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Congenic Analysis of the NKT Cell Control Gene Nkt2 Implicates the Peroxisomal Protein Pxmp4

Julie M. Fletcher, Margaret A. Jordan, Sarah L. Snelgrove, Robyn M. Slattery, François D. Dufour, Konstantinos Kyparissoudis, Gurdyal S. Besra, Dale I. Godfrey, and Alan G. Baxter

Type 1 NKT cells play a critical role in controlling the strength and character of adaptive and innate immune responses. We have previously reported deficiencies in the numbers and function of NKT cells in the NOD mouse strain, which is a well-validated model of type 1 diabetes and systemic lupus erythematosus. Genetic control of thymic NKT cell numbers was mapped to two linkage regions: Nkt1 on distal chromosome 1 and Nkt2 on chromosome 2. Herein, we report the production and characterization of a NOD.Nkrp1b.Nktxb congenic mouse strain, which has increased thymic and peripheral NKT cells, a decreased incidence of type 1 diabetes, and enhanced cytokine responses in vivo and increased proliferative responses in vitro following challenge with α-galactosylceramide. The 19 highly differentially expressed candidate genes within the congenic region identified by microarray expression analyses included Pxmp4. This gene encodes a peroxisome-associated integral membrane protein whose only known binding partner is Pex19, an intracellular chaperone and component of the peroxisomal membrane insertion machinery encoded by a candidate for the NKT cell control gene Nkt1. These findings raise the possibility that peroxisomes play a role in modulating glycolipid availability for CD1d presentation, thereby influencing NKT cell function. The Journal of Immunology, 2008, 181: 3400–3412.

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2 J.M.F. and M.A.J. contributed equally to this paper.

3 Address correspondence and reprint requests to Dr. Alan G. Baxter, Comparative Genomics Centre, James Cook University, Townsville, Queensland, Australia; †Monash University, Department of Immunology, Alfred Hospital Medical Research & Education Precinct, Melbourne, Victoria, Australia; ‡Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria, Australia; and §School of Biosciences, University of Birmingham, Edgbaston, Birmingham, United Kingdom.

4 Abbreviations used in this paper: α-GalCer, α-galactosylceramide; βα, β2-microglobulin; NPC, Niemann-Pick type C disease; PPAR, peroxisome proliferator-activated receptor; SAP, SLAM-associated protein; SP, single positive.

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SLAM expression was of functional importance, as it affected both TCR-stimulated proliferation as well as cytokine production. Significantly, thymocytes and CD4\(^+\)/H11001 splenocytes from NOD.Nkrp1b mice produced less IL-4, and slightly more IFN-\(\gamma\)/H9253 (12), in a manner analogous to the cytokine phenotypes of Slamf1/H11002/H11002/H11002 and Sap/H11002/H11002/H11002 targeted mutant mice (14–16).

The second locus identified in the genome-wide linkage scan (9) was Nkt2, which mapped between D2mit490 and D2mit280 on chromosome 2, in the same region as Idd13, a NOD-derived diabetes susceptibility gene identified in (NOD/Lt\times NOR)F2 segregation analyses (17, 18). NOR is a recombinant congenic strain in which limited regions of the NOD/Lt genome on chromosomes 1, 2, 4, 5, 7, 11, 12, and 18 have been replaced by alleles from the C57BL/6J strain. C57BL/6J itself is a recombinant congenic strain, presumably resulting from genetic contamination of C57BL/6 by DBA/2J (19), and the chromosome 2 segment it contributes to NOR is of C57BL/6 origin (17). The NOR strain is completely resistant to type 1 diabetes, despite sharing 88% of their genome with NOD/Lt mice (including the diabetes-associated H2\(^{q7}\) MHC haplotype), and most of their diabetes resistance can be attributed to the expression of the C57BL/6-derived Idd13b allele (17, 18). To improve localization of Idd13, Serreze et al. (20) generated a panel of subcongenic lines. Two of these lines, which carried only slightly overlapping segments, were only moderately...
resistant to diabetes, compared with the original recombinant congenic NOD line bearing the whole of the Idd13 congenic segment. The simplest explanation for this result is that at least two loci contribute to Idd13 (20).

Based on allelic differences in binding of a conformation-dependent anti-β2-microglobulin (β2-m) mAb, Serreze et al. (20) proposed B2m (which encodes β2-m) as a candidate gene contributing to the effects of Idd13. This hypothesis was formally tested by Hamilton-Williams et al. (21), who used allelic transgenic constructs to rescue expression of the NOD-associated a allele or the C57BL/6-associated b allele of B2m in B2m–/– targeted mutant NOD mice. Consistent with B2m playing a critical role in the diabetes susceptibility associated with Idd13, mice expressing the b allele were protected from diabetes, while a significant number of those expressing the a allele progressed to disease.

B2m is also a good candidate for Nkr2, because it acts as the light chain of CD1d, the selection and restriction ligand for type 1 NKT cells (1). In an attempt to identify the genetic sequences that control NKT cell numbers and to explore those expressing the Idd13 allele were protected from diabetes, while a significant number of NOD mice. Consistent with B2m, we produced and characterized a novel congenic segment.

Table I. List of genetic markers tested to confirm genetic homogeneity of NOD.Nkrp1b.Nkt2bb mouse line

<table>
<thead>
<tr>
<th>Organ Strain</th>
<th>No. (×10^-6)</th>
<th>% NKT Cells</th>
<th>No. NKT Cells (×10^-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus</td>
<td>NOD.Nkrp1b</td>
<td>118 ± 9</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>NOD.Nkrp1b.Nkt2bb</td>
<td>124 ± 12</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>Spleen</td>
<td>NOD.Nkrp1b</td>
<td>88 ± 13</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>NOD.Nkrp1b.Nkt2bb</td>
<td>96 ± 7</td>
<td>0.36 ± 0.03</td>
</tr>
<tr>
<td>Liver</td>
<td>NOD.Nkrp1b</td>
<td>12.8 ± 2</td>
<td>12.8 ± 2</td>
</tr>
<tr>
<td></td>
<td>NOD.Nkrp1b.Nkt2bb</td>
<td>3.3 ± 0.4</td>
<td>14.5 ± 2</td>
</tr>
</tbody>
</table>

*An asterisk (*) indicates the presence of C57BL/6-derived alleles.

Materials and Methods

Mice

NOD.Nkrp1b, C57BL/6J, and congenic mice were maintained at the Immunogenetics Research Facility at the James Cook University in specific pathogen-free conditions. The NOD.Nkrp1b strain carries B6-derived alleles at the natural killer complex on chromosome 6 (from D6mit105 to D6mit135), permitting the use of the NK1.1 marker (22, 23). β2-microglobulin transgenic mice, generated as previously described (21), were maintained at the animal facility of the Alfred Hospital Medical Research and Education Precinct (Melbourne, Victoria, Australia). NOD.Nkrp1b.Nkt2bb mice were produced by intercrossing NOD.Nkrp1b and C57BL/6J mice and performing serial backcrosses to NOD.Nkrp1b to N10, before intercrossing and selection of homozygous congenic founders. These studies have been reviewed and approved by the James Cook University Institutional Animal Care and Ethics Committee.

DNA preparation

Extraction of genomic DNA from NOD.Nkrp1b, NOD.Nkrp1b.Nkt2bb, and C57BL/6J mouse strains was conducted using the CAS-1810 X-TractorGene (Corbett Robotics) and the XTR2 X-TractorGene solid sample and reagent pack (Sigma-Aldrich), which is based on a method developed in this laboratory. Briefly, DNA was extracted by digesting 11 mm tail in 400 μl digest buffer (100 mM Tris-HCl (pH 8), 10 mM EDTA, 100 mM NaCl, 0.5% SDS, 50 mM DTT, 100 mM protease K), O/N, 56°C, 40 rpm in a VORTEMP 56EVC (Labnet). Samples were lysed by addition of 700 μl 0.4% SDS, 50 mM DTT, 100 μg/ml RNase A, 100 μg/ml Proteinase K, 56°C, 1 h. DNA was purified by the phenol-chloroform extraction method.

Table II. Thymic, hepatic, and splenic NKT cell numbers at 6 wk

<table>
<thead>
<tr>
<th>Organ</th>
<th>Strain</th>
<th>n</th>
<th>No. (×10^-6)</th>
<th>% NKT Cells</th>
<th>No. NKT Cells (×10^-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus</td>
<td>NOD.Nkrp1b</td>
<td>9</td>
<td>118 ± 9</td>
<td>0.09 ± 0.01</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>NOD.Nkrp1b.Nkt2bb</td>
<td>6</td>
<td>124 ± 12</td>
<td>0.18 ± 0.01</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>Spleen</td>
<td>NOD.Nkrp1b</td>
<td>5</td>
<td>88 ± 13</td>
<td>0.27 ± 0.02</td>
<td>0.24 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>NOD.Nkrp1b.Nkt2bb</td>
<td>6</td>
<td>96 ± 7</td>
<td>0.36 ± 0.03</td>
<td>0.35 ± 0.01</td>
</tr>
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<td>Liver</td>
<td>NOD.Nkrp1b</td>
<td>5</td>
<td>12.8 ± 2</td>
<td>12.8 ± 2</td>
<td>0.27 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>NOD.Nkrp1b.Nkt2bb</td>
<td>6</td>
<td>3.3 ± 0.4</td>
<td>14.5 ± 2</td>
<td>0.47 ± 0.08</td>
</tr>
</tbody>
</table>
FIGURE 3. Thymic and splenic subsets of NKT cells in the NOD.Nkrp1<sup>b</sup> and NOD.Nkrp1<sup>b</sup>.Nkt2b<sup>b</sup> congenic mouse lines. Flow cytometric analyses of thymic CD1d<sup>+</sup>/GalCer tetramer-binding NKT cell subset proportions of mice aged 2 and 4 wk (A, top panels) and thymic, splenic, and hepatic type 1 NKT cells at 7 wk of age (A, bottom panels) from the NOD.Nkrp1<sup>b</sup>.Nkt2b<sup>b</sup> congenic and the NOD.Nkrp1<sup>b</sup> parental line as defined by the CD44, CD4, and NK1.1 markers are illustrated. Histograms illustrating means ± SEM of thymic (B, left panel), splenic (B, middle panel), and hepatic (B, right panel) type 1 NKT cell numbers for 7-wk-old mice. Values from NOD.Nkrp1<sup>b</sup> mice are indicated by open columns and those for NOD.Nkrp1<sup>b</sup>.Nkt2b<sup>b</sup> mice are indicated by filled columns. Significance values report Mann-Whitney U test and are uncorrected for multiple hypothesis testing.

RNA preparation and microarray expression analyses

To minimize activation of the apoptosis cascade, thymi were removed from 4-wk-old female mice and placed in RNAlater (Qiagen) within 120 s of the mouse being placed in CO<sub>2</sub> for asphyxiation. In our hands, this procedure substantially improved the signal-to-noise ratio of expression analyses of thymic CD44<sup>lo</sup>CD44<sup>hi</sup>CD44<sup>hi</sup>CD44<sup>hi</sup> NK1.1<sup>+</sup> NK1.1<sup>+</sup> hepatic type 1 NKT cells at 7 wk of age (A, bottom panels) from the NOD.Nkrp1<sup>b</sup>.Nkt2b<sup>b</sup> congenic and the NOD.Nkrp1<sup>b</sup> parental line as defined by the CD44, CD4, and NK1.1 markers are illustrated. Histograms illustrating means ± SEM of thymic (B, left panel), splenic (B, middle panel), and hepatic (B, right panel) type 1 NKT cell numbers for 7-wk-old mice. Values from NOD.Nkrp1<sup>b</sup> mice are indicated by open columns and those for NOD.Nkrp1<sup>b</sup>.Nkt2b<sup>b</sup> mice are indicated by filled columns. Significance values report Mann-Whitney U test and are uncorrected for multiple hypothesis testing.

Genotyping

Identification of the congenic segment boundaries and the background screen were conducted by genotyping the extracted tail DNA using simple sequence repeats chosen from the Whitehead Institute simple sequence length polymorphism library (Cambridge, MA), as well as markers designed in-house on the basis of PCR product length polymorphisms between C57BL/6 and NOD/Lt strains, as previously described (9).

RNA preparation and microarray expression analyses

To minimize activation of the apoptosis cascade, thymi were removed from 4-wk-old female mice and placed in RNAlater (Qiagen) within 120 s of the mouse being placed in CO<sub>2</sub> for asphyxiation. In our hands, this procedure substantially improved the signal-to-noise ratio of expression analyses of thymic CD44<sup>lo</sup>CD44<sup>hi</sup>CD44<sup>hi</sup>CD44<sup>hi</sup> NK1.1<sup>+</sup> NK1.1<sup>+</sup> hepatic type 1 NKT cells at 7 wk of age (A, bottom panels) from the NOD.Nkrp1<sup>b</sup>.Nkt2b<sup>b</sup> congenic and the NOD.Nkrp1<sup>b</sup> parental line as defined by the CD44, CD4, and NK1.1 markers are illustrated. Histograms illustrating means ± SEM of thymic (B, left panel), splenic (B, middle panel), and hepatic (B, right panel) type 1 NKT cell numbers for 7-wk-old mice. Values from NOD.Nkrp1<sup>b</sup> mice are indicated by open columns and those for NOD.Nkrp1<sup>b</sup>.Nkt2b<sup>b</sup> mice are indicated by filled columns. Significance values report Mann-Whitney U test and are uncorrected for multiple hypothesis testing.

First-strand cDNA synthesis

First-strand cDNA was synthesized from 5 µg total RNA using oligo(dT) primers and SuperScript III reverse transcriptase following the manufacturer’s instructions (Invitrogen).

Real-time quantitative PCR

Primers were designed to verify microarray data on independent samples of RNA from NOD.Nkrp1<sup>b</sup> and NOD.Nkrp1<sup>b</sup>.Nkt2b<sup>b</sup> mice. All PCR were conducted on Rotor-Gene 3000 or Rotor-Gene 6000 (Corbett Robotics) and PCR mixes set up using a CAS1200 liquid handling platform (Corbett Robotics). Each 25 µl reaction contained 12.5 µl...
Platinum SYBER Green qPCR Supermix UDG (Invitrogen), 0.25 µM of each primer (5 µM), and 5 µl cDNA. *Pmp4* expression values were normalized against *Gapdh*, as previous microarray expression analyses had shown that this gene was not differentially expressed between NOD. *Nkrp1* and NOD. *Nkrp1*Nkt2b mice. The primers used for quantitation were:

*Gapdh*, F primer, 5’-TGCCGCCTGGAGAAACCTGCCAAGTATG-3’/R primer, 5’-TGGAAGAGTGGGAGTTGCTTGAGAAGT-3’;

*Pmp4* (target sequence 1455438_at), F primer, 5’-TAAA GACACAGTCTGAGCCCTGCCC-3’/R primer, 5’-ACTCGCTATGCGAAGTCACTGGTA-3’;

*Pmp4* (target sequence 1422780_at), F primer, 3’-TCAGCTTCAGGTCATTCACTTCAGG-5’/R primer, 3’-TTGACAGTAGGGCTCCAGAACTTCT-5’.

Analyses of unknown samples were conducted by comparison to a standard curve for both the gene of interest and the housekeeper. Template standards were prepared by PCR amplification of cDNA from C57BL/6 thymi using primers flanking those used for quantitation:

*Gapdh*, F primer, 5’-ACCACAGTCCATGCCATCACT-3’/R primer: 5’-TCCACCACCCCTGTTGCTGTA-3’;

*Pmp4* (target sequence 1455438_at), F primer, 5’-AAGACGGTGACTGCTGTTGAACTA-3’/R primer, 5’-GCACCTGAAGGAAAACACGGGCTTCAA-3’;

*Pmp4* (target sequence 1422780_at), F primer, 3’-TCAGCTTCAGGTCATTCACTTCAGG-5’/R primer, 3’-TTGACAGTAGGGCTCCAGAACTTCT-5’.

**Cell suspension preparation**

Thymocyte cell suspensions were prepared by gently grinding the thymus between frosted microscope slides in MACS buffer (PBS containing 2 mM...
EDTA; Amresco) and 0.5% (w/v) BSA (ICN Biomedicals). Spleens were disrupted using a 26-gauge needle and forceps. Livers were perfused in situ with cold PBS (10 ml) via the portal vein, removed, cut into small pieces, and gently pushed through fine mesh. The resulting suspension was washed twice in cold PBS and a 33.75% Percoll (Amersham Biosciences) density gradient was used to isolate the lymphocytes. Both spleen and liver cell suspensions were treated with RBC lysing buffer (Sigma-Aldrich).

Flow cytometric analysis

For flow cytometric analyses, cells were labeled with anti-CD3-APC (clone 145-2C11), FITC- or APC-conjugated anti-β2TCR (clone H57-597), PerCP-Cy5.5- or Pacific Blue-conjugated anti-CD4 (clone RM4-5), anti-NK1.1-PE-Cy7 (clone PK136), anti-CD44-FITC (clone IM7), anti-CD8-FITC (clone 53-6.7), and anti-CD1d-Biotin (clone 1B1), all from BD Pharmigen. Biotinylated Abs were detected with streptavidin-PE (BD Pharmigen). Mouse CD1d tetramer, conjugated to PE and loaded with β2GalCer, was produced in house as previously described (10) using recombinant baculovirus encoding his-tagged mouse CD1d and mouse β2m, originally kindly provided by Prof. M. Kronenberg’s laboratory (La Jolla Institute for Allergy & Immunology, San Diego, CA).

For surface staining, Abs were diluted in MACS buffer. Cells were preincubated for 15 min with CD16/32 (clone 93, eBioscience) followed by a further 20 min incubation with 10% mouse serum to prevent FcR binding, before addition of surface staining Ab cocktails. Viable lymphocytes were identified by the forward and side scatter profile and in some cases by propidium iodide exclusion. A forward scatter-area against forward scatter-height gate was used to exclude doublets from analysis. Where possible, an empty fluorescent channel was used to exclude autofluorescent cells. Flow cytometry was performed on a FACSCalibur (BD Biosciences) or a CyanADP flow cytometer (DakoCytomation), and data were analyzed using either CellQuest Pro (BD Biosciences) or Summit 4.3 software (DakoCytomation).

Assessment of insulitis and diabetes incidence

Cohorts of mice from each strain were bled fortnightly by retro-orbital venepuncture from 12 to 36 wk of age. Random blood glucose readings were obtained using the glucose oxidase method using CareSens test strips and glucometer (Life Bioscience). Mice were declared diabetic following two consecutive readings ≥11.1 mM or a single reading of ≥11.1 mM. Pancreata were excised from 36-wk-old female mice, fixed in 10% saline-buffered formalin (Sigma-Aldrich), and embedded in paraffin. Three serial 6-μm sections were taken at 100-μm intervals and stained with H&E. Sections were assigned insulitis scores as follows: 0, no evidence of infiltration; 1, <25% infiltration; 2, 50% infiltration; 3, 75% infiltration; 4, completely infiltrated or burnt-out. At least 20 islets per mouse were examined by a blinded assessor.
**Table III. List of highly differentially expressed genes on chromosome 2**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC053994</td>
<td>cDNA sequence BC053994</td>
<td>Unknown; wide intracellular expression, including peroxisomal</td>
</tr>
<tr>
<td>CI qr1</td>
<td>Complement component 1, q subcomponent, receptor 1</td>
<td>Receptor for C1q, mannose-binding lectin (MBL2), and pulmonary surfactant protein A (SPA). May mediate the enhancement of phagocytosis in monocytes and macrophages upon interaction with soluble defense collagen</td>
</tr>
<tr>
<td>NAPB</td>
<td>N-ethylmaleimide-sensitive fusion protein attachment protein β</td>
<td>Component of a SNARE-like complex. Required for vesicular transport between the endoplasmic reticulum and the Golgi apparatus</td>
</tr>
<tr>
<td>LOC245174</td>
<td>Hypothetical protein LOC245174</td>
<td>Unknown</td>
</tr>
<tr>
<td>Access1 (was Acass2l)</td>
<td>Acyl-CoA synthetase short-chain family member 1</td>
<td>Converts acetate to acetyl-CoA so that it can be used for oxidation through the tricarboxylic cycle to produce ATP and CO2. Mitochondrial part of the GINS complex, which plays an essential role in the initiation of DNA replication</td>
</tr>
<tr>
<td>Gins1</td>
<td>GINS complex subunit 1 (Psf1 homolog)</td>
<td>A casein kinase. The α and α' chains contain the catalytic site. Participates in Wnt signaling</td>
</tr>
<tr>
<td>Csnk2a1</td>
<td>Casein kinase II, α 1 polypeptide</td>
<td>Disrupts insulin signaling by binding directly to Akt kinases and blocking their activation. Binds to ATF4 and inhibits its transcriptional activation activity. Interacts with the NF-κB transactivator p65 RELA and inhibits its phosphorylation and thus its transcriptional activation activity. Interacts with MAPK kinases and regulates activation of MAP kinases. May play a role in programmed neuronal cell death but does not appear to affect non-neuronal cells. Does not display kinase activity</td>
</tr>
<tr>
<td>Trib3</td>
<td>Tribbles homolog 3</td>
<td>Role of Tribbles homolog 3 in cell migration and anchoring them at centrosomes. May play a role in cell migration</td>
</tr>
<tr>
<td>BDLR1</td>
<td>p55 and DNA damage-regulated 1</td>
<td>Role of BDLR1 in cell migration and anchoring them at centrosomes. May play a role in cell migration</td>
</tr>
<tr>
<td>Ass1l</td>
<td>Additional sex combs-like 1</td>
<td>PCG proteins act by forming multiprotein complexes, which are required to maintain the transcriptionally repressive state of homeotic genes throughout development, probably via methylation of histones</td>
</tr>
<tr>
<td>Dmnt3b</td>
<td>DNA methyltransferase 3B</td>
<td>Required for genome wide de novo methylation and is essential for development</td>
</tr>
<tr>
<td>Mapre1</td>
<td>Microtubule-associated protein, RP/EB family, member 1</td>
<td>Role of Mapre1 in microtubule polymerization, and spindle function by stabilizing microtubules and anchoring them at centrosomes. May play a role in cell migration</td>
</tr>
<tr>
<td>Cdk5rap1</td>
<td>CDK5 regulatory subunit associated protein 1</td>
<td>Probable regulator of CDK5 activity. May inhibit CDK5 function via its interaction with CDK5R1</td>
</tr>
<tr>
<td>Pomp4</td>
<td>Peroxisomal membrane protein 4</td>
<td>Peroxisomal multi-pass membrane protein; interacts with Pex19</td>
</tr>
<tr>
<td>8030497103Rik</td>
<td>RIKEN cDNA 8030497103 gene</td>
<td>Unknown</td>
</tr>
<tr>
<td>Access2 (was Acass2)</td>
<td>acyl-CoA synthetase short-chain family member 2</td>
<td>Role of Access2 in cell migration</td>
</tr>
<tr>
<td>Nfs1</td>
<td>Nitrogen fixation gene 1 (S. cerevisiae)</td>
<td>Cysteine desulfurase; cytoplasmic, nuclear and peroxisomal</td>
</tr>
<tr>
<td>2010100012Rik</td>
<td>RIKEN cDNA 2010100012 gene</td>
<td>Unknown; wide intracellular expression, including peroxisomal</td>
</tr>
<tr>
<td>Pfb20</td>
<td>PHD finger protein 20</td>
<td>Possible transcription factor</td>
</tr>
</tbody>
</table>


*See http://www.harvester.fzk.de/harvester/.

**In vitro proliferation assay**

Liver leukocytes (10^5/well) from NOD.Cd1d^{−/−}, NOD.Nkpr1^{b}, Nk2b^{b}, and NOD.Nkpr1^{b} mice were cultured in the presence of 10 ng/ml recombinant IL-7 (BD Pharmingen) with or without 0.5 μg/ml α-GalCer (Alexis Biochemicals) for 5 days. Proliferation was assayed by the addition of 0.25 μCi of [6-3H]-labeled thymidine per 200 μl well (GE Healthcare) 16 h before harvesting, and the cells were harvested with a Tomtec Harvester 96 Mach IIIM and the emission scintillated with MultiLex A melt-on scintillator sheets (Wallac) and detected with a Wallac 1450 MicroBeta JET liquid scintillation counter.

**In vivo treatment with α-GalCer**

α-GalCer (Alexis Biochemicals) was prepared in glycolipid dilution medium (10 ml distilled H_2O, 560 mg sucrose (Invitrogen), 75 mg t-Histidine (Sigma-Aldrich), 50 mg Tween 20 (Sigma-Aldrich)). Mice were injected i.v. with 4 μg of α-GalCer or control vehicle (total volume of 100 μl). Blood samples were collected by retro-orbital venepuncture from treated and control mice at 4 h following injection. Samples were centrifuged and the plasma was stored at −80°C for later analysis of cytokine levels by bead array (Bender MedSystems).

**Measurement of cytokines by bead array**

Plasma cytokine levels were determined using the Mouse Th1/Th2 10plex FlowCytomix Multiplex (Bender MedSystems) according to the manufacturer’s instructions. Serial dilutions of the provided cytokine standards were prepared and assayed as described above. Standard curves were generated and samples quantified using the Flow Cytomix Pro 2.2 software (Bender MedSystems).
Results

**NOD.Nkrp1/Nkt2** congenic mice

A NOD.Nkrp1b/Nkt2b congenic mouse line carrying a C57BL/6-derived chromosomal segment spanning 12.6 Mb of the 95% confidence interval of Nkt2 was produced by serial backcrossing to the NOD.Nkrp1b strain to N10, followed by intercrossing and selection for Nkt2b homozygotes. The proximal boundary of the congenic segment lies between D2mit422 and D2mit404, and the distal boundary is between D2mit412 and D2mit528 (Fig. 1A). A background screen of 156 polymorphic loci distributed throughout the rest of the autosomal genome failed to detect any residual C57BL/6-derived genomic contamination (Table I). Flow cytometric analyses of thymic NKT cell numbers and proportions, as determined by CD1d/α-GalCer tetramer binding, confirmed that thymi from the NOD.Nkrp1b/Nkt2b congenic line have larger proportions (Fig. 1B and Table II).
II) and numbers (Fig. 1C and Table II) of type 1 NKT cells than those from the NOD. Nkrp1b parental strain controls.

NOD. Idd13 NOR mice, which bear a NOR-derived 70-Mb chromosomal segment spanning the entire 95% confidence interval of the Nkt2 locus, have numbers of thymic type 1 NKT cells exceeding those of both the NOD. Nkrp1b and NOD. Nkrp1bbb lines (Fig. 1B). This result is consistent with more than one locus within the Nkt2 linkage region controlling thymic type 1 NKT cell numbers: one within the Nkt2b congenic segment, and at least one other locus within the Idd13 congenic segment.

Homozygous congenic NOD. Idd13NOR, NOD. Nkrp1b.Nkt2bb, and control NOD. Nkrp1b mice were bled at 2-wk intervals from 12 to 36 wk by retro-orbital venepuncture and random blood glucose levels determined by the glucose oxidase technique. At 36 wk of age, 3 of 25 (12%) female NOD. Idd13NOR congenic mice, 16 of 42 (38%) female NOD. Nkrp1b.Nkt2bb congenic mice, and 24 of 38 (63.2%) female control mice had developed diabetes (p < 0.05, 4-fold table χ² test; Fig. 1D). At the same age, 3 of 21 (14%) male NOD. Idd13NOR congenic mice, 12 of 39 (30.8%) male NOD. Nkrp1b.Nkt2bb congenic mice, and 9 of 24 (37.5%) male control mice had developed diabetes (NS, 4-fold χ² table test; data not shown). Similarly, the severity of insulitis observed at 36 wk in the pancreata of NOD. Nkrp1b.Nkt2bb female mice was intermediate between that observed in female NOD. Idd13NOR and control NOD. Nkrp1b mice (Fig. 1E).

NOD. B2mα and NOD. B2mβ transgenic mice

B2m, which encodes β2m (the light chain of MHC class I products and CD1d) is a candidate for Nkt2 because it lies close to the 95% confidence limits for Nkt2 (9) and because CD1d acts as the selection and restriction ligand for type 1 NKT cells (1). As the Nkt2b congenic segment excludes the B2m locus, the phenotype of this line cannot be attributed to allelic variation at B2m. However, as NOD. Idd13NOR mice show a greater increase in CD1d/α-GalCer tetramer binding thymic type 1 NKT cells than do NOD. Nkrp1b.Nkt2bb congenic mice, it remained possible that the C57BL/6 allele of B2m increased NKT cell numbers.

**FIGURE 7.** The effects of the Nkt2b congenic region on the ability to present glycolipid and activate type 1 NKT cells were studied. The levels of expression of CD1d on CD4⁺CD8⁺βTCR⁺ thymocytes of NOD. Nkrp1b.Nkt2bb congenic and NOD. Nkrp1b parental control mice were similar (A). Hepatic NKT cells from NOD. Nkrp1b.Nkt2bb congenic mice (n = 5; two replicates per mouse) make greater proliferative responses to α-GalCer stimulation in vitro than do those from NOD. Nkrp1b parental control mice (n = 7; two replicates per mouse; B). Liver leukocytes from control NOD. Cd1−/− mice (n = 3; two replicates per mouse) failed to respond. In vivo cytokine production (means ± SEM) 4 h after injection i.v. with 4 μg of α-GalCer of NOD. Nkrp1b.Nkt2bb congenic (n = 6) was greater than that of NOD. Nkrp1b parental control mice (n = 8; C). NOD. Cd1−/− mice (n = 4) failed to respond.

[Graphs and tables showing data from experiments are not included in this text representation.]
in addition to a second linked locus lying within the Nkt2b congenic region. To formally test this possibility, comparisons of thymic NKT cell numbers and proportions between NOD.B2m<sup>-/-</sup>/C57BL/6 congenic strains that transgenically express either the a or b allele of B2m (21) were performed. No significant increase in either the proportions or numbers of NKT cells was found in the transgenic line bearing the C57BL/6 B2m<sup>+</sup> allele (Fig. 2), formally excluding the possibility that B2m contributes to the increase in type 1 NKT cell numbers conferred by C57BL/6-derived alleles in the Nkt2 linkage region.

**NKT cell subsets in NOD.Nkrp1<sup>b</sup>.Nkt2b<sup>b</sup> congenic mice**

Thymic NKT cell subsets, which are related to each other by a developmental pathway, can be defined by the cell surface markers CD4, CD44, and NK1.1 (24, 25). Flow cytometric analyses of thymic NKT cells from NOD.Nkrp1<sup>b</sup>.Nkt1<sup>b</sup> mice, which are congenic for the chromosome 1 NKT cell control gene, revealed that most additional NKT cells were of the relatively immature CD<sup>4</sup><sup>+/</sup>CD44<sup>hi</sup>NK1.1<sup>-</sup> population (12), raising the possibility that the Nkt2 locus contributed to NKT cell thymic maturation, in addition to affecting total type 1 NKT cell numbers. Consistent with this view, a comparison of NOD.Nkrp1<sup>b</sup>.Nkt2b<sup>b</sup> mice with NOD.Nkrp1<sup>b</sup> parental control mice indicated that all type 1 NKT subsets are increased approximately 2-fold in the thymi of congenic mice, while in the periphery there is a disproportionate increase in the numbers of the developmentally mature NK1.1<sup+</sup> subset that is most apparent in the liver (Fig. 3).

**Microarray gene expression analysis**

As the identification of genes contributing to the phenotype of NOD.Nkrp1<sup>b</sup>.Nkt1<sup>b</sup> mice was greatly assisted by transcriptomic analysis of thymocytes (12), the same approach was taken to identify a list of candidate genes that could contribute to the phenotype observed in NOD.Nkrp1<sup>b</sup>.Nkt2b<sup>b</sup> mice. Microarray gene expression analysis was performed on thymi of 4-wk-old NOD.Nkrp1<sup>b</sup> (n = 9) and NOD.Nkrp1<sup>b</sup>.Nkt2b<sup>b</sup> mice (n = 7; Fig. 4A), following procedures to minimize activation of the apoptosis cascade. Thymic RNA was extracted, labeled, and hybridized to Affymetrix mouse 430 series 2.0 expression microarrays, which were scanned using an Affymetrix 7G scanner. Data were imported into Avadis Prophetic using an RNA summarization algorithm. The statistical significance threshold was set by permutative analysis (100,000 permutations) and a Mann-Whitney U test applied. A total of 52 genes were identified as being highly differentially expressed (i.e., those with a p < 0.0002), of which 19 mapped to the Nkt2b congenic region (< 0.6% of genome; χ² = 1087; df = 1; p < 10<sup>-200</sup>; χ² one sample test; Fig. 4B–D). All 19 of the highly differentially expressed genes mapping to the Nkt2b congenic region lay within the 95% confidence limits obtained in the original linkage analysis (9) (Fig. 5). Their physical positions and expression fold-change are shown in Fig. 5D, and their identities are given in Table III.

While further work is required to characterize the genes on this shortlist, one potential candidate for control of NKT cell numbers is Pomp<sub>4</sub>, which encodes a 24-kDa peroxisomal integral membrane protein of unknown function. The only molecule to which it is known to bind is the dual compartment (cytoplasmic/peroxisomal), chaperone/membrane transporter Pex19 (26), which is encoded by a gene that lies within the Nkt1 linkage region (9) and is highly differentially expressed in NOD.Nkrp1<sup>b</sup>.Nkt1<sup>b</sup> congenic mice (12). Validation of Pomp<sub>4</sub> microarray data was obtained by quantitative RT-PCR of the sequences probed by the array on an independent sample set (Fig. 6, n = 6–7; p < 0.02; Mann-Whitney U test).

Although a role for peroxisomes in NKT cell biology has not been previously proposed, they play a critical role in glycolipid metabolism and phospholipid biosynthesis, and intersect with the endosomal processing pathway (see Discussion), consistent with a role in CD1-mediated glycolipid presentation. Certainly, natural and targeted deletion mutants of a relatively broad range of genes that affect fatty acid metabolism express severe deficiencies in thymic type 1 NKT cell numbers (27–31).

**Effects of the Nkt2b congenic interval on glycolipid presentation**

As targeted mutant β-hexosaminidase B-deficient mice (a model of Sandhoff disease; Ref. 32) and natural mutant BALB/cNctr-Npc1<sup>+/</sup>/Npc1-deficient mice (a model of Niemann-Pick type C disease; Ref. 33) show defects in their ability to positively select and stimulate type 1 NKT cells (31), the effects of the Nkt2b congenic interval on NKT cell stimulation were studied. The levels of expression of CD1d on CD4<sup>+</sup>CD8<sup>+</sup>β<sup>+</sup>TCR<sup>+</sup> thymocytes of NOD.Nkrp1<sup>b</sup>.Nkt2b<sup>b</sup> congenic and NOD.Nkrp1<sup>b</sup> parental control mice were similar (Fig. 7A). An attempt was made to study presentation by irradiated thymocytes from NOD.Nkrp1<sup>b</sup>.Nkt2b<sup>b</sup> congenic and parental control mice in vitro, but in our hands, thymocytes presented glycolipid poorly and inhibited autopresentation of α-GalCer by hepatic leukocytes. In the absence of additional filler cells, the proliferative responses of hepatic leukocytes from NOD.Nkrp1<sub>b</sub>.Nkt2b<sup>b</sup> congenic mice were greater than those from NOD.Nkrp1<sub>b</sub> parental mice when stimulated with α-GalCer (p < 0.05; Mann-Whitney U test; n = 5–7; two replicates per mouse; Fig. 7B).

Consistent with the in vitro findings of increased autostimulation of hepatic leukocytes from NOD.Nkrp1<sub>b</sub>.Nkt2b<sup>b</sup> congenic mice, in vivo production of IL-2, IL-4, and IFN-γ 4 h after injection i.v. of NOD.Nkrp1<sub>b</sub>.Nkt2b<sup>b</sup> congenic with 4 μg of α-GalCer was significantly higher than that of NOD.Nkrp1<sub>b</sub> parental control mice (n = 6–8; p < 0.03; Mann-Whitney U test; Fig. 7C).

**Discussion**

NOD mice have fewer type 1 NKT cells than do C57BL/6 or BALB/c mice, as determined by CD1d/α-GalCer tetramer staining (22). A major aim of this laboratory is to identify the genetic coding sequences responsible for the difference in thymic type 1 NKT cell numbers between the NOD and C57BL/6 mouse strains, taking advantage of the NOD.Nkrp1<sub>b</sub> congenic line, which expresses the NKT cell developmental marker NK1.1 (22, 23). Identification of genes that control type 1 NKT cell number is of interest because of the broad range of immune functions regulated by this cell population. The approach taken here (i.e., linkage analysis in a mouse model and the production of congenic lines) follows the traditional route that resulted in the identification and characterization of the role of the MHC in immune responses.

Two genetic regions affecting thymic type 1 NKT cell numbers in this system were identified by linkage analysis. The first, Nkt1, mapped near D1mit15 on distal chromosome 1 (9), in the same region as the NOD mouse lupus susceptibility gene Babs2/Bana3 (11), and the second locus identified, Nkt2, mapped between D2mit490 and D2mit280 on chromosome 2 (9), in the same region as the diabetes susceptibility gene Idd13 (17, 18). The locations of Nkt1 and Nkt2 have been confirmed by the production of congenic lines and the demonstration of partial correction of type 1 NKT cell numbers in each case (Ref. 12 and data presented herein). Comparison of the numbers of CD1d/α-GalCer tetramer-binding type 1 NKT cells in NOD mice bearing the C57BL/6-derived Nkt2b congenic segment and those bearing the larger NOR-derived (b haplotype) Idd13 congenic segment provided evidence of at
least two loci within the Nkt2 region affecting type 1 NKT cell numbers: one within the Nkt2b region, and the other more proximal. The possibility that the second locus causing a decrease in type 1 NKT cells numbers in NOD mice is B2m was formally excluded by analysis of allelic B2m transgenic lines produced on a B2m-targeted mutant background (21). The degree of protection from type 1 diabetes seen in the two congenic lines was consistent with at least two loci within the Idd13 region also controlling this phenotype, as has been previously reported (20). Although one of the diabetes susceptibility loci has been formally demonstrated to be B2m, it is clear that an additional locus lies within the Nkt2b congenic region. This raises the intriguing possibility that the protection from diabetes provided by the Nkt2b segment is mediated by its effects on NKT cell numbers and development.

Thymic NKT cell subsets, which are related to each other by a developmental pathway, can be defined by the cell surface markers CD4, CD44, and NK1.1 (24, 25). A comparison of C57BL/6 and NOD mice revealed that in addition to an ~3–4-fold reduction in thymic NKT cell number, those type 1 NKT cells that developed in the thymi of NOD mice were relatively developmentally retarded, with approximately one third fewer expressing the maturation NK1.1 marker. While both the Nkt1 and Nkt2b congenic lines produced increased numbers and proportions of thymic (and peripheral) NKT cells, most of the increase in NOD.Nkrp1b.Nkt1b mice was due to the relatively immature NK1.1- populations, suggesting that this locus supported the entry of larger numbers of thymocytes into the NKT cell developmental pathway, without greatly affecting their maturation. In contrast, the Nkt2b segment increased the numbers of type 1 NKT cells of all developmental stages. Indeed, in the liver, almost all of the increase in type 1 NKT cell number was due to increased numbers of the relatively mature NK1.1+ type 1 NKT cells. Another significant difference between the Nkt1l and Nkt2l regions is that expression of C57BL/6 alleles at the Nkt1l locus failed to provide protection from type 1 diabetes (Ref. 34 and our unpublished data), while the NOD.Nkrp1b.Nkt2b line expressed a significantly reduced incidence of disease. It is possible that the increased numbers of NKT cells found in the NOD.Nkrp1b.Nkt2b congenic mice were functionally immature, consistent with their relatively immature cell surface phenotype. Although Rocha-Campos et al. (34) reported improved cytokine (IL-4 and IFN-γ) secreting performance by type 1 NKT cells from NOD.Nkt1 congenic mice, recent experiments have raised the possibility that diabetes protection conferred by type 1 NKT cells in the NOD mouse model can be mediated by a cell-cell contact-dependent mechanism in the absence of IL-4 (35).

The C57BL/6 allele of Nkt2b is associated with increased NKT cell proliferation and cytokine responses to α-GalCer as well as with increased numbers of thymic NKT cells of all stages of maturity. The simplest explanation for these data is that the C57BL/6 allele confers a stronger stimulatory capacity, resulting in increased thymic-positive selection of developing NKT cells. A directly analogous argument was applied to explain the increase in NKT cell numbers in NOD.Nkrp1b.Nkt2b congenic mice.

The characterization of mouse lines for both Nkt1l and Nkt2 has been greatly assisted by the application of microarray expression analyses, which have reduced the number of candidate genes of interest from potentially thousands of loci to one or two dozen. A striking feature of both this manuscript and our previous study of the Nkt1 congenic region (12) is the extremely high signal-to-noise ratio (χ2 of approximately 105) and the resulting resolving power of the studies. Factors contributing to this success appear to be the maturity of the technology, use of an adequate number of replicates, choice of biological system, and avoidance of apoptosis and activation of experimental samples. This approach has provided for the Nkt2b region a list of 19 candidates based on genomic location and highly significant differences in thymic expression between the NOD.Nkrp1b.Nkt2b congenic line and the NOD.Nkrp1b parental control line. While further work is required to characterize the candidates on this short list, at this stage the most prominent candidate appears to be Pxm4.

Pxm4 (was termed PMP24) was originally isolated as a 24-kDa polypeptide from rat liver peroxisome membranes (36). Although of unknown function, it contains two putative membrane-spanning domains and, consistent with its role as a peroxisomal intrinsic membrane protein, it lacks the peroxisome targeting sequences (both type 1 and type 2) that mediate transport into the peroxisome lumen via binding Pex5 and Pex7 (36). Instead, it contains the Pex19BS motif VxxFxxR (http://www.peroxisomedb.org), which mediates peroxisomal membrane insertion via Pex19 (26). Remarkably, the gene Pex19 lies within the Nkt1 linkage region (9) and is highly differentially expressed in NOD.Nkrp1b.Nkt1b congenic mice, which also have a partial correction of the deficiency in numbers of thymic NKT cells (12) that is characteristic of NOD mice (4, 5).

Peroxisomes were first described by Nobel Laureate (1974) Christian René de Duve (37). They are ubiquitous cytoplasmic phospholipid bilayer-delimited organelles in eukaryotes, where they play critical roles in fatty acid β- and α-oxidation (degradation) and etherphospholipid biosynthesis. Peroxosomes are rich in glycosidases, including α-galactosidase and especially β-galactosidase (38), deficiencies of which are associated with glycosphin-golipid storage diseases and, in some studies, reduced numbers of type 1 NKT cells (27, 31, 39). Peroxisomes self-assemble from a specialized compartment of the endoplasmic reticulum and are able to increase their size, number, and enzymic activity in response to stimulation via peroxi-some proliferator-activated receptors (PPAR), which are members of the nuclear steroid hormone receptor superfamily of ligand-activated transcription factors.

Oxidized low-density lipoproteins are natural activators of PPARα and PPARγ, and can increase generation of all-trans retinoic acid from retinol, resulting in retinoic signaling via activation of the retinoic acid receptor α (RARα) (40). In human dendritic cells, activation of RARα acutely up-regulates CD1d expression, enhancing type 1 NKT cell activation (41). The rate and efficiency of lipid metabolism in peroxisomes defines the steady-state levels of many of these signaling lipids, including long chain fatty acids and retinoid acid (42). Modulation of this pathway is unlikely to account for the differences in NKT cell numbers and activities in NOD.Nkrp1b.Nkt2b congenic mice, however, as CD1d expression remains unchanged in the line.

PPARα activation also leads to up-regulation of Npc1 and Npc2, the genes that are mutated in Niemann-Pick type C disease (NPC) (43). Although NPC belongs to the group of lysosomal storage diseases characterized by an accumulation of cholesterol and sphingomyelin in lysosomes, studies of BALB/cNctr-Npc1m1J mice, a spontaneous mutant mouse model of Npc1 deficiency, suggest additional effects on peroxisome function. A sizeable decrease of peroxisomal β-oxidation of fatty acids and catalase activity was observed in mouse NPC 18 days before the onset of signs of disease, while lysosomal enzymatic function did not decline until 6 days afterward (44). BALB/cNctr-Npc1m1J mice also express CD1d at lower levels in their thymi than do wild-type mice, and they lack type 1 NKT cells (30). Despite these observations, the molecular basis of any role for peroxisomes in CD1d/NKT cell biology remains unclear.

Although the function of Pxm4 is unknown, its translocation into the peroxisomal membrane by Pex19 suggests that it is likely
to play a role in one or other of the major metabolic pathways of peroxisomes. As increased message for Pxm4p is associated with the NOD allele that confers weaker NKT cell stimulatory capacity, one possibility is that Pxm4p contributes to the biosynthesis of a nonstimulatory etherphospholipid ligand for CD1d. Most etherphospholipids are phosphoglycerolipids similar in structure to phosphatidyl choline, which was the major component of the organic phase extracted from recombinant murine CD1d (45), or phosphatidyl ethanolamine, which was responsible for the NKT cell-stimulatory activity of a polar lipid fraction of a tumor cell extract (46), but carry an ether linkage instead of an ester linkage for one of the fatty acyl chains. The synthesis of etherphospholipids is entirely dependent on peroxisomal enzymes for multiple bio-synthetic steps. Indeed, the first step, the esterification of dihydroxyacetone-phosphate, which is catalyzed by the peroxisomal matrix protein dihydroxyacetonephosphate acyl-transferase, is also required for the synthesis of phosphatidyl choline and phosphatidyl ethanolamine (47). Such ligands could be introduced from peroxisomes into the endosomal pathway for loading into CD1d by at least two mechanisms. First, peroxisomes fuse with, and discharge some of their contents into, phagosomes (48), consistent with their classical antimicrobial role (49). Second, proliferated peroxisomes undergo autophagy, in which they are engulfed by a vesicle termed an autophagosome, which fuses with lysosomes, resulting in peroxisome proteolysis (50).

Alternatively, Pxm4p may be a component in a fatty acid degradation pathway that catalyzes stimulatory CD1d ligands. Peroxisomes play a critical role in the β- and α-oxidation of very long chain fatty acids (i.e., longer than C20) (47), which results in the cleavage of any carbohydrate head group from the acyl chains of chain fatty acids (i.e., longer than C20) (47), which results in the presentation.

be involved in modulating glycolipid availability for CD1d.

Insightful discussions.

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