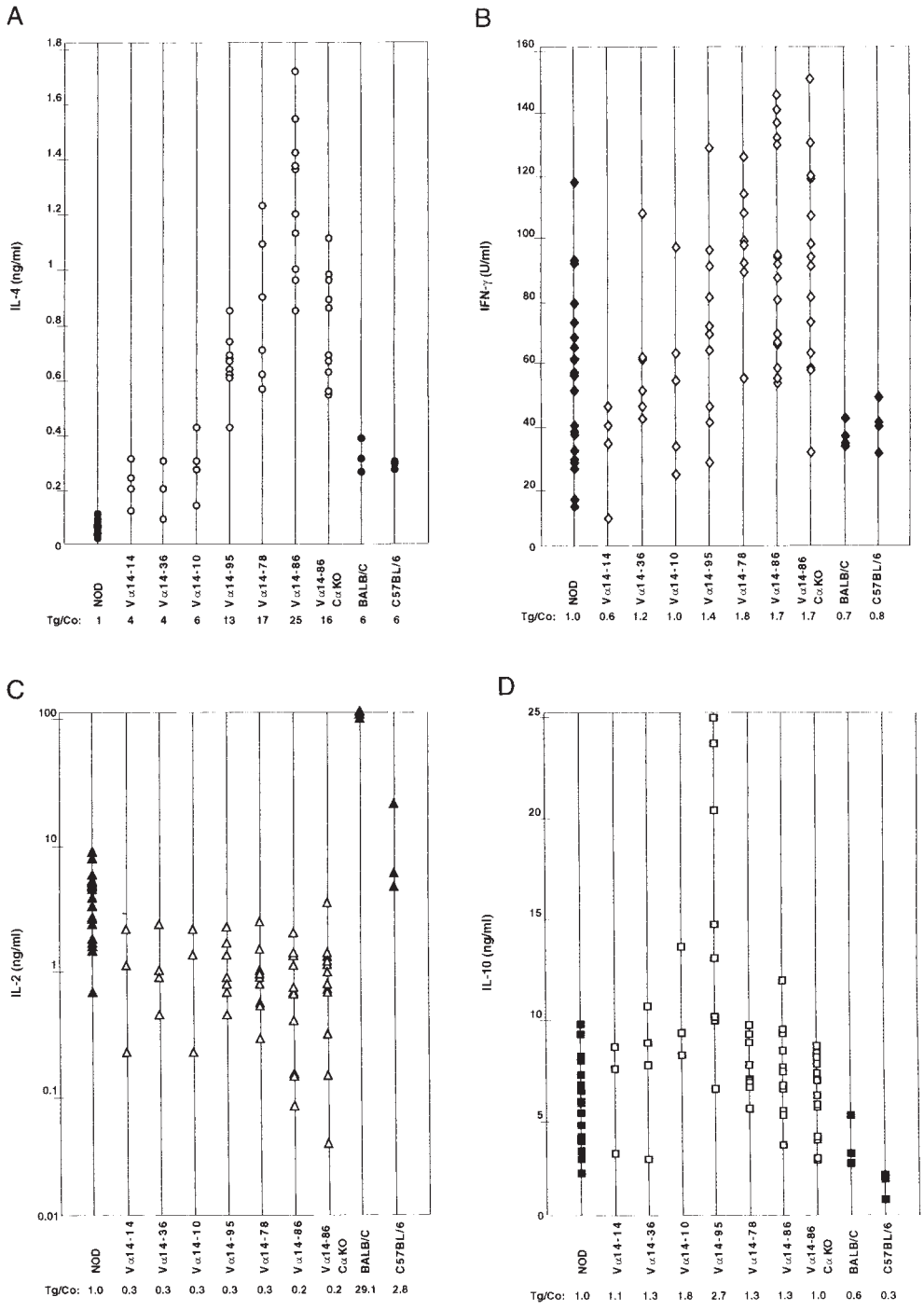

Figure 3. Cytokine production by splenocytes from Vα14-Jα281 transgenic mice. Levels of cytokines produced in vitro by splenocytes after in vivo anti-CD3 stimulation. (*A*) IL-4 production was measured in supernatants after 2 h in culture without further stimulation, and (*B*) IFN-γ, (*C*) IL-2, and (*D*) IL-10 production was measured after 24 h in culture on anti-CD3–coated plates. Females from 6 transgenic lines, line 86 $C\alpha^{-/-}$, 20 negative NOD littermates, and controls (BALB/c and C57BL/6) were analyzed at 10 wk of age. Each point represents an individual mouse; *filled symbols*, control mice; *open symbols*, transgenic NOD mice.

burst. Line 86 was the best protected ($P < 0.0001$, log rank test), followed by line 78 ($P = 0.004$) and line 95 ($P = 0.04$). The incidence of diabetes in lines 10, 14, and 36 (intermediate numbers of NK T cells) was not significantly different from that in control NOD mice. The incidence rates were compared with those in transgene-negative littermates of all six lines, and to that in a larger group of NOD mice housed in the same facilities (data not shown). Among males, only those from line 86 (Fig. 5 *B*) showed a reduced incidence of the disease ($P = 0.04$). Males from lines 95 and 78 had the same incidence as their control littermates. Histologic studies were performed on the pancreas of 11–12-wk-old females of line 86 and their negative littermates (data not shown). Islet infiltration was present in both groups but was less invasive in transgenic than in control mice, resulting in a slightly lower percentage of healthy islets in the latter (39 vs. 52%). Therefore, histological findings were consistent with clinical findings.

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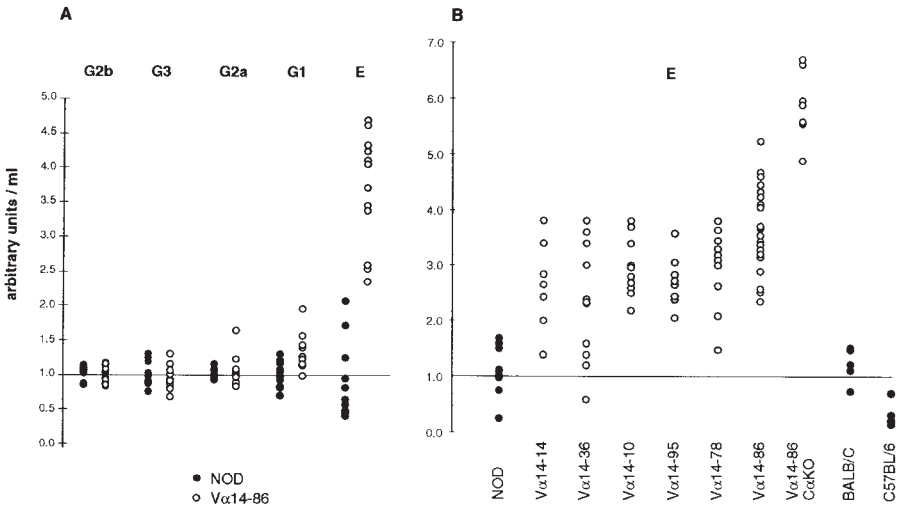

Figure 4. Increased levels of Th2-controlled Ig isotypes in Vα14-Jα281 transgenic mice. Serum levels of Ig isotypes were measured in 10–16-wk-old females. The average level of each isotype in control female NOD mice is set arbitrarily at 1 U/ml. (*A*) Serum Ig isotypes of female transgenics from line 86 and their negative littermates. (*B*) Serum IgE in females from the six Vα14-Jα281 transgenic lines, line 86 $C\alpha^{-/-}$, NOD, BALB/c, and C57BL/6 mice.

Diabetes Incidence in Males after Cyclophosphamide Injection. Diabetes can be accelerated in 10-wk-old NOD males by a single injection of cyclophosphamide (300 mg/kg). In two consecutive experiments, males of line 86 appeared to be resistant to diabetes acceleration by cyclophosphamide, whereas males from line 36 (intermediate $NK1.1^+$ T cell numbers) developed diabetes at the same frequency as wild-type NOD controls (Fig. 5 *C*). This suggested that resistance to cyclophosphamide-induced diabetes was only achieved when NK T cells reached a given threshold count.

Adoptive Transfer. To gain further insight into the mode of action of NK T cells, enriched populations of splenocytes from $C\alpha^{-/-}$ transgenic mice belonging to line 86 were coinjected with 2×10^6 diabetogenic T cells. The addition of such a population significantly enriched in NK T cells delayed the development of diabetes in scid NOD recipients, whereas a control population of $C\alpha^{-/-}$ but non–Vα14-Jα281 transgenic splenocytes had no effect (Fig. 6 *A*). Interestingly, when the number of diabetogenic T cells was reduced to 10^6, diabetes transfer was more efficiently inhibited, pointing to a quantitative relationship between pathogenic cells and actively protecting cells (Fig. 6 *B*).

To determine if mice of transgenic line 86 which escaped overt diabetes nevertheless harbored diabetogenic T cells, splenocytes from 1-yr-old clinically silent transgenic donors were transferred into NOD scid recipients. For comparison, splenocytes from nondiabetic wild-type littermates and splenocytes from recently diagnosed diabetic NOD mice were injected in parallel. The results demonstrated the presence of diabetogenic T cells in transgenic mice. Splenocytes from Vα14-Jα281 transgenic mice were as efficient as those from diabetic NOD mice in inducing diabetes in scid recipients (Fig. 7 *A*). Similar results were obtained when the experiment was performed with splenocytes from transgenic males of line 86 (Fig. 7 *B*). Thus, diabetogenic T cells develop normally in diabetes-free transgenic mice but are actively suppressed by NK T cells.

Discussion

Enforced expression of the Vα14-Jα281 TCR α chain in NOD mice results in overexpression of NK T cells as reported previously in C57BL/6 mice (16). This augmentation can be visualized phenotypically in the spleen and thymus by measuring the percentage of DN $NK1.1^+$ α/β T cells, and functionally by the burst of IL-4 release shortly after an intravenous injection of anti-CD3 mAb. The significant increase in serum IgE provides further confirmation that NK T cells, which can produce massive amounts of IL-4 shortly after triggering of their TCR, promote the production of Th2-controlled Ig isotypes in vivo, with no particular stimulation of the immune system. It is not certain that the subset of α/β DN spleen cells, which was increased markedly in all the transgenic lines, corresponds entirely to NK T cells. α/β DN cells can be found in various TCR transgenic mice (28, 29), even when TCR transgenes are not specific for CD1 and are not related to NK T cells. We prefer a more conservative definition of NK T cells, i.e., cells which are positive for the NKRP1 receptor (NK1.1) and α/β TCR. Even with this definition, the increase in NK T cells remained impressive in our Vα14-Jα281 transgenic mice (7.9% of all α/β cells vs. 1% in controls).

Interestingly, the increase in NK T cell numbers in the spleen was variable in the six transgenic lines analyzed in this study, ranging from 3 to 7.9%. The production of this panel of transgenic lines allowed us to determine if the increase in NK T cell numbers up to and beyond the levels in BALB/c and C57BL/6 mice modified the incidence of diabetes. We found that the three transgenic lines (lines 36, 14, and 10), which expressed 2.5–3.5 times more NK T cells than control NOD mice, produced similar amounts of IL-4 on in vivo stimulation by anti-CD3 mAb to those found in BALB/c and C57BL/6 mice, and elevated serum IgE levels compared with NOD mice. However, these transgenic lines had a similar incidence of diabetes as wild-type NOD mice. Conversely, the other three transgenic

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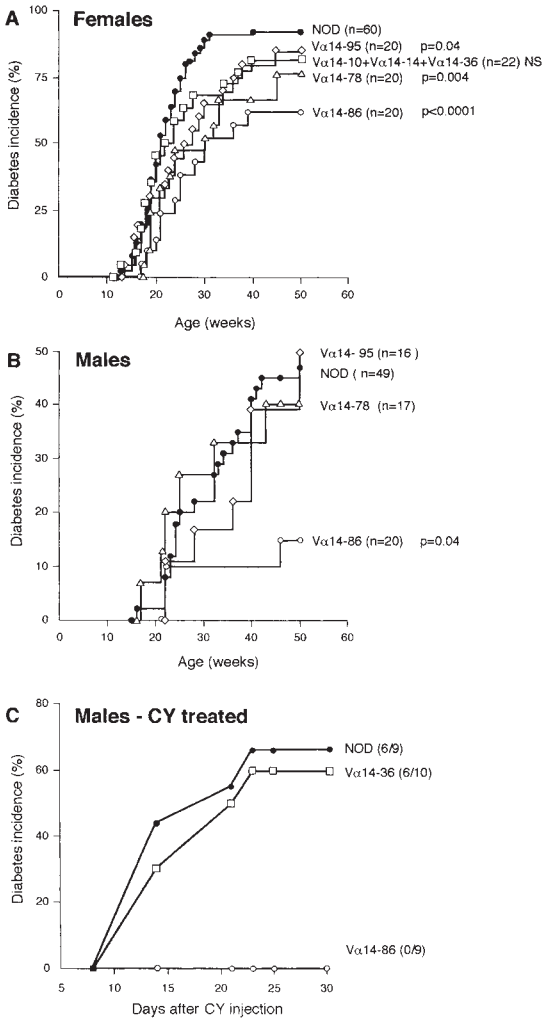

Figure 5. Overexpression of NK T cells protects against diabetes development. (*A*) Incidence of spontaneous diabetes in Vα14-Jα281 transgenic females of lines 86, 78, 95, and 10 plus 14 plus 36, compared with negative littermates. The incidence in the latter three lines was not significantly different from each other, and values were thus pooled. (*B*) Incidence of spontaneous diabetes in males from lines 95, 78, and 86 compared with negative littermates. (*C*) Diabetes incidence after one injection of cyclophosphamide (*CY*; 300 mg/kg) in 10-wk-old males from Vα14-Jα281 transgenic line 86 and line 36 and their negative littermates.

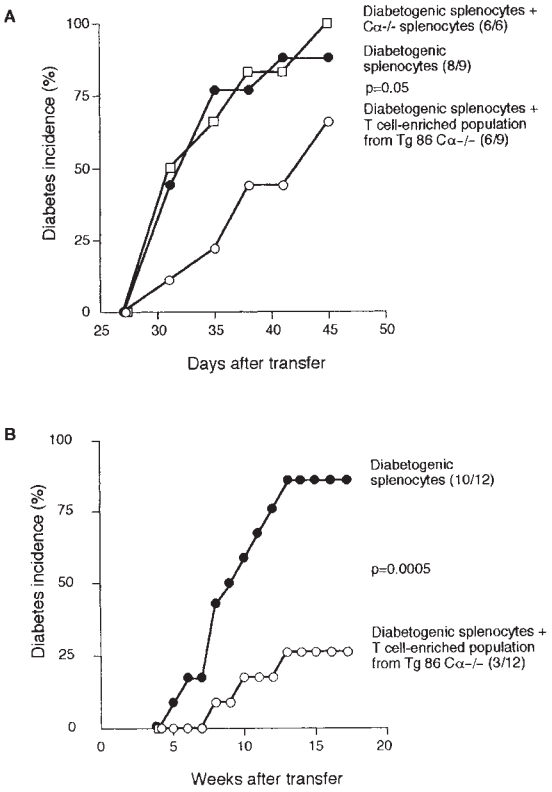

Figure 6. Active protection by cotransfer of NK T cell–enriched populations from transgenic donors. NOD scid males (5-wk-old) were injected intravenously with a mixture of cells containing diabetogenic T cell–enriched lymphocytes from freshly diagnosed diabetic mice (2×10^6 in *A*, 10^6 in *B*), and 10^7 T cell–enriched lymphocytes from $C\alpha^{-/-}$ transgenic mice and the same number of splenocytes from $C\alpha^{-/-}$ nontransgenic mice. T cells were purified by panning on anti-μ–coated plates; the percentage of T cells in each population varied from 75 to 80%.

lines, with larger increases in NK T cell numbers, manifested, at least in females, a significantly reduced disease incidence compared with their control littermates. This was even more marked in the case of cyclophosphamide-accelerated disease. Thus, against a polygenic background conducive to diabetes, the disease incidence can be reduced only by raising the number of functional NK T cells.

The possibility that the protection achieved in three independent lines of NOD transgenic mice could be due to insertional interference of the transgene on genes required for diabetes development is unlikely. More importantly, defects such as incomplete T cell repertoire build-up, crippled T cell ontogeny, and abnormal subset development, which could possibly account for the reduced diabetes incidence in TCR α chain transgenic mice, were excluded by transfer experiments. First, cotransfer of Vα14-Jα281 T cells with diabetogenic splenocytes from nontransgenic diabetic female NOD mice prevented the development of diabetes in scid recipients. This suggests that Vα14-Jα281 T cells can suppress pathogenic autoreactive T cells. Second, in lines 95 and 78, males clearly generated sufficient amounts of diabetogenic T cells to cause clinical disease. Only females were partially protected in these lines. Third, splenocytes from old (>1 yr) nondiabetic Vα14-Jα281 transgenic females and males belonging to line 86 contained diabetogenic T cells, as revealed by transfer into NOD scid mice. Surprisingly, these splenocytes were as efficient at transferring diabetes as splenocytes from diabetic mice. This suggests that diabetogenic T cells accumulate in the spleen of aging transgenic mice and are inhibited by the increased number of NK T cells. After transfer to scid recipients, diabetogenic splenocytes seem to escape from immunoregulation by NK T cells, probably because the ratio between protective and pathogenic cells is modified in the scid recipients. The importance of the ratio between the two populations was clear in the cotransfer experiments

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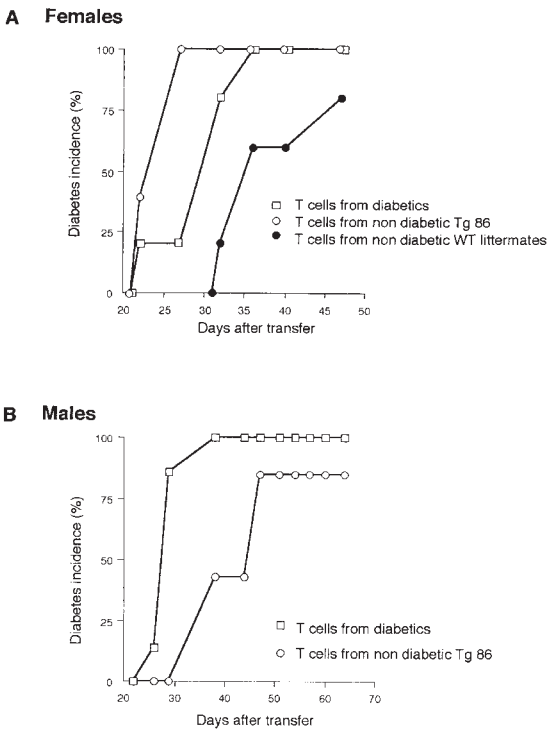

Figure 7. Healthy NOD mice from the protected transgenic line Vα14-Jα281 line 86 contain diabetogenic T cells. 2×10^6 T cell–enriched splenocytes from 1-yr-old transgenic or wild-type nondiabetic mice (*A*, females; *B*, males) were injected intravenously into 5-wk-old female or male NOD scid recipients ($n = 5$). T cell–enriched splenocytes from freshly diagnosed diabetic females were used as positive controls. T cell enrichment was performed as in the legend to Fig. 6.

(Fig. 6). Altogether, these results show that (*a*) TCR α chain transgenic mice develop a T cell repertoire with diabetogenic precursors, and that (*b*) Vα14-Jα281 T cells can regulate diabetogenic T cells.

The implication of NK T cells in autoimmune conditions does not seem to be restricted to NOD mice. Human IDDM patients have a reduced number of Vα24-JαQ$^+$ DN peripheral blood lymphocytes, and these cells show deficient IL-4 production (13). In other models of autoimmunity, such as lpr/lpr, NZB, and BxW lupus mice, there is a decrease in NK T cell numbers at the onset of disease (30, 31). SJL mice, which have a profound deficiency in NK T cells, are highly susceptible to experimental allergic encephalomyelitis, a Th1-mediated autoimmune disease (32).

To explain the impact of NK T cells on diabetes development, we postulate that these cells induce a shift in the balance of antiislet T cells from the pathogenic Th1 phenotype toward a less harmful Th2 phenotype. Our preliminary analysis of intraislet cytokine mRNA from transgenic females of line 86 supports this view, by clearly showing a decrease in IFN-γ mRNA and an increase in IL-4 mRNA (data not shown). This result is reminiscent of previous observations on the role of Th1 cells as inducers of diabetes, and the protective role of IL-4. Indeed, diabetes is accelerated by systemic treatment with IL-12 and delayed by systemic treatment with anti–IFN-γ mAb, IL-12 antagonist $(p40)_2$, or IL-4, or by local islet expression of IL-4 in insulin promoter–IL-4 transgenic mice (3–6, 33, 34). This local action of NK T cells will require further investigation. It is, for instance, possible that NK T cells are turned on locally by increased expression of their ligand, CD1. Alternatively, other signals initiated by the inflammatory process, such as B7.1 and B7.2, CD40, or even more upstream signals such as those postulated in the case of necrotic cell death (35), may locally alert NK T cells. Like other members of the innate immune system, NK T cells may respond to a series of ill-defined predetermined stimuli via TCR and NK receptors (16, 36, 37). In conclusion, our observations support the particular role of NK T cells in immunoregulation and their potential to control autoimmune disorders.

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