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Prevention of Diabetes in Nonobese Diabetic Mice Mediated by CD1d-Restricted Nonclassical NKT Cells

Nadia Duarte, Martin Stenström, Susana Campino, Marie-Louise Bergman, Marie Lundholm, Dan Holmberg, and Susanna L. Cardell

A role for regulatory lymphocytes has been demonstrated in the pathogenesis of type 1 diabetes in the NOD mouse but the nature of these cells is debated. CD1d-restricted NKT lymphocytes have been implicated in this process. Previous reports of reduced diabetes incidence in NOD mice in which the numbers of NKT cells are artificially increased have been attributed to the enhanced production of IL-4 by these cells and a role for classical NKT cells, using the Vα14-Jα18 rearrangement. We now show that overexpression in NOD mice of CD1d-restricted TCR Vα3.2*Vβ9+ NKT cells producing high levels of IFN-γ but low amounts of IL-4 leads to prevention of type 1 diabetes, demonstrating a role for nonclassical CD1d-restricted NKT cells in the regulation of autoimmune diabetes. The Journal of Immunology, 2004, 173: 3112-3118.

Type 1 diabetes (T1D) is an autoimmune disease where lymphocytes destroy the insulin-producing β cells of the pancreatic islets of Langerhans. The development of T1D is influenced by multiple genetic and environmental factors of which most remain unknown. The NOD mouse strain (1) spontaneously develops a disease very similar to human T1D, and constitutes a major animal model used for investigating the cause of autoimmune diabetes (2). At 3–4 wk of age, the pancreas of NOD mice starts to be infiltrated by immune cells. Subsequently, the insulitis becomes invasive and destructive, and diabetes occurs from around 12 wk of age.

The effect of regulatory cells of lymphoid origin is well established in the control of the autoimmune disease including the diabetes pathogenesis of NOD mice (3). However, the nature of this cell population(s) remains largely elusive, and multiple cellular subsets have been ascribed the capacity to inhibit diabetes development. The original demonstration of a regulatory role of CD4+ T cells in the NOD pathogenesis (4, 5) has been followed by several reports on different subsets with capacity to inhibit diabetes development including TCRγδ T cells (6), CD25+CD4+ T cells (7), and CD62L+CD4+ (8, 9) T cells.

More recently, CD1d-restricted NKT lymphocytes have been implicated in the regulation of autoimmune disease in humans and mice (10). NKT cells are a subset of TCRαβ cells, most of which recognize lipid Ags presented on the MHC class I-like molecule, CD1d, (11) and which coexpress surface markers normally found on NK cells (12). NOD mice (13, 14), like human diabetes patients (15, 16), have a small and functionally defective NKT cell population lending support to the notion that this cellular subset may have important regulatory functions in T1D. Further, NOD mice lacking CD1d and CD1d-dependent NKT cells display an accelerated onset and increased incidence of disease (17–19). Conversely, transfer of double negative (DN) TCRαβ+ thymocytes, a population enriched for CD1d-restricted T cells, into young NOD mice prevents the development of diabetes in the recipient (14).

Although the regulatory influence of CD1d-restricted cells in autoimmune diseases is well established, the mechanism of disease regulation, as well as the precise nature of the regulatory NKT cell subset, remains less well understood. CD1d-restricted cells make up a heterogeneous population with a large proportion of CD1d-restricted NKT cells displaying a semiconserved, canonical TCR (20) consisting of an invariant Vα14-Jα18 TCRβ chain, combined with diverse TCRβ chains using a limited number of Vβ regions (12). These cells display CD69 on the surface and are efficient producers of both IL-4 and IFN-γ upon stimulation. A second set, called nonclassical CD1d-restricted NKT cells, have diverse TCR (21–23), produce high levels of IFN-γ, low levels of IL-4, and are CD69+CD49b+ (24). The potential of the canonical CD1d-restricted subset to regulate diabetes was shown in a series of elegant experiments using TCR-transgenic mice (25–27). However, to date, the role in autoimmune diabetes of the nonclassical CD1d-restricted T cell population with diverse TCR has not been addressed. To investigate the capacity of the latter subset to regulate autoimmune diabetes, we have analyzed a TCR-transgenic mouse model, carrying CD1d-restricted TCR of the diverse type on the NOD genetic background. Diabetes development was found to be efficiently suppressed in this transgenic line and transgene-expressing NKT cells could prevent diabetes in adoptive transfer experiments, demonstrating, for the first time, a role for nonclassical

Abbreviations used in this paper: T1D, type 1 diabetes; CD1d-tet, CD1d-tetramer; DN, double negative.

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4 Abbreviations used in this paper: T1D, type 1 diabetes; CD1d-tet, CD1d-tetramer; DN, double negative.
CD1d-restricted NKT cells in the regulation of autoimmune diabetes.

Materials and Methods

Mice

C57BL/6, NOD, and 24αβNOD mice were bred and maintained in a specific-pathogen-free animal facility at the Gulbenkian Institute for Science, the Biomedical Center at Lund University (Lund, Sweden), and at the Umeå Center for Molecular Medicine at Umeå University (Umeå, Sweden). The 24αβ TCR-transgenic mice were made directly on a NOD genetic background using TCR expression constructs described before (28), encoding a CD1d-reactive TCR. Each TCR chain construct (containing transgenic TCRα and TCRβ regions, respectively) was microinjected alone to create single chain transgenic mice. Transgenic founders were screened by flow cytometry for transgene expression on PBL (see further below). Mice positive for the transgenic TCRα and β chains were intercrossed to obtain 24αβNOD mice expressing the complete transgenic TCR. The mice were between 6 and 16 wk of age when used in the experiments, unless otherwise indicated. Female NODscid mice were used for transfer experiments at the age of 5–9 wk.

Cell preparation

The mice were killed by cervical dislocation and single cell suspensions were prepared from the spleen, lymph nodes, thymus, and bone marrow in HBSS (Invitrogen Life Technologies, Paisley, U.K.) containing 1% HEPES. To obtain liver mononuclear cells, the livers were pressed through a cell strainer and suspended in 25 ml of PBS. After being washed once with PBS, the cells were fractionated by discontinuous (27 and 80%) percoll gradient centrifugation for 20 min at 850 g. The interface was harvested, and cells were washed with PBS and used for experiments. Viable lymphocytes were counted after trypan blue staining. Where indicated, B cells were depleted by panning. Plate-bound rabbit anti-mouse Ig Ab were used to deplete spleen cells of B cells as follows: petri dishes (Labora Laboratorie-produkter, Partille, Sweden) were coated with polyclonal rabbit anti-mouse Ig Abs. Spleen cells were added, 25 × 10^6 cells in 3 ml HBSS-FCS. The plates were incubated for 90 min at 4°C before nonadherent cells were carefully harvested. After panning, the cell suspensions contained 2.0–2.5% B cells.

Flow cytometry

Before staining, the cells were incubated with the 2.4G2 (anti-CD16/CD32) Ab to block nonspecific binding. The following mAbs or second step conjugates were bought from BD Pharmingen (San Diego, CA): Vα3.2-biotin, Vβ9-FITC, TCRβ-CyChrome, TCRβ-APC, TCRβ-FITC, CD8α-PerCP, CD4-PerCP, CD4-biotin, CD44-PE, CD69-PE, CD69-FITC, CD122-FITC, CD49b(DX5)-PE, CD49b-FITC, B220-PE, NK1.1-PE, IL-4-APC, IL-4-PE, IFN-γ-FITC, streptavidin-APC, streptavidin-PerCP and isotype controls. Streptavidin-PE was from Southern Life Science (Little Chalfont, U.K.). Invariant CD1d-restricted cells were detected with a PE-labeled CD1d-tetramer (CD1d-tetramer; Ref. 29) loaded with α-galactosylceramide (αGalCer; Ref. 30), kindly provided by Dr. M. Kronenberg (La Jolla Institute of Allergy and Immunology, San Diego, CA). The stained cells were analyzed by four-color flow cytometry on a FACSCalibur (BD Biosciences, San Jose, CA) using CellQuest software (BD Biosciences).

In vitro stimulation and immunofluorescence staining of intracellular cytokines

Cell suspensions from spleens were depleted from B cells as described above. B cell-depleted spleen cells or thymocytes were stimulated in vitro for 4 h with ionomycin and PMA in the presence of brefeldin A at 1 × 10^6 cells/ml in complete RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FCS, 1 mM sodium pyruvate, 10 mM HEPES buffer, 100 U/ml penicillin/streptomycin, and 50 mM 2-ME (all supplements from Invitrogen Life Technologies). The cells were harvested and stained for surface expression as described above. After surface staining, the cells were fixed in 2% paraformaldehyde in PBS, and kept in FACS wash (2% FCS and 0.05% NaN3 in PBS) overnight at 4°C. The cell membranes were permeabilized in 0.5% saponin, 1% BSA, 0.1% NaN3 in PBS, and intracellularly stained for IL-4 and IFN-γ production. The cells were washed and analyzed as described above.

Diabetes incidence

From 10 wk of age, mice were analyzed weekly for glucosuria, using Clinistix (Bayer, Pittsburgh, PA). Mice that had two consecutive positive glucosuria tests were scored as diabetic and sacrificed.

Histology

Insulitis was evaluated on 8-μm thick, frozen sections of the pancreas taken at several levels throughout the organ. The sections were stained with H&E dyes and lymphocytic infiltration was scored for each islet. A minimum of 30 islets were analyzed for each animal. Peri-insulitis was recorded when islets showed inflammatory cells in their surroundings but no invasion had yet occurred. Mild insulitis was scored when inflammatory

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FIGURE 1. Expression of transgenic TCR chains. Surface expression on CD4+, CD8+, and CD4-CD8- (DN) splenocytes (A) and thymocytes (B) of the TCR-transgenic Vα3.2 and Vβ9 chains. Cells from 24αβ transgenic (upper rows) and wild-type (Wt) littermate mice (lower rows) were stained for CD4, CD8, Vα3.2, and Vβ9 expression as described in Materials and Methods. CD4 and CD8 expression on total splenocytes (A) and thymocytes (B) is shown in the far left panels. CD4+, CD8+, and CD4-CD8- cells (for thymus only) were gated and analyzed for the expression of the transgenic TCR chains as indicated. Numbers show frequencies of cells in respective quadrants.
cells had invaded <25% of the islet and severe insulitis was recorded when inflammatory cells had invaded >25% of the islet. For statistical analysis, the nonparametric Mann-Whitney U test was used to compare the proportion of infiltrated islets (the total score of mild insulitis and severe insulitis) between NOD control and 24αβNOD transgenic mice.

**Cotransfer experiments**
The 24αβ cells were obtained from 24αβNOD spleens. B cells were depleted by panning, as described above. The depleted cell suspensions had B cell contamination of 1.5-3.5% B220+ cells. Diabetogenic splenocytes were obtained from diabetic wild-type NOD mice (13-16 wk of age), diagnosed by two consecutive positive glucosuria tests. For the test of diabetes inhibition, female NODscid mice were i.v. injected with 2 × 10⁶ sex-matched diabetogenic splenocytes, either alone or cotransferred with a number of B cell-depleted splenocytes corresponding to 10³ sex-matched TCRβ+ 24αβNOD splenocytes (equivalent of 2.5-3.6 × 10⁶ 24αβ T cells). Donor 24αβNOD mice were non diabetic and 11-23 wk of age. The cells were suspended in PBS and injected in a volume of 200 μl per mouse. Recipients were tested every week for diabetes and diagnosed as described above. NODscid recipients were injected at 5-9 wk of age. For statistical analysis the log-rank test was used to compare the cumulative incidence of diabetes between the two groups of mice.

**Results**

**NOD mice transgenic for a variant CD1d-restricted TCR**
To investigate the regulatory function of nonclassical CD1d-restricted NKT cells on the development of autoimmune diabetes, we established NOD mice transgenic for the 24αβ TCR. The CD1d-reactive 24αβ TCR uses rearranged Vα3.2 and Vβ9 segments (21), and was previously shown on a C57BL/6 (B6) genetic background to direct the development of CD49bhigh NK1.1+ T cells producing high amounts of IFN-γ, and low amounts of IL-4, upon activation (24, 28). For the present investigation, novel transgenic lines were made separately for each of the two TCR chains directly on the NOD background, and the two lines were intercrossed to produce 24αβNOD mice expressing the complete CD1d restricted TCR. Transgenic TCR chains were detected with Abs specific for the Vα3.2 and Vβ9 regions on the transgenic TCRα and β chains respectively. Both transgenic TCR chains were expressed in CD4+ and CD8+ T lymphocytes in the single chain transgenic mice. The transgenic TCRα chain was expressed by 20 and 40% of CD4+ and CD8+ T cells, respectively, while the TCRβ chain was expressed by 70 and 90% of CD4+ and CD8+ T cells, respectively (data not shown). Thus, neither of the transgenes prevented the production of cells using endogenous TCR chains. In 24αβNOD mice expressing both transgenic TCR chains, the CD4/ CD8 ratios of spleen cells and thymocytes were normal (Fig. 1). A population of cells carrying both transgenic TCR chains was found among CD4+ and CD4−CD8− cells in both organs, while this population was less manifest among CD8+ cells (Fig. 1). In comparison, populations expressing the transgenic β-chain but not the transgenic α-chain were more frequent. Importantly, invariant CD1d-restricted NKT cells were not detectable in 24αβNOD spleen using a CD1d-tet loaded with αGalCer (29), a synthetic ligand (30) for these cells (data not shown).

**Transgenic NKT cells**
We next analyzed whether the T lymphocytes carrying the transgenic 24αβ TCR had the typical characteristics of NKT cells. NKT cells can be identified by the expression of TCRαβ and NK1.1 in B6 mice (12). In the absence of the NK1.1 Ag in the NOD mouse strain, we used a panel of other markers known to be expressed by NKT cells (Fig. 2). Transgenic T cells in the spleen expressed CD122 as well as high levels of the late activation marker, CD44, as expected for NKT cells. Most of the cells (around 70%) were CD4−CD8−, although a CD4+ population and a minor (<10%) CD8+ population were present. Further, most transgenic cells were negative for the CD69 marker, while around 50% of the cells were CD49b+ and around 30% were Ly49G2+2. This surface marker profile is essentially identical with that found on 24αβ transgenic T cells on the B6 background, cells associated with high production of IFN-γ but very little IL-4 (24, 28). This phenotype contrasts to that of invariant CD1d-restricted cells in both NOD and B6 mice that were CD69+ and CD49blow (Fig. 2C). As illustrated in Fig. 3, transgenic cells were most frequent among T lymphocytes in spleen and liver, followed by pancreatic lymph nodes, and lower in bone marrow and inguinal lymph nodes.

![Figure 2](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/)
Cytokine profile of transgenic NKT cells

Transgenic lymphocytes were stimulated polyclonally to establish their cytokine production profile. Single cell analysis of intracellular cytokines revealed that a high proportion of the cells displaying both transgenes on the surface expressed intracellular IFN-γ, while a relatively low frequency of these cells contained IL-4 (Fig. 4). This was found in both splenocytes and thymocytes. This pattern of cytokines is similar to that found in noncanonical NKT cells in normal B6 mice, as well as to that found in transgenic NKT cells in 24αβ B6 mice (24), while a large fraction of invariant CD1d-restricted T cells in both NOD and B6 mice synthesized IL-4 and IFN-γ. Simultaneous analysis of IL-4 and IFN-γ production in 24αβNOD cells suggested that the two cytokines were primarily made by distinct cells within the population. Thus, the transgenic 24αβ TCR allowed the development of NKT cells in NOD mice, with the typical surface markers and cytokine profile of noncanonical NKT cells.

24αβNOD mice were protected from diabetes but not insulitis

To determine whether the transgenic expression of a nonclassical CD1d-restricted TCR influenced the incidence of disease, we followed diabetes development in 24αβNOD mice (Fig. 5). In female transgene-negative control mice, the incidence of diabetes reached a level close to 75% at 27 wk of age. Strikingly, only 2 of 30 female 24αβNOD mice followed for 45 wk developed disease, demonstrating a dramatic effect by expression of the transgenic CD1d-restricted TCR. Just over 30% of male littermate control NOD mice were scored diabetic during this time, while only 1 of 37 tested 24αβNOD transgenic male mice became diabetic (data not shown).

To assess the degree of lymphocyte infiltration in the protected 24αβNOD mice, pancreata from 12-wk-old NOD-transgenic and NOD control female mice were examined histologically and scored for insulitis (Fig. 6). We observed islet invasion and destruction in the 24αβNOD transgenic mice, which demonstrates that these animals harbor autoaggressive cells able to mount an autoimmune response. However, when compared with NOD control mice the proportion of infiltrated islets was significantly lower in the transgenic 24αβNOD (p < 0.03).

24αβNOD splenocytes prevented diabetes after transfer

The finding that 24αβNOD mice develop severe infiltration at 12 wk of age, despite being protected from disease until at least 45 wk...
of age, suggested that autoaggressive T cells developed in these mice but were prevented from full destruction of the insulin-producing β cells. Therefore, we determined whether the transgenic 24αβNOD cells could actively prevent diabetes. A total of 2 × 10⁶ diabetogenic spleen cells isolated from female diabetic NOD mice were injected into female NODscid mice alone, or together with B cell-depleted spleen cells from female transgenic 24αβNOD mice. The number of transgenic cells injected contained 10⁷ total T lymphocytes of which ~30% expressed both transgenic TCR chains. Among mice receiving only diabetogenic cells, all animals developed diabetes between 5 and 11 wk after transfer (Fig. 7). In contrast, during the 15-wk experimental period, 5 of 13 mice which had also received transgenic cells did not develop disease, and in the 8 mice that were scored diabetic, disease was delayed and occurred 7–14 wk after transfer. The difference between the two groups was highly significant (p < 0.0001, log-rank test). This demonstrated that B cell-depleted transgenic splenocytes can actively prevent or delay diabetes induction by diabetogenic spleen cells when transferred into NODscid mice.

Discussion

It is well established that the development of T1D is affected by regulatory cells, and defects in regulatory populations have been proposed to contribute to disease susceptibility in NOD mice which serve as an important model for human T1D (2). A diversity of regulatory cell types has been reported to reduce or prevent disease in the NOD system. Several studies in mice and humans have reported the regulatory T cell subset (13–16, 25, 31), although there are reports which have questioned this hypothesis (32–34). We show here that transgenic expression of a CD1d-restricted, Vα3.2-Vβ9 TCR in NOD mice leads to an increase in nonclassical CD1d-restricted NKT cells, and prevents development of T1D. Whereas this is similar to the effect previously seen in NOD mice transgenically expressing a canonical CD1d-restricted TCR (25), it demonstrates for the first time a role for nonclassical CD1d-restricted NKT cells in the regulation of autoimmune diabetes. Our data show that transgenic expression of a noncanonical CD1d-restricted TCR can direct the development of NKT cells characterized by expression of CD122, inhibitory Ly49 receptors, CD44, and on the B6 background NK1.1 (24, 28). This resembles the effect of transgenic expression of the canonical Vα14-Jα18 TCR (25, 35). However, while transgenic Vα14-Jα18 TCRα chain expression results in an NKT cell population producing high amounts of IL-4 (24, 25, 35), the transgenic 24αβ T cells produce only limited amounts of IL-4 but high amounts of IFN-γ (24, 28). Moreover, while the former cells express CD69 but low levels of CD49b on the surface, the latter express only low levels of CD69 but high levels of CD49b (24). We denote these cells NKT2 (the Vα14-subset) and NKT1 (with diverse TCR), respectively. We have recently described that the two transgenic NKT subpopulations have the characteristic functional capacity and phenotypic markers of canonical and noncanonical NK1.1⁺TCRβ⁺ splenocytes, respectively, in normal B6 mice (24). Recent publications have implied a CD49b⁺CD4⁻ T (36) or CD49b⁻NK1.1⁺ T (37) cell population in the prevention...
of autoimmune disease, including in a model for autoimmune diabetes (36). It is tempting to speculate that these studies concern regulatory nonclassical CD1d-restricted cells of the NKT1-type. Our data demonstrate that CD1d-restricted TCR other than the canonical Vα14-TCR can be used by regulatory T cells in the NOD diabetes model. There is a difference in specificity of the CD1d-restriction between the two TCR, but their natural selecting or activating ligands have not been identified. However, both the canonical and the noncanonical CD1d-restricted TCR showed autoactivity to CD1d (20, 21), suggesting that a relatively high reactivity to an endogenous ligand might be a common feature.

IL-4 and/or IL-10 have been suggested by several investigators to be crucial mediators of regulatory cells, including NKT cells, in the NOD model (3, 14, 26). These cytokines would favor a shift from an anti-islet T cell response of Th1 phenotype to a Th2 phenotype, thereby converting an autoregressive response to a more harmless process (3). In support of this notion, activation of the canonical subset of CD1d-restricted NKT cells by administration of harmless process (3). In support of this notion, activation of the nonobese diabetic (NOD) model (3, 14, 26). These cytokines would favor a shift to be crucial mediators of regulatory cells, including NKT cells, in accordance with the observation that administration of IL-4 prevents diabetes onset in this mouse strain (3). The production of IL-4 by transgenic 24αBNOD cells was low compared with that of invariant CD1d-restricted cells in NOD mice, however, this does not exclude IL-4 secretion as a mechanism through which transgenic 24αBNOD cells mediate their regulatory capacity. In contrast, it is also possible that the two NKT cell subsets prevent diabetes through different mechanisms, or through a common mechanism independent of IL-4. Other regulatory mechanisms such as IFN-γ production, which characterizes both NKT cell subsets, have been indeed suggested to be responsible for the prevention of disease (39). A possible cellular target for NKT cells may be dendritic cells. NKT cells may act through the regulation of their function (18), indirectly preventing the activation of diabetogenic T cells.

CD1d-restricted NKT cells are generally thought to mediate their effects early during immune responses, due to their rapid cytokine secretion upon activation in vivo (12). However, while diabetes development was almost completely inhibited in the late stage in the autoimmune process. This is further supported by the observation that transgenic 24αBNOD cells are able to inhibit the development of diabetes induced in NODscid mice by spleen cells transferred from diabetic NOD donors. In line with this, transgenic NOD Vα14-cells were able to interfere with functional maturation, scored as reduced IFN-γ secretion and prevention of disease induction, but not initial activation and expansion of diabetogenic cells (27). This is reminiscent of the recently reported regulatory effect of CD49b+CD4+ T cells carrying endogenous TCR on the diabetes which occur in BDC2.5 TCR-transgenic mice. Here the regulatory cells were found to execute control of autoaggressive cells or to direct them into an anergic state, rather than to prevent their development (36). With the present report, it is now clear that both classical and nonclassical NKT cells can modulate autoimmunity. However, it remains open whether the CD1d-restricted NKT cell subsets are equally efficient, and whether they use the same mode of action to mediate this regulatory effect.

Future experiments aiming to elucidate these issues will be important for the understanding of the role of NKT cells in diverse immunological situations including in the regulation of autoimmunity. The 24αBNOD mice provide a useful tool for the elucidation of how nonclassical CD1d-restricted cells intervene in the autoimmune process. Knowing the detailed mechanisms of NKT cell activation and function, it may be possible to develop strategies to induce NKT cells selectively to reduce harmful autoreactivity, and to successfully prevent autoimmune disease development at an early stage, before irreversible damage has been done to crucial tissues.

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