Cytometric and functional analyses of NK and NKT cell deficiencies in NOD mice


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Abstract
Defects in NK and NKT cell activities have been implicated in the etiology of type 1 (autoimmune) diabetes in NOD mice on the basis of experiments performed using surrogate phenotypes for the identification of these lymphocyte subsets. Here, we have generated a congenic line of NOD mice (NOD.b-Nkrp1b) which express the allelic NK1.1 marker, enabling the direct study of NK and NKT cells in NOD mice. Major deficiencies in both populations were identified when NOD.b-Nkrp1b mice were compared with C57BL/6 and BALB.B6-Cmv1r mice by flow cytometry. The decrease in numbers of peripheral NK cells was associated with an increase in their numbers in the bone marrow, suggesting that a defect in NK cell export may be involved. In contrast, the most severe deficiency of NKT cells found was in the thymus, indicating that defects in thymic production were probably responsible. The deficiencies in NK cell activity in NOD mice could only partly be accounted for by the reduced numbers of NK cells, and fewer NKT cells from NOD mice produced IL-4 following stimulation, suggesting that NK and NKT cells from NOD mice shared functional deficiencies in addition to their numerical deficiencies. Despite the relative lack of IL-4 production by NOD NKT cells, adoptive transfer of αβ TCR+c9059/NK1.1+c9059 syngeneic NKT cells into 3-week-old NOD recipients successfully prevented the onset of spontaneous diabetes. As both NK and NKT cells play roles in regulating immune responses, we postulate that the synergistic defects reported here contribute to the susceptibility of NOD mice to autoimmune disease.

Introduction
NOD mice are widely used as a spontaneous model of type 1 (autoimmune) diabetes mellitus (1). In addition to diabetes, NOD mice develop signs of systemic autoimmunity such as antinuclear autoantibodies (ANA) and hemolytic anemia (HA) in old age (2). Furthermore, exposure to killed mycobacteria results in the precipitation of an autoimmune disease which resembles systemic lupus erythematosus (SLE) in humans, and is characterized by enhancement of ANA, precipitation of HA and the induction of immune complex glomerulonephritis (3,4). This multiplicity of autoimmune phenomena in NOD mice suggests the presence of a fundamental defect in immune control affecting their ability to induce and maintain immunological tolerance (1).

New evidence has recently thrown light on the role of the innate immune system in regulation of adaptive responses. Of particular interest are the roles of two families of immunoregulatory lymphocytes, the NK and NKT cells. NK cells are large granular lymphocytes which secrete several proinflammatory cytokines, including IFN-γ, and mediate cytotoxicity against a broad range of targets, such as MHC class I-deficient virally infected cells and tumor cells (5). NKT cells are a population of lymphocytes which co-express the αβ TCR, as well as receptors characteristic of NK cells and rapidly produce large amounts of cytokine, including IL-4 (6,7). The TCR repertoire expressed by NKT cells is unusually restricted—to Vα14–Jα281 and Vβ8, Vβ7 or Vβ2 in the mouse.

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(8,9) and \( V_\gamma 24, J_\gamma Q \) and \( V_\beta 11 \) in humans (10). NKT cells are activated on recognition of the class I-like, \( \beta_2 \) microglobulin- \( \beta_2 m \)-dependent leukocyte surface antigen, CD1d (11,6,12), which appears to act as a restriction element for the presentation of glycolipids such as glycosylphosphatidylinositol (GPI) (13) and phosphatidylinositolmannosides (PIM) (14).

Kataoka et al. (15) reported that splenocytes from NOD mice exhibit a defect in NK cell cytotoxic function as evidenced by decreased killing of YAC-1 target cells in vitro. The presence of this defect has been confirmed with the same assay by others (16). Although FACS analysis of splenocytes from NOD mice revealed a complete absence of NK1.1+ lymphocytes (16), this was subsequently demonstrated to be caused by the absence of the NK1.1 allelic marker in this strain (17–19). The defect in NK activity in NOD mice cannot be attributed to the lack of the NK1.1 allele, because BALB/c mice, which also lack the NK1.1 allele, have functional NK cells (20). In the absence of the NK1.1 marker, it is therefore difficult to determine if the apparent defect in NK cell activity in NOD mice results from a functional, numerical or combined deficiency.

The absence of the NK1.1 marker in NOD mice also complicates the study of NKT cells in this strain. Using surrogate phenotypes for NKT cells [\( \alpha \beta \) TCR\(^+\)CD4-CD8- (21,19); HSA-\(^{+}\), CD8- \( \alpha \beta \) TCR\(^{+}\), Mel-14- or CD44\(^{+}\) (17)] we and others found that NOD mice have a deficiency of NKT cells in the thymus (17,21) and, to a lesser extent, in the periphery (19). Furthermore, in adoptive transfer experiments we have shown that diabetes in NOD mice can be prevented by the injection of \( 2 \times 10^8 \) \( \alpha \beta \) TCR\(^+\) double-negative thymic NKT cells and that this protection is mediated by an IL-4-dependent mechanism (22). Gombert et al. (17) also studied NKT cell function in vivo in NOD mice by measuring the production of IL-4 following injection of anti-CD3 mAb, as described by Yoshimoto and Paul (23). They found that at the age of 3–5 weeks, NOD mice produced no more IL-4 in response to this challenge than did NKT cell-deficient NOD.B6m\(^{-}\) mutant mice. Furthermore, a similar deficit was found following stimulation of HSA-CD8\(^{+}\) thymocytes in vitro, suggesting the possibility that NKT cells from NOD mice may be deficient in IL-4 production on a per cell basis—a finding supported by us using intracellular cytokine staining of \( \alpha \beta \) TCR\(^+\) DN thymocytes (22).

Lehuen et al. (24) subsequently confirmed the protective role of NKT cells in NOD mice by increasing their numbers through the introduction of a transgene encoding the NKT cell-associated TCR \( V_\gamma 4-J_\gamma 281 \) chain. Mild protection from spontaneous diabetes and a more robust protection from cyclophosphamide-precipitated diabetes was seen in the transgenic lines with the most NKT cells. In order to determine the numbers of NKT cells in these transgenic lines, the authors crossed them to NOD.b-Nkrp\(^{16}\) mice (referred to as NOD.NK1.1 congenic mice in the manuscript cited), a novel line of NOD mice congenic for the NK-related complex on chromosome 6. Unfortunately, the manuscript contains very little information on the characterization of this line. As NOD.b-Nkrp\(^{16}\) mice offer enormous advantages in the study of NK and NKT cells in NOD mice, we have independently generated another line of these mice by the traditional method of backcrossing and phenotypic selection. The data presented here therefore represent the first detailed study of NK and NKT cell numbers and function in NOD mice.

**Methods**

**Mice**

NOD/Lt, BALB/c, BALB.B6-Cmv\(^{17}\) and C57BL/6J mice were obtained from the Animal Resource Centre (Canning Vale, WA, Australia). BALB.B6-Cmv\(^{17}\) mice are a BALB/c-based congenic strain which carries the NK-related complex from C57BL/6 mice (25). The breeding of specific crosses was performed within the animal facility at the Centenary Institute (Sydney, Australia). Mice were housed in clean conditions and sentinel mice were tested by serology at 4-monthly intervals for the following pathogens: mouse hepatitis virus, rotavirus, salmonella, mouse cytomegalovirus, polyoma virus, murine adenovirus, lymphocytic choriomeningitis virus, mouse pneumonia virus, reovirus, Sendai virus, Thiele's murine encephalitis viruses, Bacillus pertiformis, Mycoplasma pulmonis, Bordetella bronchiseptica, Corynebacterium kutscheri, klebsiella species, Pasteurella multocida, Pasteurella pneumotropica, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumoniae, Citrobacter freundii and Salmonella species. No sentinel mice tested positive for any of these pathogens.

**Production of NOD.b-Nkrp\(^{16}\) mice**

A BALB.B6-Cmv\(^{17}\) female mouse was crossed with a NOD/Lt male mouse and the progeny were serially back-crossed to NOD/Lt mice to generation N10. At each generation after F\(_1\), breeders were selected by flow cytometry for expression of the B6-associated NK1.1 allelic marker on peripheral blood lymphocytes. At N10, heterozygous mice were intercrossed and their progeny analyzed by flow cytometry and PCR of D6Mit135 (Research Genetics, Huntsville, AL), a simple sequence repeat (SSR) tightly linked to the NK-related complex on chromosome 6. Homozygous mice carrying the B6 allele at this locus were used to found the new congenic line, while heterozygous mice were maintained for 8 months to assess the incidence of spontaneous diabetes.

**Flow cytometric analysis**

**Antibodies.** Anti-Pan-NK (clone DX5) conjugated to phycoerythrin (PE), CD161/NK1.1 (clone PK136) conjugated to biotin or PE, anti-CD4 (clone RM4-5) conjugated to biotin or PE, anti-CD8\(_\alpha\) (clone 53-6.7) conjugated to PE, CD85 (clone 53-6.7) conjugated to PE, biotin or allophycocyanin (APC), anti-TCR\(_\beta\) (clone H57-597) conjugated to FITC or APC, anti-IL-4 (clone 11B11) conjugated to PE and anti-IFN-\(\gamma\) (clone XMG1.2) conjugated to FITC were all obtained from PharMingen (San Diego, CA). Purified rabbit anti-asialo-GM\(_1\) was obtained from Wako Pure Chemical Industries (Richmond, VA), streptavidin–Texas Red was obtained from Molecular Probes (Eugene, OR) and goat anti-rabbit–FITC was obtained from Southern Biotechnology (Birmingham, AL).

**CD\(_1\)-\(\alpha\)-GalCer tetramer.** CD\(_1\)-\(\alpha\)-GalCer tetramer was kindly provided by Dr Mitchell Kronenberg and was prepared as previously described (26).
Fig. 1. The incidence of diabetes in female (A) and male (B) NOD.b-Nkrp1b heterozygous (squares) and non-congenic (diamonds) mice.

**Preparation of lymphocytes.** Single-cell suspensions of lymphocytes from blood, bone marrow, lymph nodes (pooled inguinal, cervical and axillary), spleen and thymus were prepared by standard techniques (19). Livers were pressed through a 200 gauge stainless steel mesh screen and washed at least twice. Lymphocytes were then separated out using a 37.5% Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient. The cells were then treated with red blood cell lysing buffer (Sigma, St Louis, MO).

**Cell surface staining.** Staining was carried out in 96-well U-bottom plates. Cells were initially incubated with anti-CD16/CD32 (anti-FcγRIII/II; clone 2.4G2) to prevent non-specific binding of subsequent mAb and then incubated with the appropriate mixture of mAb for 30 min at 4°C. Secondary staining reagents were then added when necessary and incubated as before. The cells were analyzed on a FACScan or FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA).

**Intracellular cytokine staining.** Twenty-four-well flat-bottom plates were coated with anti-CD3 (KT3, 10 µg/ml in pH 9.1 carbonate buffer). The cells were then added at 2×10^5 cells/well in 1.5 ml T cell medium [RPMI 1640 (Gibco/BRL, Grand Island, NY) with 10% fetal calf serum (FCS), 1% l-glutamine (Sigma) and 5×10^-5 M 2-mercaptoethanol (Sigma)] and the plates were incubated at 37°C in 5% CO₂ for 1–2 h; Brefeldin A (5 µg/ml; Sigma) was subsequently added and the plates incubated for a further 4 h before the cells were harvested and washed. Control samples were prepared from cells which were cultured without anti-CD3 stimulation or the addition of Brefeldin A. Samples were surface stained as described above, fixed in 4% formaldehyde in PBS and then stained intracellularly with anti-IL-4 and/or anti-IFN-γ or the appropriate mixture of mAb and then incubated with the appropriate mixture of mAb.

**In vivo NK cell cytotoxicity assay**

NK cell cytotoxicity of freshly isolated splenocytes from C57BL/6, NOD/Lt and NOD.b-Nkrp1b mice was examined in a 4-h 51Cr-release assay. Target cells were generated by stimulating splenocytes from C57BL/6, C57BL/6.β2m−/−, NOD/Lt or NOD.β2m−/− mice with phorbol myristate acetate (1 ng/ml; Sigma) and ionomycin (1 µg/ml; Sigma) for 24 h. Target cells (2.0×10⁵/well) were mixed with freshly isolated effector splenocytes (2.0×10⁴/well) and incubated as before. The cells were analyzed on a FACScan or FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA).

**Fig. 2.** Flow cytometric analysis of splenocytes from C57BL/6, NOD/Lt and NOD.b-Nkrp1b demonstrating (top row) the appropriate expression of NK1.1 on NK cells (NK1.1/αβ TCR) and NKT cells (NK1.1/αβ TCR) in NOD.b-Nkrp1b mice, and (bottom row) the appropriate and equivalent binding of CD1α-GalCer tetramer by NKT cells from NOD and NOD.b-Nkrp1b mice. Values represent the means ± SD of the proportions (n = 5).
were recovered over a Histopaque-1083 (Sigma) gradient and then directly labeled at 5×10^5 cells/ml with 2 μM carboxyfluorescein diacetate succinimidyl esters [CFSE (28); Molecular Probes, Eugene, OR] in PBS at room temperature for 10 min. Labeled cells were washed and injected i.v. into the tail veins of lethally irradiated (940 rad) recipient mice (5×10^5/mouse). After a period of 2–5 days, the recipient mice were killed, and the femurs, tibias and spleens taken for quantification of donor cells by flow cytometry. By prior experimentation, it was determined that a 3-day period of engraftment ensured maximum spread of CFSE brightness amongst dividing cells without the labeled population merging into the background of unstained cells.

Statistical analyses

Qualitative differences between samples were examined using the 4-fold table (χ^2) test unless the expected value in any cell was <5, in which case the Fisher’s exact test was used. Quantitative differences between samples were compared using the Mann–Whitney U (rank-sum) test or ANOVA as appropriate. A goodness of fit (χ^2) test was used to compare proportions of affected animals with those predicted by modeling.

Results

Incidence of diabetes in NOD.b-Nkrp1^b congic mice

Genotypic analysis of N10 and N10F1 (first intercross of N10) mice was performed on tail cuttings by PCR amplification of the SSR D6mit135, which is tightly linked to the Nkrp1 (NK1.1) locus. Nkrp1^b homozygous mice were used to found the new congenic strain, which is now maintained at the Animal Resources Centre (Perth, WA, Australia). At F_3, genotypic analysis was performed to determine the segment boundaries. The proximal boundary lay between D6Mit323 and D6Mit105, and the distal boundary lay between D6Mit135 and D6Mit259. This segment included proximal BALB/c-derived flanking sequences in addition to the B6-derived NKC segment. Non congenic and heterozygous mice were bled at two-weekly intervals from 12 to 36 weeks by retro-orbital venepuncture and random blood glucose levels determined by the glucose oxidase technique. At 36 weeks of age, 10 of 14 (71%) female heterozygous mice and 18 of 33 (55%) female non-congenic mice had developed diabetes (NS, 4-fold χ^2 table test; Fig. 1A). At the same age, four of 24 (17%) male heterozygous mice and four of 17 (24%) male non-congenic mice had developed diabetes (NS, 4-fold χ^2 table test; Fig. 1B). Under the housing conditions at the Centenary Institute, the typical incidences of diabetes in NOD/Lt mice are 70 and 20% for female and male mice respectively.

Flow cytometric comparison of NK cells in NOD.b-Nkrp1^b, C57BL/6 and BALB.B6-Cmv1^f congenic mice

Expression of NK1.1 on splenocytes from C57BL/6, NOD/Lt and NOD.b-Nkrp1^b was examined by flow cytometry, and was found to be appropriately expressed on both NK cells (NK1.1^αβTCR^) and NKT cells (NK1.1^αβTCR^) in NOD.b-Nkrp1^b mice (Fig. 2).

A flow cytometric comparison was then performed on lymphocytes from thymus, spleen, peripheral lymph nodes, liver, blood and bone marrow from 8-week-old female NOD.b-Nkrp1^b (n = 5), C57BL/6 (n = 5) and BALB.B6-Cmv1^f (n = 5) mice. With the exception of the liver and blood, total numbers of lymphocytes obtained from each organ did not differ significantly between strains. More lymphocytes were obtained from the livers of BALB/c and B6 mice and from the blood of B6 mice than from those of NOD.b-Nkrp1^b mice (Table 1).

The peripheral blood, spleen and lymph nodes of NOD.b-Nkrp1^b mice were significantly deficient in NK (NK1.1^αβTCR^) cells (Table 1). The numbers of NK cells in the livers of NOD.b-Nkrp1^b mice were also reduced, although the proportion of hepatic leukocytes that were NK cells in the NOD.b-Nkrp1^b mice differed significantly from that of BALB but not that of B6 mice. In stark contrast to the deficiency of NK cells in the peripheral organs, NOD.b-Nkrp1^b mice had twice as many NK cells in the bone marrow as B6 mice and 5 times that of BALB mice (Table 1). These findings may reflect either a block in NK cell export or a compensatory increase in NK cell production by the bone marrow.

Flow cytometric comparison of NKT cells in NOD.b-Nkrp1^b, C57BL/6 and BALB.B6-Cmv1^f congenic mice

The thymuses and lymph nodes of NOD.b-Nkrp1^b mice were profoundly deficient in NK1.1^αβTCR^ NKT cells (Table 1). The numbers of NKT cells in the other organs was similar between the NOD.b-Nkrp1^b and BALB mice, although the proportions of NKT cells in spleen, liver and blood from B6 mice were significantly higher than from the other two strains (Table 1).

Comparison of NK cells in NOD/Lt and NOD.b-Nkrp1^b congenic mice

In order to determine if the Nkrp1^b congenic segment affected the number or function of NK cells in the NOD.b-Nkrp1^b congenic mice, the expression of two surrogate markers for NK cells (DX5 and asialo-GM_1) and the cytotoxic activities of NK cells in the two NOD lines were compared.

Thymus, spleen, peripheral lymph node, liver blood and bone marrow leukocytes from NOD/Lt, NOD.b-Nkrp1^b and C57BL/6 female mice were stained with DX5 and asialo-GM_1 antibody and examined by flow cytometry. No significant differences were found in the numbers of DX5^ among dividing cells without the labeled population merging
Table 1. Flow cytometric analysis of NK (NK1.1+αβTCR+) and NKT (NK1.1+αβTCR+) cells in thymus, spleen, peripheral lymph nodes, liver, blood and bone marrow of 8-week-old female NOD.b-Nkrt10, C57BL/6 and BALB/c-b-Nkrt10 mice (results given as mean ± SD of five mice)

<table>
<thead>
<tr>
<th></th>
<th>Total cells</th>
<th>NK1.1+ αβTCR+</th>
<th>NK1.1+ αβTCR−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus</td>
<td>×10^8</td>
<td>×10^6 (% of total)</td>
<td>×10^4 (% of total)</td>
</tr>
<tr>
<td>NOD</td>
<td>1.38 ± 0.18</td>
<td>1.22 ± 0.24 (0.09 ± 0.02)</td>
<td>7.02 ± 3.28 (0.05 ± 0.02)</td>
</tr>
<tr>
<td>B6</td>
<td>1.68 ± 0.58</td>
<td>5.58 ± 1.13 (0.36 ± 0.13)c</td>
<td>2.70 ± 1.24 (0.02 ± 0.01)b</td>
</tr>
<tr>
<td>BALB</td>
<td>1.41 ± 0.23</td>
<td>4.22 ± 0.58 (0.31 ± 0.09)b</td>
<td>4.25 ± 1.28 (0.03 ± 0.01)a</td>
</tr>
<tr>
<td>Spleen</td>
<td>×10^7</td>
<td>×10^5 (% of total)</td>
<td>×10^6 (% of total)</td>
</tr>
<tr>
<td>NOD</td>
<td>7.27 ± 0.87</td>
<td>2.80 ± 0.35 (0.39 ± 0.03)</td>
<td>1.52 ± 0.17 (2.11 ± 0.27)</td>
</tr>
<tr>
<td>B6</td>
<td>8.57 ± 1.77</td>
<td>17.2 ± 3.9 (2.14 ± 0.92)c</td>
<td>2.90 ± 0.75 (3.36 ± 0.41)c</td>
</tr>
<tr>
<td>BALB</td>
<td>8.66 ± 1.04</td>
<td>3.26 ± 0.40 (0.39 ± 0.09)</td>
<td>3.46 ± 0.37 (4.04 ± 0.39)d</td>
</tr>
<tr>
<td>Lymph node</td>
<td>×10^7</td>
<td>×10^4 (% of total)</td>
<td>×10^5 (% of total)</td>
</tr>
<tr>
<td>NOD</td>
<td>4.68 ± 0.97</td>
<td>1.51 ± 0.58 (0.03 ± 0.01)</td>
<td>0.95 ± 0.21 (0.20 ± 0.03)</td>
</tr>
<tr>
<td>B6</td>
<td>5.41 ± 1.56</td>
<td>12.6 ± 3.44 (0.23 ± 0.01)d</td>
<td>3.03 ± 0.99 (0.56 ± 0.08)d</td>
</tr>
<tr>
<td>BALB</td>
<td>6.20 ± 0.91</td>
<td>7.67 ± 3.61 (0.12 ± 0.06)c</td>
<td>2.67 ± 0.50 (0.43 ± 0.04)d</td>
</tr>
<tr>
<td>Liver</td>
<td>×10^6</td>
<td>×10^2 (% of total)</td>
<td>×10^5 (% of total)</td>
</tr>
<tr>
<td>NOD</td>
<td>4.94 ± 1.83</td>
<td>0.43 ± 0.14 (8.80 ± 2.07)</td>
<td>0.46 ± 0.15 (9.38 ± 0.94)</td>
</tr>
<tr>
<td>B6</td>
<td>9.56 ± 2.9α</td>
<td>2.04 ± 0.79 (21.08 ± 5.35)c</td>
<td>1.25 ± 0.49 (12.97 ± 3.09)</td>
</tr>
<tr>
<td>BALB</td>
<td>14.7 ± 3.27d</td>
<td>1.11 ± 0.35 (7.64 ± 2.27)</td>
<td>2.31 ± 0.81 (15.63 ± 4.13)c</td>
</tr>
<tr>
<td>Blood (ml)</td>
<td>×10^−6</td>
<td>×10^1 (% of total)</td>
<td>×10^5 (% of total)</td>
</tr>
<tr>
<td>NOD</td>
<td>1.92 ± 0.38</td>
<td>0.98 ± 0.18 (0.53 ± 0.15)</td>
<td>0.65 ± 0.09 (3.53 ± 0.94)</td>
</tr>
<tr>
<td>B6</td>
<td>3.40 ± 0.10b</td>
<td>2.50 ± 0.68 (0.75 ± 0.10)b</td>
<td>3.06 ± 1.14 (9.13 ± 3.09)c</td>
</tr>
<tr>
<td>BALB</td>
<td>2.48 ± 0.37</td>
<td>1.05 ± 0.22 (0.42 ± 0.03)</td>
<td>3.36 ± 0.85 (13.71 ± 3.95)c</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>×10^−7</td>
<td>×10^1 (% of total)</td>
<td>×10^5 (% of total)</td>
</tr>
<tr>
<td>NOD</td>
<td>2.15 ± 0.25</td>
<td>0.53 ± 0.74 (2.06 ± 0.05)</td>
<td>5.59 ± 0.82 (2.60 ± 0.27)</td>
</tr>
<tr>
<td>B6</td>
<td>1.74 ± 0.60</td>
<td>14.8 ± 3.78 (1.39 ± 0.45)d</td>
<td>2.62 ± 1.95c (1.39 ± 0.45)d</td>
</tr>
<tr>
<td>BALB</td>
<td>1.44 ± 0.66</td>
<td>2.30 ± 1.11 (0.16 ± 0.03)</td>
<td>1.16 ± 0.70c (0.77 ± 0.17)d</td>
</tr>
</tbody>
</table>

aP < 0.05, ANOVA. 
bP < 0.01, ANOVA. 
cP < 0.001, ANOVA. 
dP < 0.0001, ANOVA.

Table 2. Comparison of proportions of DX5+ asialo-GM1+ cells in tissues from NOD/Lt and NOD.b-Nkrt10 10-week-old female mice (n = 5; mean ± SD)

<table>
<thead>
<tr>
<th>DX5+ asialo-GM1+ (%)</th>
<th>NOD/Lt</th>
<th>NOD.b-Nkrt10</th>
<th>C57BL/6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus</td>
<td>0.15 ± 0.07</td>
<td>0.13 ± 0.03</td>
<td>0.06 ± 0.01a</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.94 ± 0.11</td>
<td>1.55 ± 0.07</td>
<td>2.37 ± 0.96b</td>
</tr>
<tr>
<td>Lymph node</td>
<td>0.32 ± 0.06</td>
<td>0.36 ± 0.05</td>
<td>0.67 ± 0.11a</td>
</tr>
<tr>
<td>Blood</td>
<td>4.79 ± 1.01</td>
<td>4.24 ± 0.93</td>
<td>5.43 ± 0.91</td>
</tr>
<tr>
<td>Bone Marrow</td>
<td>6.70 ± 2.27</td>
<td>6.14 ± 1.01</td>
<td>5.47 ± 1.24</td>
</tr>
</tbody>
</table>

aP < 0.01, Mann−Whitney U-test. 
bP < 0.05, Mann−Whitney U-test.

presence of NK cell activity in vivo in NOD mice (Fig. 3). Furthermore, the numbers recovered differed between NOD/Lt and NOD.b-Nkrt10 mice (9.0 ± 3.3×10^5 versus 2.4 ± 0.6×10^4 respectively, n = 5, P < 0.05, Mann−Whitney U-test), suggesting that the Nkrt10 congenic segment contained genes which decreased NK cell-mediated cytotoxicity of class I MHC-deficient hemopoietic cells in this model. These findings were confirmed by the analysis of donor cells in the spleens of irradiated recipients (data not shown).

The validity of this in vivo measure of NK cell-mediated cytotoxicity was confirmed by comparing the efficacy of killing following transfer of NOD.β2m− bone marrow into NOD.scid and NOD/Lt mice. In line with the previous experiment, while 9.2 ± 2.5% of bone marrow cells recovered from NOD/Lt recipients of NOD/Lt marrow were CFSE labeled, only 2.9 ± 0.5% of bone marrow cells from NOD/Lt recipients of NOD.β2m− bone marrow were. Similarly, while 13.2 ± 5.5% of bone marrow cells recovered from NOD/scid recipients of NOD/Lt marrow were CFSE labeled, only 2.8 ± 1.0% of bone marrow cells from NOD.scid recipients of NOD.β2m− bone marrow were, indicating that cytotoxicity of class I MHC-deficient marrow was not mediated by T cells, including NKT cells (n = 5; NS, NOD/Lt versus NOD.scid recipients of NOD.β2m− bone marrow. Mann−Whitney U-test).

As cytokine production can contribute to the activation of NK cells in vivo, and in order to quantitate the difference in NK cell lytic activity between the NOD lines and to compare their levels of NK cell cytotoxicity with that of C57BL/6 mice, freshly isolated splenocytes from C57BL/6, NOD/Lt and NOD.b-Nkrt10 mice were tested in a 4-h 51Cr-release assay. The presence of NK cell activity in NOD mice was confirmed by the ability of splenocytes from both NOD/Lt and NOD.b-Nkrt10 mice to lyse labeled target splenocyte blasts from NOD.β2m− mice (Fig. 4). This in vitro analysis did not identify any substantial difference in cytotoxic activity between splenocytes from the two NOD lines and both showed less lytic activity (expressed as lytic units at an ET ratio of 20:1; LU20) against NOD.β2m− blasts or B6.β2m− blasts than splenocytes from C57BL/6 mice (Table 3). The lytic activities
Table 3. NK cell cytotoxicity by unmanipulated splenocytes from C57BL/6, NOD/Lt and NOD.b-Nkrp1b mice was compared in vitro in a 51Cr-release assay against NOD/Lt, NOD.β2m−/−, C57BL/6 and C57BL/6 β2m−/− splenic blasts. Results are presented as LU20 for 10^7 splenocytes (A) or 10^6 NK cells (B).

<table>
<thead>
<tr>
<th>Responder</th>
<th>Target</th>
<th>(A)</th>
<th>(B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD/Lt</td>
<td>Rx</td>
<td>β2m^+/+</td>
<td>β2m^+/+</td>
</tr>
<tr>
<td>NOD/Lt</td>
<td>–</td>
<td>ND</td>
<td>9.5</td>
</tr>
<tr>
<td>NOD.b-Nkrp1b</td>
<td>–</td>
<td>ND</td>
<td>8.5</td>
</tr>
<tr>
<td>NOD.b-Nkrp1b</td>
<td>αNK1.1</td>
<td>ND</td>
<td>ND</td>
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</tr>
<tr>
<td>C57BL/6</td>
<td>–</td>
<td>ND</td>
<td>28.9</td>
</tr>
</tbody>
</table>

Target background: NOD/Lt

Target background: C57BL/6

Lysis of wild-type targets was not detected (ND) and lytic activity was eliminated by prior in vivo treatment with PK136 (anti-NK1.1 Rx).

Fig. 3. NK cell cytotoxicity by NOD/Lt and NOD.b-Nkrp1b mice was compared in vivo by following the fate of CFSE-labeled NOD.β2m−/− bone marrow cells 3 days after adoptive transfer into 940 rad-αβ TCR−. By this calculation, the lytic activity of NOD NK cells irradiated C57BL/6, NOD/Lt and NOD.b-Nkrp1b mice. The proportions was about half that of C57BL/6 NK cells (Table 3B). (A) and numbers (B) of donor bone marrow cells, as well as the progeny of those cells were identified by the presence of the CSFE label. Values represent the means ± SD of the proportions and numbers of donor cells recovered.

Fig. 4. NK cell cytotoxicity by unmanipulated splenocytes from C57BL/6, NOD/Lt and NOD.b-Nkrp1b mice was compared in vitro in a 51Cr-release assay. Comparison of NKT cells in NOD/Lt and NOD.b-Nkrp1b mice

In order to determine if the Nkrp1b congenic segment affected the number or function of NKT cells in the NOD.b-Nkrp1b congenic mice, the numbers of, and IL-4 production by, thymic NKT cells in NOD/Lt and NOD.b-Nkrp1b mice were compared. As the NK1.1 marker cannot be used to identify NKT cells in NOD/Lt mice, the surrogate phenotype αβTCR+ DN was used initially (21). No significant difference was found between the numbers of αβTCR+ DN cells in the thymuses of 8-week-old female NOD/Lt mice and those of NOD.b-Nkrp1b mice (1.21 ± 0.23 x 10^5 versus 1.19 ± 0.25 x 10^5; n = 5, NS, Mann–Whitney U-test), which in both cases was a third the numbers of αβTCR+ DN cells found in C57BL/6 mice (3.6 ± 1.4 x 10^5).

Similar studies comparing numbers of CD1-α-GalCer tetramer-binding lymphocytes (26) failed to identify any significant difference in NKT cell numbers in spleen (Fig. 2), thymus, liver, blood or lymph node between NOD and NOD.b-Nkrp1b mice.

The proportion of CD4+ CD8− [double-negative (DN)] thymocytes producing IL-4 after 6 h stimulation with plate-bound anti-CD3 mAb was determined by intracellular cytokine staining and flow cytometry. There was no significant difference of NK cells from C57BL/6 and NOD.b-Nkrp1b mice were compared on a per cell basis by correcting the LU20 values for the proportions of splenocytes that were NK cells (NK1.1+).
in the proportions of DN thymocytes which produced IL-4 after stimulation between NOD/Lt and NOD.b-Nkrp1P mice (0.47 ± 0.18 versus 0.64 ± 0.17%; n = 5, NS, Mann–Whitney U-test), which in both cases was less than a 10th the proportion of C57BL/6 DN thymocytes that produced IL-4 (8.55 ± 2.90%; Fig. 5).

The proportion of DN thymocytes producing IFN-γ after 6 h stimulation with plate-bound anti-CD3 mAb was also determined by intracellular cytokine staining and flow cytometry. There was no significant difference in the proportions of DN thymocytes which produced IFN-γ after stimulation between NOD/Lt and NOD.b-Nkrp1P mice (2.3 ± 0.5 versus 2.8 ± 0.7%; n = 5, NS, Mann–Whitney U-test), which in both cases was significantly less than proportion of C57BL/6 DN thymocytes that produced IFN-γ (4.4 ± 1.3%; P < 0.05; Mann Whitney U-test; Fig. 5).

In conclusion, there was no evidence that the Nkrp1P congenic segment altered the number or function of NKT cells in the NOD.b-Nkrp1P congenic line.

Production of IL-4 by NOD.b-Nkrp1 NKT cells

Using surrogate markers, Gombert et al. (17) found evidence for a deficiency in IL-4 production by the NKT cells of 3- to 5-week old NOD mice, a finding supported by our own data (22). The existence of the NOD.b-Nkrp1P line permits, for the first time, an accurate assessment of the severity of this deficit. Thymocytes from 5-week old C57BL/6 and NOD.b-Nkrp1P mice were stimulated with plate-bound anti-CD3 mAb for 6 h, with Brefeldin A present for the last 4 h. The cells were then surface stained with anti-NK1.1 and anti-CD4, fixed, permeabilized, and stained for intracellular IL-4 and IFN-γ. While 25.9 ± 1.9% of NK1.1+ thymocytes from C57BL/6 mice contained IL-4, only 2.9 ± 1.3% of NK1.1+ thymocytes from NOD.b-Nkrp1P mice did so (Fig. 6; P < 0.01, Mann–Whitney U-test). This deficiency in IL-4 production by NK1.1+ thymocytes was shared equally between the CD4int and CD4+ subpopulations (data not shown). It is relevant to consider the synergistic effect of the functional deficit of thymic NKT cells together with the numerical deficiency of these cells in NOD mice. At 5 weeks of age, NOD.b-Nkrp1P mice had 3.8 ± 0.7 × 10^5 NK1.1+ thymic cells while C57BL/6 mice had 9.7 ± 1.1 × 10^5 of these cells (P < 0.01, Mann–Whitney U-test) ~3-fold difference. Thus the thymuses of NOD.b-Nkrp1P mice contained ~20-fold fewer IL-4-producing NK1.1+ T cells than C57BL/6 mice.

Protection from diabetes by NKT cells from NOD.b-Nkrp1P mice

The deficiencies observed in the numbers and function of NK1.1+αβTCR+ DN NKT cells in NOD.b-Nkrp1P mice highlight the question of whether the diabetes-preventing activities previously attributed to NKT cells from NOD mice (19,22) were correctly attributed or were actually due to the presence of contaminating lymphoid precursors of an αβTCR+CD62L−CD4+ immunoregulatory subset, as has been proposed by Herbelin et al. (29). NK1.1+αβTCR+ NKT thymocytes were therefore compared to unmanipulated thymocytes in female 3-week-old NOD/Lt mice to examine for evidence of residual protective activity in the DN thymocyte population which could not be
attributable to NKT cells. This group of mice developed diabetes normally (four of five, 80%).

Discussion

In addition to contributing to immune responses against pathogens such as viruses (30) and protozoa (31), NK cells play a role in inhibiting inappropriate immune responses in mouse models of multiple sclerosis (EAE) (32) and colitis (33). Similarly, NKT cells are involved in responses to a wide range of pathogens [Escherichia coli (34), Salmonella choleraesuis (35), Listeria monocytogenes (36), Mycobacterium tuberculosis (14), Plasmodium and Trypanosoma (13)] and appear to play an immunoregulatory role in autoimmune disease, since NKT cell deficiency is associated with autoimmunity in C57BL/6/lpr mice, MRL/lpr mice, C3H/ldl mouse, and (NZB×NZWF)F1, mice (37,38).

Here, a new congenic mouse line was generated to study the roles of NK and NKT cells in the autoimmune diseases of NOD mice, since putative deficiencies in both these cell populations have been reported to be present in these mice [NK (15,16) and NKT (17,19,21,22,24)]. The NOD.b-Nkrp1b line was produced by transferring the genomic segment containing the NK-related complex, including Nkrp1 (the gene encoding NK1.1), from BALB.B6-Cmv1b mice to the NOD background by serial backcrossing. The BALB.B6-Cmv1b strain contains the NK-related complex of C57BL/6 mice and has been previously used to study NK anti-viral responses (25). It was used as the segment donor instead of using B6 directly, so that a pair of BALB-based control strains exist for comparisons of NK cell activity. In order to further aid such comparisons, a NOD.c-H2b congenic strain was simultaneously generated from the same cross.

The NKC lies adjacent to the genetic region containing the Idd19 diabetes susceptibility gene, which was identified in a backcross with the PWK feral mouse strain (39). In the NOD.b-Nkrp1b congenic strain, this region (as defined by D6mit25) was still segregating at F5, with two-thirds of alleles tested being B6 type and one-third NOD. While it is not known if B6 alleles of Idd19 can affect the incidence of diabetes in NOD mice, there was no evidence of such an effect in the heterozygous congenic mice studied here.

Flow cytometric comparison of NOD.b-Nkrp1b, BALB.B6-Cmv1b, and C57BL/6 mice demonstrated for the first time that NOD mice have a relative deficiency of peripheral NK cells, which was associated with increased numbers of these cells in the bone marrow—suggesting that a defect in NK cell export may be involved. As the NK-related complex contains a series of gene clusters which control the activity and specificity of NK cells, the cytolytic activity of NK cells in NOD/Lt and NOD.b-Nkrp1b mice was compared in two assays. In the first, a conventional in vitro 51Cr-release assay, the splenocytes of both NOD lines were found to generate comparable levels of cytotoxicity against NOD.B6(m/+) splenic blasts, although these levels were substantially lower than that provided by splenocytes from C57BL/6 mice against either NOD.B6(m/+) or C57BL/6.B6(m/+) splenic blasts. This difference in NK cell activity between NOD and C57BL/6 mice could only partly be accounted for by the reduced numbers of NK cells in the periphery of NOD mice.

The second assay of NK cell cytotoxic activity was an in vivo assay adapted from Bix et al. (27) and involved tracking the survival of β2m(m/+) mutant bone marrow cells following injection into lethally irradiated recipients. In this case, donor cells were identified by prior labeling with CFSE, a fluorescent cytoplasmic dye. Because this system identified the progeny of labeled cells, small differences in cytotoxic activity were amplified by exponential expansion of surviving donor cells. Using this sensitive assay, NOD.b-Nkrp1b mice were consistently shown to have lower NK cell activity than NOD/Lt mice, although both lines had significantly less activity than C57BL/6 mice. This difference between the two NOD lines can probably be attributed to the NK-related complex and dissection of its significance will require comparison of NK cell responses to a variety of other targets.

Previous studies of NKT cells in NOD mice by Gombert et al. (17,18) and our own group (19,21,22) relied on surrogate phenotypes to identify this lymphocyte population: HSA−, CD8−, αβTCR+, Med-14− or CD44+ in the case of Gombert et al., and αβTCR+ DN in our case. Although the use of surrogate phenotypes was supported by evidence of a Vq8 bias in the TCR repertoire of these populations (17,19), it did not identify mutually inclusive subpopulations of lymphocytes (data not shown) and is therefore problematic. The NOD.b-Nkrp1b line reported here resolves this problem. When the numbers and distribution of NKT cells in a wide range of lymphoid tissues, were compared between NOD.b-Nkrp1b, BALB.B6-Cmv1b, and C57BL/6 mice, a profound deficiency of NKT cells in the thymuses and lymph nodes of NOD mice was confirmed. In addition, anti-CD3 stimulation of NK1.1+ thymocytes revealed a further deficit in IL-4 production by NOD NKT thymocytes. The combined deficiencies in NKT cell number and IL-4 production result in a 20-fold reduction in IL-4 producing NKT cells in the thymuses of NOD.b-Nkrp1b mice compared to those of C57BL/6 mice.

We have previously shown that a single i.v. injection of 1.0×106 CD45(bright)CD8− or DN thymocytes from female (BALB/c×NOD)F1, or syngeneic donors protected intact NOD mice from the spontaneous onset of clinical autoimmune diabetes (19,22). Further dissection of this thymocyte subset demonstrated that αβTCR+ DN cells were responsible for this activity, indicating that T cells were definitely involved and suggesting that NKT cells where most likely to be responsible (22). Herbelin et al. (29) challenged this interpretation of our data, suggesting that the results were due to the presence of contaminating lymphoid precursors of an αβTCR+CD62L−CD4+ immunoregulatory subset within the αβTCR+ DN population. Here, we have directly addressed this concern by comparing the protective activities of two subsets of DN thymocytes: one, which co-expressed both αβTCR and NK1.1 markers; and the other, from which αβTCR+ NK1.1+ NKT cells had been removed by FACS. While very low numbers (2.5×102) of thymocytes expressing the αβTCR and NK1.1 protected NOD mice from diabetes when injected into unmanipulated female NOD/Lt mice at 3 weeks of age, the remaining population contained no protective activity. This experiment demonstrated conclusively that NKT cells, or a subset of NKT cells, are responsible for the protection from diabetes afforded by DN thymocytes.

Carnaud et al. (40) recently reported evidence of cross-
talk between NK and NKT cells following injection of α-galactosylceramide, and proposed that such intercellular communication may play an important role in physiological activation of these cells. As NOD mice have major defects in both NK and NKT cell mediated functions, these problems may be expected to compound each other. Furthermore, since both populations appear to play important roles in antitumor responses (41) and immunoregulation (reviewed in 7), the combination of these defects may act synergistically to contribute to autoimmunity in NOD mice and tumor development in NOD.scid mice (42). Perhaps of even greater significance is the possibility that common defects are responsible for the deficiencies identified in these two similar lymphocyte subsets. An interesting candidate for such a defect is the expression of lymphotxin, since this molecule appears to play an important role in the development of both NK and NKT cells (43,44,45).

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Abbreviations

α-GalCer α-galactosylceramide  
β2m β2-microglobulin  
AβA antisense nucleotides  
APC allophycocyanin  
CFSE carboxyfluorescein diacetate succinimidyl esters  
DN double negative  
EAE experimental allergic encephalomyelitis  
FCS fetal calf serum  
GPI glycosylphosphatidylinositol  
HA hemolytic anemia  
PE phycoerythrin  
PIM phosphatidylinositolmannoside  
SLE systemic lupus erythematosus  
SSR simple sequence repeats

References


