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Slamf1, the NKT Cell Control Gene Nkt11

Margaret A. Jordan, Julie M. Fletcher, Daniel Pellicci, and Alan G. Baxter

Invariant NKT (iNKT) cells play a critical role in controlling the strength and character of adaptive 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. In this study, we report the production and characterization of a NOD.Nkprp1b.Nkt1b congenic mouse strain, apply microarray expression analyses to limit candidate genes within the 95% confidence region, identify Slamf1 (encoding signaling lymphocyte activation molecule) and Slamf6 (encoding Ly108) as potential candidates, and demonstrate retarded signaling lymphocyte activation molecule expression during T cell development of NOD mice, resulting in reduced expression at the CD4+CD8+ stage, which is consistent with decreased NKT cell production and delayed tolerance induction in NOD mice. The Journal of Immunology, 2007, 178: 1618–1627.

Invariant NKT (iNKT) cells are an immunoregulatory population of lymphocytes that plays a critical role in controlling the adaptive immune system and contributes to the regulation of autoimmune responses (1–3). We have previously reported deficiencies in the numbers and function of NKT cells in the NOD mouse strain (4, 5), which is a well-validated model of type 1 diabetes (6) and systemic lupus erythematosus (7, 8), and mapped genetic control of thymic NKT cell numbers in a first backcross (BC1) from C57BL/6 to NOD.Nkprp1b mice (9). The numbers of thymic NKT cells of 320 BC1 mice were determined by fluorescence-activated cell analysis using CD1d/α-galactosylceramide (CD1d/α-GalCer) tetramer (10). Tail DNA of 138 female BC1 mice was analyzed for PCR product length polymorphisms at 181 simple sequence repeats, providing >90% coverage of the autosomal genome with an average marker separation of 8 cM. Two loci exhibiting significant linkage to NKT cell numbers were identified; the most significant (Nkt1; log-likelihood ratio 6.82) was mapped near D1mit15 on distal chromosome 1 (9) in the same region as the NOD mouse lupus susceptibility gene Babs2/Bana3 (11). The second locus (Nkt2; log-likelihood ratio 4.90) was mapped between D2mit490 and D2mit280 on chromosome 2 (9) in the same region as Idd13, a NOD-derived diabetes susceptibility gene (12). In an attempt to identify the genetic sequences on chromosome 1 that control NKT cell numbers, we produced and characterized a NOD mouse line congenic for the C57BL/6 allele at the Nkt1 locus.

Materials and Methods

Mice

NOD.Nkprp1b, C57BL/6d, and congenic mice were maintained at the Immunogenetics Research Facility at the James Cook University in specific pathogen-free conditions. The NOD.Nkprp1b strain carries B6-derived alleles at the NK complex on chromosome 6 (from D6mit105 to D6mit135), permitting the use of the NK1.1 marker (13, 14). NOD.Nkprp1b.Nkt1b mice were produced by intercrossing NOD.Nkprp1b and C57BL/6d mice and performing serial backcrosses to NOD.Nkprp1b 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.Nkprp1b, NOD.Nkprp1b.Nkt1b, and C57BL/6d mouse strains was conducted using the CAS-1810 X-TractorGene (Corbett Robotics) and the XTR2 X-tractor gene solid sample reagent pack (Sigma-Aldrich), which is based on a method developed in this laboratory. Briefly, DNA was extracted by digesting an 11-mm tailtip in 400 µl of digest buffer (100 mM Tris-HCl (pH 8), 10 mM EDTA, 100 mM NaCl, 0.5% SDS, 50 mM DTT, and 100 mM proteinase K), O/N, 56°C, 40 rpm in a VORTEMP 56EVC (Labnet). Samples were lysed by addition of 700 µl of 5.25 M guanidine thiocyanate lysis buffer (5.25 M guanidine thiocyanate, 10 mM Tris-HCl (pH 6.5), 20 mM EDTA, 0.4% Triton X-100, and 64.8 mM DTT), loaded on a glass filter (GF/B) polypropylene microplate (Whatman International), and washed twice in protein wash buffer and once in 100% ethanol. Samples were eluted in 150 µl of elution buffer. The DNA yield was quantified spectrophotometrically.

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, as well as markers designed in-house on the basis of PCR product length polymorphisms between C57BL/6 and NOD/Lt strains, as described previously (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 RNA-later (Qiagen) within 120 s of the mouse being placed in CO2 for asphyxiation. In our hands, this procedure substantially improved the signal to noise ratio of expression analysis, greatly reducing the numbers of differentially expressed genes identified. The thymi were individually homogenized in the RLT buffer of an RNeasy kit (Qiagen), with contamination minimized by extensive washing.
Characterization of the NOD.Nkrp1p/Nkt1p congenic mouse line. The boundaries of the Nkt1 congenic segment on distal chromosome 1 are indicated (A). Proportions (B) and absolute numbers (C) of thymic NKT cell numbers in 5-wk-old mice from the congenic line and the NOD.Nkrp1p parental line as determined by CD1d/α-GalCer tetramer binding are shown. Values for NOD.Nkrp1p mice are indicated by □ whereas those for NOD.Nkrp1p/Nkt1p mice by ●. Proportion means and SEM are shown, whereas for numbers, individual values and statistical analysis (Mann-Whitney U test) are given.

First-strand cDNA synthesis

First-strand cDNA was synthesized from 5 μg of total RNA using oligo(dT) primers and Superscript II reverse transcriptase following manufacturer’s instructions (Invitrogen Life Technologies).

Real-time quantitative PCR

Primers were designed to verify microarray data on independent samples of NOD mice. The primers used for quantitation were as follows: Slamf1 exon 3–5 (microarray probe 1425570_at), F primer, 5′-TAATCTTC ATCTCGTTTCTACGGC-3′, and R primer, 5′-TTGGGCAATAAAGTA AGGC-3′; Slamf1 exon 7 (microarray probe 1425569_a_at), F primer, 5′-AG ATGAAAGGAAACCAAGC-3′, and R primer, 5′-TGTTTGAGCATA AGAAAGGC-3′; Slamf6 L isoform (microarray probe 1420659_at), F primer, 5′-TCCACCACCCTGTTGCTGTA-3′, and R primer, 5′-TGGAAGAGTG 3′.

Expression microarray hybridizations were performed by the Australian Genome Research Facility using the one-cycle cDNA synthesis kit (Affymetrix) and Affymetrix 430 2.0 mouse gene microarray, which contains 45,000 probe sets, representing 34,000 well-substantiated mouse genes.

The probe arrays were scanned using the GeneChip Scanner 3000, and the images (.dat files) were processed using GeneChip Operating System (Affymetrix) and imported into Avadis Prophetic3.3 (Strand Genomics) for further analysis. The statistical significance threshold was set by permutation analysis (10,000 permutations) and a Kruskal-Wallis test. A conservative significance threshold of p < 0.001 was set; this value coincided with a lack of overlap in signal values between the two groups (n = 7/group).

Cell suspension preparation

Thymocyte cell suspensions were prepared by gently grinding the thymus between two frosted microscope slides in MACS buffer (PBS containing 2 mM, 1 mM, and 0.1 mM of cDNA. Slamf1 and Slamf6 expression values were normalized against Gapdh, as microarray expression analyses had shown that this gene was not differentially expressed between NOD.Nkrp1p and NOD.Nkrp1p/Nkt1p mice. Presence of C57BL/6-derived alleles.

**Table 1. List of genetic markers tested to confirm genetic homogeneity of NOD.Nkrp1p/Nkt1p mouse line**

<table>
<thead>
<tr>
<th>Genetic Marker</th>
<th>Homozygous C57BL/6 Carrier Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1mit58</td>
<td>D1mit72, D1mit279, D1mit124, D1mit180, D1mit438, D1mit306, D1mit494, D1mit348, D1mit445, D1mit103, D1mit199, D1mit199, D1mit102, D1mit449, D1mit208, D1mit369, D1mit396*</td>
</tr>
<tr>
<td>D1mit58</td>
<td>D1mit458, D1mit406, *D1mit209, D1mit555</td>
</tr>
</tbody>
</table>

* Presence of C57BL/6-derived alleles.
mM EDTA (Amresco) and 0.5% (w/v) BSA (ICN Biomedicals). Spleens were disrupted using a 26-gauge needle and forceps and the resulting cell suspension treated with RBC lysing buffer (Sigma-Aldrich).

Flow cytometric analysis

For flow cytometric analyses cells were labeled with anti-/H9252 TCR-FITC (clone H57-597), anti-CD3-FITC (clone 145-2C11), anti-CD3-allophycocyanin (clone 145-2C11), anti-CD3-allophycocyanin-Cy7 (clone 145-2C11), anti-CD4-allophycocyanin (clone GK1.5), anti-CD4-PerCP-Cy5.5 (clone RM 4-5), anti-NK1.1-PE-Cy7 (clone PK136), anti-CD8-FITC (clone 53-6.7), anti-CD45R/B220-allophycocyanin (clone RA3-6B2), anti-CD44-FITC (clone IM7), all from BD Pharmingen, and anti-CD150(SLAM)-PE (clone TC15-12F12.2; Biolegend). Mouse CD1d tetramer, conjugated to either PE or PE-Cy7 and loaded with /H9251 α-GalCer, was produced in house as previously described (10) using recombinant baculovirus encoding his-tagged mouse CD1d and mouse /H9252 β2-microglobulin, provided by Prof. M. Kronenberg’s laboratory (La Jolly Institute for Allergy and 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 an additional 20-min incubation with 10% mouse serum to prevent FcR binding, before addition of surface staining Ab mixtures. 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 outliers.

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

<table>
<thead>
<tr>
<th>Organ</th>
<th>Strain</th>
<th>n</th>
<th>Total Cell Number (×10⁶)</th>
<th>% NKT Cells</th>
<th>Number NKT Cells (×10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus</td>
<td>NOD.Nkrp1</td>
<td>7</td>
<td>113 ± 7</td>
<td>0.09 ± 0.01</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>F1</td>
<td>7</td>
<td>143 ± 9</td>
<td>0.16 ± 0.02</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>NOD.Nkrp1.Nkt1</td>
<td>4</td>
<td>184 ± 7</td>
<td>0.17 ± 0.01</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>Spleen</td>
<td>NOD.Nkrp1</td>
<td>5</td>
<td>75 ± 3</td>
<td>0.30 ± 0.02</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>F1</td>
<td>4</td>
<td>65 ± 1</td>
<td>0.44 ± 0.03</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>NOD.Nkrp1.Nkt1</td>
<td>5</td>
<td>62 ± 3</td>
<td>0.55 ± 0.02</td>
<td>3.4 ± 0.2</td>
</tr>
</tbody>
</table>
autofluorescent cells. In general, flow cytometry was performed on a FACSVantage SE with FACSDiVa option (BD Biosciences) and data analyzed using either CellQuest Pro or FACSDiVa software (BD Biosciences). The data in Fig. 8E were acquired on a CyAn ADP flow cytometer (DakoCytomation) and analyzed with Summit 4.3 software (DakoCytomation).

**Proliferation assays**

Single-cell suspensions were cultured in triplicate at 37°C, 5% CO₂ for 3–5 days in RPMI 1640 medium with l-glutamine (Invitrogen Life Technologies) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 50 μM 2-ME. Stimulation was achieved by the addition of Dynabead Mouse CD3/CD28 T cell expander beads (Dynal Biotech) in varying proportions. Some cultures were established in the presence of 100 μg/ml of the blocking signaling lymphocyte activation molecule (SLAM) peptide 132–146 (FCKQLKLYEQVSPPE; Auspep), 100 μg/ml of the nonblocking SLAM peptide 83–97 (DLSKGSYPDHLEDGY; Auspep) or inhibiting concentrations (6.25 μg/ml) of the TC15 anti-SLAM mAb (Biolegend). Proliferation was assayed by the addition of 0.25 μCi of 6-3H-labeled thymidine per 200-μl well (GE Healthcare) 8–16 h before harvesting. At termination, plates were spun to pellet cells, 100 μl of supernatant was removed for cytokine assays, and the cells were harvested with a Tomtec Harvester 96Mach IIIM, the emission scintillated with MeltiLex A melt-on scintillator sheets (Wallac) and detected with a Wallac 1450 Microbeta Jet liquid scintillation counter.

**Cytokine measurement**

Cytokine levels in cell culture supernatants were determined using Mouse Th1/Th2 Cytokine Cytometric Bead Array (BD Biosciences). Capture beads (30 μl, specific for IL-2, IL-4, IL-5, IFN-γ, and TNF) together with 30 μl of culture supernatant samples and 30 μl of PE detection reagent, were incubated for 2 h in 96-well plates. Beads were washed twice with 200 μl of wash buffer, resuspended, and data were acquired using a FACSCalibur (BD Biosciences). Serial dilutions of the provided cytokine standards were prepared and assayed as described above. Standard curves were generated and samples quantified using the BD CBA software (BD Biosciences).

**Results**

A NOD.Nkrd⁺·Nktb⁺ congenic mouse line carrying a C57BL/6-derived chromosomal segment spanning the 95% confidence interval of Nktb was produced by serial backcrossing to the NOD.Nkrd⁻ strain to N10, followed by intercrossing and selection for Nktb homozygotes. The proximal boundary of the congenic segment lies between D1mit369 and D1mit396 and the distal boundary is distal to the most telomeric marker available, D1mit155 (Fig. 1A). A background screen of 136 polymorphic loci distributed throughout the rest of the autosomal genome failed to

**FIGURE 3.** Averaged log signal intensity of Affymetrix Mouse 430 series 2 expression microarray profiling of thymi from NOD.Nkrd⁺ and NOD.Nkrd⁺·Nktb⁺ mice (n = 7/group). A. Results of genes for which a p < 0.05 was obtained are illustrated. Genes that are highly differentially expressed (experiment-wise permutative analysis threshold of p < 0.001; Kruskal-Wallis test). Diagonal lines indicate 2-fold differential expression. Numbers indicate gene identities as listed in Fig. 4. The linkage data from Ref. 9 are presented transformed to physical distances (B), the location of the Nkr1 congenic interval (indicated by the solid line) presented on the same scale (C), and the locations of the highly differentially expressed genes displayed as a histogram (D). The probability of 21 of the 28 locatable highly differentially expressed genes mapping to the Nktb congenic region by chance is p < 10⁻²⁰⁰ (χ² one sample test).
The proportions of thymic NKT cell numbers in (NOD.Nkrp1b/Nkrp1b) congenic line is a product of both a higher proportion of NKT cells strain controls. The increase in thymic NKT cell numbers in the NOD.Nkrp1b/Nkrp1b confirmed that thymi from the NOD.Nkrp1b/Nkt1b mice are intermediate between the congenic and parental strains. Microarray gene expression analysis

To identify a subset of candidate genes within the Nkt1 linkage 95% confidence interval, microarray gene expression analysis was performed on thymi of 4-wk-old NOD.Nkrp1b/Nkt1b and NOD.Nkrp1p/Nkrp1p mice (n = 7/group; Fig. 3A), following procedures to minimize activation of the apoptosis cascade. Thymic RNA was extracted, hybridized and scanned by Affymetrix Mouse 430 series 2 expression microarrays performed by the Australian Genome Research Facility, and data imported into Avadis Prophetic using an RMA summarization algorithm. The statistical significance threshold was set by permutative analysis (10,000 permutations) and a Kruskal-Wallis test applied. A total of only 28 genes were identified as being highly differentially expressed (i.e., those with a p < 0.001), of which 21 mapped to the Nkt1 congenic region (~1.6% of genome; χ² = 986; df = 1; p < 10⁻²⁰⁰, χ² one sample test; Fig. 3B–D). This result is indicative of an extremely good signal-to-noise ratio.

Only 15 of the 21 highly differentially expressed genes mapping to the Nkt1 congenic region lie within the 95% confidence limits obtained in the original linkage analysis (Ref. 9; Fig. 4). Their physical positions and expression fold changes are shown in Fig. 4D, and their identities given in the figure legend. Of these genes,
the most prominent candidates for control of NKT cell numbers are Slamf1 and Slamf6 because signaling through SLAM-associated protein (SAP) appears to be essential for thymic positive selection of NKT cells (reviewed in Ref. 17; see Discussion).

The expression data from the thymi of NOD.Nkrp1b and NOD.Nkrp1b.Nkt1b mice for Slam family members 1–9 are shown in Fig. 5. Only Slamf1 and Slamf6 were significantly differentially expressed in the congenic line compared with the parental line (p < 0.001; Mann-Whitney U test).

Characterization of SLAM expression in thymus and spleen

Validation of Slamf1 and Slamf6 microarray data was obtained by quantitative RT-PCR of the sequences probed by the array on an independent sample set (Fig. 6, n = 6–9; Fig. 7, n = 5–9). Validation of SLAM expression on thymic and splenic lymphocytes was also performed by flow cytometry (Fig. 8). Consistent with microarray and RT-PCR quantitation of thymic SLAM expression, thymocytes from NOD.Nkrp1b.Nkt1b congenic mice expressed significantly more SLAM on their surfaces than those of the parental strain (Fig. 8A). The cell surface markers CD3, CD4, and CD8 can be used to define the developmental pathway of T cells from the least mature CD4–CD8– (double-negative) CD3–, through a double-positive (DP) intermediate stage, to the most mature CD4+ or CD8+ single-positive (SP) subsets immediately before thymic export (Fig. 8B). Flow cytometric analysis of SLAM expression on thymocytes from each developmental stage revealed major differences in the developmental program of thymic SLAM expression between the NOD.Nkrp1b and NOD.Nkrp1b.Nkt1b strains. While the NOD.Nkrp1b.Nkt1b mice and C57BL/6 mice (data not shown) express high levels of SLAM on DP thymocytes, with a relatively lower level expressed on mature SP cells, expression of SLAM on the developing T cells of NOD.Nkrp1b mouse is retarded, reaching its peak of expression only at the mature SP stage (Fig. 8C, D). At each developmental stage, the levels of expression of SLAM on the thymocytes of (NOD.Nkrp1b × NOD.Nkrp1b.Nkt1b)F1 mice were intermediate between the two parental strains (data not shown). Levels of expression of SLAM on mature thymic NKT cells are similar (Fig. 8E).

Consistent with the levels of SLAM expression on mature SP thymocytes, splenic expression was relatively similar on both T and B cells of both strains and the (NOD.Nkrp1b × NOD.Nkrp1b.Nkt1b)F1 mice (Fig. 8, F–I; data for F1 mice not shown).

Functional consequences of differences in SLAM expression

To determine whether the difference in SLAM expression on DP thymocytes between the NOD.Nkrp1b and NOD.Nkrp1b.Nkt1b strains was sufficient to have functional effects, an assay of SLAM function was established. As SLAM acts as a costimulator through homotypic interactions, and the difference in levels of expression was largely restricted to the DP population of thymocytes, SLAM function was assessed by measuring TCR-stimulated proliferation of whole thymocytes. Whole thymocytes or purified CD4+ splenocytes were stimulated in vitro with anti-CD3/anti-CD28 coated beads and the proliferative response detected by thymidine incorporation five or three days later, respectively. The validity of this system as a surrogate measure of SLAM function was confirmed

FIGURE 6. Validation of expression microarray profiling of Slamf1 expression in thymi from NOD.Nkrp1b and NOD.Nkrp1b.Nkt1b mice. Gene structure and exon structure of the three SLAM isoforms are shown together with the relative locations of microarray probe targets and RT-PCR primer sites (A). The microarray results (B; n = 7) and RT-PCR validation (C; n = 6–9) performed on an independent sample are shown.
by the inhibition of proliferation by the addition of 100 μg/ml of the blocking SLAM peptide 132–146 or inhibiting concentrations (6.25 μg/ml) of the TC15 anti-SLAM mAb (Fig. 9A).

Consistent with varying expression levels of SLAM on DP thymocytes modulating the signal threshold of the responding mature SP thymocytes, greater proliferation was seen in cultures of thymocytes from NOD.Nkrp1b.Nkt1b mice than from those of NOD.Nkrp1b mice (Fig. 9B; n = 6; p < 0.05; Mann-Whitney U test). The addition of exogenous IL-2 eliminated this difference between the strains, which is consistent with SLAM’s role as a costimulator. No significant difference in TCR-stimulated proliferation of splenic CD4+ T cells was observed, which is consistent with the absence of a difference in the levels of SLAM expression on this population.

The supernatants from the cultures were then assayed for cytokines. Thymocytes from NOD.Nkrp1b mice produced significantly less IL-4 and IL-5, and slightly more IFN-γ, in a manner analogous to the cytokine phenotypes of Slamf1−/− and Sap−/− targeted mutant mice (18–20). A similar deviation in IL-4 production was seen in cultures of CD4+ splenocytes (Fig. 9, C–E).

Discussion

The production and characterization of the NOD.Nkrp1b.Nkt1b mouse strain described here formally confirmed the location of a major NKT cell control gene on distal chromosome 1, as the congenic mice had a 2-fold increase in proportions, and a three-fold increase in absolute numbers, of thymic NKT cells at six weeks of age. While conventional NOD lines lack the NK1.1 developmental marker, the NOD.Nkrp1b parental line used in these studies is congenic for Nkrp1b, the allele encoding NK1.1, and was specifically developed by us to allow analysis of the major NKT cell subsets (13, 14). The presence of C57BL/6-derived alleles at the Nkrp1 locus on chromosome 6 does not affect either the numbers of NKT cells, nor the strain’s susceptibility to autoimmune disease (14). Flow cytometric analyses of thymic and splenic NKT cell subsets defined by the cell surface markers CD44, CD4, and NK1.1 (15, 16, 21) indicated that the majority of the additional NKT cells found in NOD.Nkrp1b.Nkt1b mice belonged to the CD4+ NK1.1− population, which is considered to be developmentally relatively immature (15, 16). This finding suggests that the addition of the C57BL/6-derived allele is sufficient to increase the number of cells entering the NKT cell developmental pathway, but insufficient to push those cells through to maturity. The functional characteristics of these cells, as well as the additional effects of Nkt2, the other NKT cell control locus, are issues of great interest.

Two strategies were applied to reduce the number of Nkt1 candidate genes under consideration. The first was the use of gene expression microarrays. As a generalization, this has not been a particularly helpful strategy in the past, and reports of hundreds or thousands of differentially expressed genes in congenic mice have been published (e.g., Ref. 22). In our experience, a dramatic improvement in signal-to-noise ratio could be attained by avoiding engagement of the activation and apoptosis cascades. In this specific case, thymi were removed from mice within 120 s of the induction of anoxia and placed immediately in RNAlater. The second strategy applied was the use of a stringent statistical threshold,
rather than ad hoc fold difference thresholds, which have no obvious biological validity. As a consequence of these procedures, 21 of the 28 locatable highly differentially expressed genes mapped to the Nkt1 congenic region and only fifteen of these genes lay within the Nkt1 95% confidence interval. To our knowledge, no microarray expression analysis of congenic mice has produced a better signal to noise ratio. Of the 15 highly differentially expressed genes lying within the Nkt1 95% confidence interval, the most prominent candidates for control of NKT cell numbers are Slamf1 and Slamf6, as signaling through SAP appears to be essential for thymic positive selection of NKT cells (reviewed in Ref. 17).

Slamf1 encodes the Ig-like receptor termed SLAM or CD150, which forms homotypic interactions and modulates immune responses (17, 23). It associates with, and signals through, the Src homology (SH)2-domain containing adaptor protein SAP, which is mutated in the human inherited immunodeficiency X-linked lymphoproliferative disease (24–26), and FynT, a Src-related protein tyrosine kinase, which is recruited to SAP through a unique interaction involving the SH2 domain of SAP and the SH3 domain of FynT (27). Ligation of SLAM with mAbs enhanced TCR-stimulated proliferation and cytokine production by human and mouse T cells (28–30), which is consistent with a role as a costimulator (31). T cells from Slamf1−/− targeted mutant mice showed a similar defect in T cell-mediated IL-4 production and slightly increased IFN-γ production, compared with SAP-sufficient wild-type CD4+ T cells (19, 20, 32, 33). Significantly, SAP-deficient X-linked lymphoproliferative patients as well as mice bearing targeted deletions of SAP (34–36) or FynT (37, 38) lack NKT cells, indicating a critical role for the SAP/FynT signaling pathway, presumably following recruitment to one or more members of the SLAM family of cell surface receptors. As NKT cells are positively selected on DP thymocytes (39, 40), selection is dependent on the SAP/FynT signaling pathway (34–38), and SLAM is known to be expressed on the surfaces of DP thymocytes (41). SLAM-SLAM interactions may be responsible (42). Slamf1 lies within a haplotype block containing genes encoding nine SLAM family members, many of which contain multiple polymorphism between the minority haplotype, expressed in C57BL/6, C57L, C57BR, C57BL/10, and RF (haplotype 1) and the majority haplotype, which is expressed in 129/SvJ, A/J, AKR/J, BALB/cJ, C3H/HeJ, CBA/J, DBA/2J, MRL/Mpj, NOD/Lt, NZB/B1WJ, NZW, SJL/J, and others (haplotype 2; Ref. 43). The lupus susceptibility gene Sle1b has been localized to this region by congenic mapping and is expressed in haplotype 2 (43).

RT-PCR and flow cytometry confirmed a major difference in SLAM expression on the thymocytes of NOD.Nkrp1b.Nkt1b and NOD.Nkrp1b mice. Comparison of SLAM levels on thymic subsets revealed variation in the developmentally regulated pattern of expression between the strains. While the NOD.Nkrp1b.Nkt1b mice
express increasing levels of SLAM through T cell development to peak on DP thymocytes and then decline to relatively lower levels on mature SP cells, expression of SLAM on developing T cells of NOD.Nkrp1b mice is retarded, reaching its peak of expression only at the mature SP stage. Consistent with the levels of SLAM expression on mature SP thymocytes, splenic expression was relatively similar between the strains on both T and B cells. This difference in SLAM expression was of functional importance, as it affected both TCR-stimulated proliferation as well as cytokine production. Significantly, thymocytes and CD4+ splenocytes from NOD. Nkrp1b mice produced less IL-4, and slightly more IFN-γ, in a manner analogous to the cytokine phenotypes of Slamf1−/− and Sap−/− targeted mutant mice (18–20).

The retardation of developmentally programmed SLAM expression in NOD mice has three significant implications. First, as DP thymocytes account for >80% of the thymus, it explains the finding of differential gene expression between the strains in whole thymic RNA preparations. Second, because NKT cells are positively selected on DP thymocytes via a mechanism dependent on the SAP/FynT signaling pathway, decreased SLAM expression at this developmental stage may provide an explanation for the reduced numbers of NKT cells in NOD mice. Third, as SLAM also acts as a costimulator for conventional T cells, it is possible that the relatively lower levels of SLAM expression at the stage of negative selection (late DP stage) compared with those at maturity (SP thymocytes and in the periphery) result in a lowering of the signaling threshold of conventional T cells in the periphery. If true, this may result in an increased proportion of peripheral T cells capable of responding to self-Ags.

In conclusion, the data presented make a strong case for the hypothesis that the control of NKT cell numbers attributed to the Nkt1 gene is mediated by differential expression of Slamf1 and are
consistently with an additional contribution by Slamf6. In addition, it is possible that the retarded programmed expression of SLAM on developing conventional T cells of NOD mice may contribute to lowering their TCR signaling threshold in the periphery thereby contributing to autoimmune disease in this strain.

Acknowledgments

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References