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Genetic Control of NKT Cell Numbers Maps to Major Diabetes and Lupus Loci


Natural killer T 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. We have previously reported deficiencies in the numbers and function of NKT cells in the nonobese diabetic (NOD) mouse strain, a well-validated model of type 1 diabetes and systemic lupus erythematosus. In this study, we report the results of a genetic linkage analysis of the genes controlling NKT cell numbers in a first backcross (BC1) from C57BL/6 to NOD.Nkrp1b mice. The numbers of thymic NKT cells of 320 BC1 mice were determined by fluorescence-activated cell analysis using anti-TCR Ab and CD1d/galactosylceramide tetramer. Tail DNA of 138 female BC1 mice was analyzed for PCR product length polymorphisms at 181 simple sequence repeats, providing greater than 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 (Nkt2) was on distal chromosome 1, in the same region as the NOD mouse lupus susceptibility gene Bab2/Bana3. The second most significant locus (Nkt2) mapped to the same region as Idd13, a NOD-derived diabetes susceptibility gene on chromosome 2. The Journal of Immunology, 2003, 171: 2873–2878.

Materials and Methods

Mice

NOD.Nkrplb (17) and C57BL/6J mice were obtained from the Animal Resource Centre (Canning Vale, Australia). The NOD.Nkrp1b strain carries B6 alleles at the NKC on chromosome 6 (from D6mit105 to D6mit 115), permitting the use of the NK1.1 marker, if needed. 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 four-monthly intervals for the following pathogens: mouse hepatitis virus, rotavirus, ectomelia, mouse CMV, polyoma virus, murine adenovirus, lymphocytic choriomeningitis virus, mouse pneumonia virus, reovirus, Sendai virus, Theler’s murine encephalitis virus, Bacillus piliformis, 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.

Phenotyping

Cell suspensions of thymus from 6-wk-old mice were prepared by gently grinding the organs between the frosted ends of glass microscope slides in PBS, and stained in PBS containing 5% FCS and 0.02% azide. Cells were analyzed by multiparameter flow cytometric analysis for forward scatter, side scatter, and binding to anti-αβTCR-FITC (clone H57-597; BD PharMingen, San Diego, CA) and PE-labeled, α-GaCer loaded, or unloaded (control), mCD1d tetramers (18). Analysis was performed on a FACSCalibur or FACStar Plus (BD Biosciences, San Jose, CA).

Genotyping

DNA of BC1 progeny was extracted from tails and subjected to an autosomal genome-wide scan using simple sequence repeats (SSR) chosen from the Whitehead Institute simple sequence length polymorphism library.
ow cytometry of thymocytes stained with anti-αβ TCR-FITC and PE-labeled, α-GalCer-loaded mCD1d tetramers. Identification of NKT cell numbers among the female BC1 population.

Table II.

Proportions of thymic NKT cells was conducted using a version of Mapmaker/EXP (22) and were reamplified. Recombination distances between markers were calculated from recombination frequencies using the Mapmaker/EXP program (22). Lengths of chromosomes and order of markers were checked against published maps (19) (http://www-genome.wi.mit.edu/, http://www.informatics.jax.org/; http://www.celeradiscoverysystem.com). Interval analysis of linkage to the chromosomes and order of markers were checked against published maps (19) (http://www-genome.wi.mit.edu/, http://www.informatics.jax.org/; http://www.celeradiscoverysystem.com).

Linkage analysis

Genotyping errors were identified manually as double recombinants or by the error-checking function of Mapmaker/EXP (22) and were reamplified. Recombination distances between markers were calculated from recombination frequencies using the Mapmaker/EXP program (22). Lengths of chromosomes and order of markers were checked against published maps (19) (http://www-genome.wi.mit.edu/, http://www.informatics.jax.org/; http://www.celeradiscoverysystem.com). Interval analysis of linkage to the proportions of thymic NKT cells was conducted using a version of Mapmaker/QTLD (quantitative trait locus) 2.0b that was ported to run on the Pentium 4 under Windows 2000 by M. Butler. The output of Mapmaker provides a log-likelihood ratio for any putative QTL located at an arbitrary point between markers genotyped. Significance thresholds used were those suggested by Lander and Kruglyak (23) for analyses of mouse backcrosses; viz logarithm of odds (LOD) = 3.3 for the threshold for significant linkage and LOD ≥ 1.9 for the threshold suggestive of linkage. Quantitative differences between samples were compared using the Mann-Whitney U (rank sum) test.

Results

Production and phenotypic analysis of BC1 mice

Male and female ((C57BL/6 × NOD.Nkrp1) × NOD.Nkrp1)BC1 mice were killed between 6 and 7 wk, and their thymi were harvested for flow cytometric analysis of NKT cell numbers. Single cell suspensions were stained with CD1a/αGalCer tetramer-PE and αβ TCR-FITC and double-staining cells enumerated (Fig. 1a). As a small, but significant difference was observed between male and female BC1 mice (Table I; p < 0.0001; Mann-Whitney U test), only female mice were used in the subsequent analyses.

The distribution of numbers of thymic NKT cells among the population of female BC1 mice was bimodal, with 17% (n = 23) of mice falling into the group with high numbers of NKT cells (0.89 ± 0.02%; mean ± SE) and the remainder (n = 116) falling into the main peak with lower numbers (0.27 ± 0.01%; Fig. 1b). This result suggested that this phenotype was not a complex genetic trait, which would have approximated a normal distribution, but displayed relatively simple inheritance and was consistent with two dominant genes contributing to the expression of high numbers of NKT cells in this cross.

Table I.

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<th>Strain</th>
<th>n</th>
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<tr>
<td>C57BL/6 × NOD.Nkrp1 (female)</td>
<td>8</td>
<td>0.59 ± 0.05</td>
</tr>
<tr>
<td>BC1 males</td>
<td>113</td>
<td>0.65 ± 0.03*</td>
</tr>
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* Value of p < 0.0001, Mann-Whitney U test.

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Genotypic linkage analysis of thymic NKT cell number

A scan of the autosomal genome for QTL controlling numbers of thymic NKT cells was performed using the entire female dataset at an average marker separation of 8 cM. Interval analysis was performed using a version of the Mapmaker/QTL program (22) that was ported to the Windows 2000 operating system by M. Butler. The stringent linkage thresholds for experimental mouse backcrosses set by Lander and Kruglyak (23) for significant linkage (LOD ≥3.3) and suggestive linkage (LOD ≥1.9) were applied.

Two peaks of significant linkage were identified (Fig. 2). Strongest linkage localized to distal chromosome 1, with a log-likelihood ratio of 6.82 at D1mit15. The region indicated by this peak of linkage (in this work named Nkt1) was fine mapped using markers first reported in this study; Table III) developed in, or immediately flanking, immunologically relevant genes identified as being adjacent to the D1mit15 primer sequence in the Celera (http://www.celeradiscoverysystem.com) or public (http://www-genome.wi.mit.edu) mouse genome sequence databases. The 7-cM region containing D1mit15 also contains the genes for E-selectin (Sele), lymphotactin (Scy1), Cd3 ζ (Cd3ζ), the FcγRb2 (Fcgr2b), the FcγR3 (Fcgr3), the NK cell receptor 2B4 (Nmrk), and CD48

<table>
<thead>
<tr>
<th>Marker (N/B)*</th>
<th>Gene</th>
<th>Primer Sequences</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>D10mit87</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D4mit15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D13mit54</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D7mit355</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* N = NOD allele size; B = C57BL/6 allele size.
(Cd48), none of which demonstrated higher linkage than D1mit15 (Fig. 3).

The second locus (in this work named Nkt2) was indicated by a region of significant linkage between D2mit490 and D2mit280 on chromosome 2, with a maximum LOD score of 4.904. This 3-cM segment contains the gene encoding a heat shock protein (unnamed) and that encoding the receptor for the complement component C1Q (http://www.celeradiscoverysystem.com). The gene for /H9252 2 -microglobulin (/H9252 2 m), located by the marker D2bax205, which lies 1.8 kb downstream of the coding region, lies 16.5 cM proximal to the peak of linkage (Fig. 3). Although just outside the 95% confidence interval, it is possible that this gene contributed to the linkage peak, which is somewhat wider than that for Nkt1.

Three other genomic regions surpassed the threshold for suggestive linkage (LOD ≥1.9; Fig. 2). The first was on proximal chromosome 2, reaching a peak LOD of 1.92 between D2mit294 and D2mit458; the second was on chromosome 7, reaching a peak LOD of 1.90 at D7mit101; and the third was in the MHC at D17mit176, with a LOD of 2.00.

As a NOD.Nkrp1 congenic line was used in these studies, and this line carries C57BL/6 loci in the NKC, these data cannot exclude the possibility of a gene controlling NKT cell numbers in this region. However, because the congenic line itself expresses the same numerical deficiency of NKT cells seen in the parental NOD/Lt line (17), such a possibility is extremely unlikely.

Characterization of alleles and their interactions

To further characterize the effects of allelic variation at Nkt1 and Nkt2 on numbers of CD1/αGalCer tetramer+ αβTCR+ NKT cells, BC1 mice were sorted by the allele expressed at each of these loci and the numbers of thymic NKT cells were compared (Fig. 4). This analysis confirmed that the C57BL/6 allele at each locus was dominant in increasing the numbers of NKT cells present, and that the effects of the two loci were approximately equal.

Fitting both Nkt1 and Nkt2 with the Mapmaker/QTL map command produced a combined LOD score of 11.84. As the LOD scores of Nkt1 and Nkt2 were 6.87 and 4.87, respectively (total LOD of 11.74), this result was consistent with the loci acting in an additive fashion. These two loci together accounted for 33% of all variance in NKT cell numbers in the BC panel.

Discussion

NOD mice are a well-established model of type 1 (autoimmune) diabetes mellitus characterized by spontaneous lymphocytic infiltration of the pancreatic islets of Langerhans and specific destruction of the insulin-producing β cells, resulting in hypoinsulinaemia and disturbed glucose homeostasis (24). Extensive genetic linkage analysis of diabetes in this model has been performed, and over 20 loci affecting this phenotype have been localized (25–41). Although many of these genetic regions are associated with credible candidate genes, one of the few to have been validated is Idd13 on chromosome 2 (29), in which transgenic rescue provided substantial support for a role for β2m in contributing to the diabetes susceptibility encoded by loci within the Idd13 linkage region (42).

When exposed to mycobacteria (Mycobacterium bovis), NOD mice are protected from the onset of diabetes, but may instead rapidly develop a systemic autoimmune syndrome with several
features of systemic lupus erythematosus (SLE) (43–45), including hemolytic anaemia, antinuclear autoantibodies (46), and immune complex glomerulonephritis (47) (reviewed in Ref. 48). This syndrome has been mapped in a backcross to the BALB/c strain, and loci conferring susceptibility to autoantibody production were localized to three regions, Bana1 at the MHC on chromosome 17, Bana2 on proximal chromosome 10, and Bana3 on distal chromosome 1 (20). The latter locus maps to the same chromosomal region as genes controlling susceptibility to antinuclear autoantibodies 1 (20). The latter locus maps to the same chromosomal region to systemic autoimmunity is suggested by its involvement in two different models of autoimmune arthritis (54, 55). An even broader significance of this region to systemic autoimmunity is suggested by its involvement in two different models of autoimmune arthritis (54, 55).

NKT cells are a subset of T cells that, together with NK and dendritic cells, appear to form part of an immunological rapid response unit involved in determining, in part, the extent and character of a wide range of immune responses (56–63). We have previously reported that NOD mice have fewer thymic NKT cells than other inbred mouse strains examined (3, 4), and demonstrated by adoptive transfer that this deficiency was associated with susceptibility to disease (3, 9). Similarly, there is some evidence that such a relationship may exist between NKT cell deficiency and SLE (7). In this study, we found that genetic control of thymic NKT cell numbers mapped to the distal part of the Idd13 region on chromosome 2 and to the Bana3/Sle1/Nha2/Lbw7 region of chromosome 1. These linkages are therefore consistent with an important role of NKT cells in the regulation of autoimmune responses in diabetes and lupus, and suggest that both Idd13 and Bana3 act through control of NKT cell numbers. It may be significant that the genes encoding two critical components of the NKT cell-activating synapse, β2m and CD3ζ, lie within these regions.

Acknowledgments

We thank Michael C. Butler for debugging and recompiling Mapmaker/ QTL to run on the Pentium 4, and Rama Kandasamy, Tim Butler, Jason Coombes, Jason Wills, Jan-Marek Weislogel, and Arnout van der Plas for technical assistance. We are grateful for the encouragement and unfailing support of Mitch Kronenberg throughout these studies.

References


FIGURE 4. Box plots indicating the phenotypic effects of inheritance of a single C57BL/6 (b) allele at either Nkt1 or Nkt2, or as else at both loci.


