

Genetic and Functional Analysis of the *Nkt1* Locus Using Congenic NOD Mice

Improved V α 14-NKT Cell Performance but Failure to Protect Against Type 1 Diabetes

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Defective invariant natural killer T-cells (iNKT cells) have been implicated in the etiology of type 1 diabetes in nonobese diabetic (NOD) mice. In a genome scan of a cross between NOD and C57BL/6 mice, the most significant locus controlling the number of iNKT cells, referred to as *Nkt1*, was recently mapped to distal chromosome 1. Here, using congenic mice for this chromosomal segment, we definitively demonstrate the existence of *Nkt1* and show that introgression of the C57BL/6 allele onto the NOD background improves both the number of iNKT cells and their rapid production of cytokines elicited by α -galactosylceramide treatment, explaining at least half of the difference between the NOD and C57BL/6 strains. Using new subcongenic lines, we circumscribed the *Nkt1* locus to a 8.7-cM segment, between the NR1i3 and D1Mit458 markers, that notably includes the SLAM (signaling lymphocytic activation molecule) gene cluster, recently involved in murine lupus susceptibility. However, despite a significant correction of the iNKT cell defect, the *Nkt1* locus did not alter the course of spontaneous diabetes in congenic mice. Our findings indicate a complex relationship between iNKT cells and autoimmune susceptibility. Congenic lines nonetheless provide powerful models to dissect the biology of iNKT cells. *Diabetes* 55:1163–1170, 2006

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APC, allophycocyanin; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; α -GalCer, α -galactosylceramide; IFN- γ , γ -interferon; IL, interleukin; iNKT cell, invariant natural killer T-cell; mAb, monoclonal antibody; SLAM, signaling lymphocytic activation molecule; TCR, T-cell antigen receptor.

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The development of type 1 diabetes results from T-cell-mediated destruction of insulin-producing β -cells in the pancreas (1). Expression of effector lymphocytes seems to be caused in large part by a defect of immunoregulatory cells (2), among which invariant natural killer T-cells (iNKT cells) are currently the focus of considerable attention. This unique population of $\alpha\beta$ -T-cells shows an invariant T-cell antigen receptor (TCR) α -chain, including V α 14J α 18 in the mouse and V α 24J α Q in humans, and also expresses natural killer cell receptors (3). Most remarkably, these cells are capable of rapidly producing large amounts of cytokines on stimulation by glycolipid-type ligands that must be presented by the major histocompatibility complex class I-like CD1d molecule (4). Although α -galactosylceramide (α -GalCer), a glycolipid isolated from marine sponge, has long been used as a surrogate antigen for iNKT cells, their endogenous ligand has been only recently characterized (5).

Remarkably, mice of the nonobese diabetic (NOD) strain, the well-established murine model of spontaneous type 1 diabetes (6), exhibit both a numerical and a functional defect of their iNKT cells as early as 3 weeks of age compared with nondiabetic strains (7–9). The protective role of iNKT cells is strongly suggested by adoptive transfer experiments (10) and by the study of NOD mice transgenic for a V α 14-J α 18 TCR (11) or for CD1d overexpressed in pancreatic islets (12). Conversely, diabetes is exacerbated in CD1d knockout NOD mice, which are devoid of iNKT cells (13,14). Finally, diabetes can be prevented by in vivo treatment with α -GalCer (15,16), opening a path for the therapeutic manipulation of this cell subset.

Interstrain differences suggest that the number and function of iNKT cells might be under genetic control (8). Recently, a genome-wide screen of a cross between NOD and C57BL/6 mice localized two main loci controlling iNKT cell number: *Nkt1* on distal chromosome 1 and *Nkt2* on chromosome 2 (17). Importantly, the *Nkt2* locus overlapped with the *Idd13* locus for insulin-dependent diabetes (*Idd*) susceptibility, suggesting that the iNKT cell defect of NOD mice might be a genetically determined component of their diabetes susceptibility. Consistent with this finding, mice congenic for certain *Idd* loci, including *Idd6*, *Idd9*, and *Idd13*, showed a significant

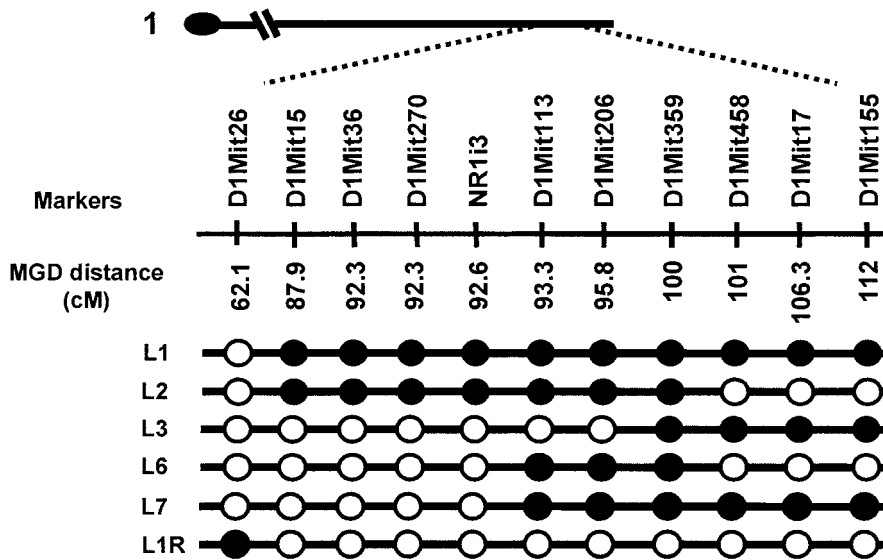


FIG. 1. Schematic representation of the genetic intervals on distal chromosome 1 defining the various congenic and subcongenic strains used in the current study. Only the most representative markers are shown. The following microsatellites were also used to strengthen the mapping data: D1Mit353, D1Mit146, D1Mit540, D1Mit149, D1Mit456, D1Mit354, D1Mit356, D1Mit355, D1Mit115, D1Mit457, D1Mit150, D1Mit403, D1Mit166, D1Mit407, D1Mit361, and D1Mit273. The genetic distances from the centromere (in cM) were obtained at the Mouse Genome Database (MGD; release 3.01, available at <http://www.informatics.jax.org>). ●, NOD allele; ○, C57BL/6 allele.

modulation of their iNKT cell number and function (18,19). In contrast, however, diabetes-free mice of the NOD-related nonobese diabetes-resistant (NOR) strain harbor the same iNKT cell defect as NOD mice, whereas lupus-prone mice demonstrate an increased number and activity of iNKT cells (18). Taken together, these observations indicate that there is no simple relationship between the genetic control of iNKT cell variation and that of autoimmune susceptibility.

Although the *Nkt1* locus does not map to a known *Idd* locus, we previously characterized a major quantitative trait locus in the same chromosomal region that controls the increased IgG₁ and IgG_{2b} serum levels of NOD mice compared with C57BL/6 mice (20). Moreover, this region was also associated with the propensity of NOD mice to express anti-nuclear autoantibodies (21). We had also derived reciprocally congenic strains for this chromosomal region. Such strains provide an essential tool both to confirm the existence of a locus controlling a complex trait as well as to undertake its detailed analysis. Here, we have used these congenic strains and new subcongenic lines to further investigate the *Nkt1* locus and its potential influence on type 1 diabetes.

RESEARCH DESIGN AND METHODS

NOD, C57BL/6J, and congenic mice were bred in our animal facility under specific pathogen-free conditions. Congenic strains for distal chromosome 1, including L1 (formerly designated NOD.C57BL/6-fcgr2) and L1R (formerly C57BL/6.NOD-fcgr2), were previously described (20) and have been backcrossed for 20 generations. Subcongenic strains, L2, L3, L6, and L7 (Fig. 1) were derived from L1 mice backcrossed with the NOD parent and by genotypic selection of mice carrying a chromosomal interval of interest. Homozygous mice were then obtained by brother-sister matings. Genomic DNA was genotyped for chromosome 1 microsatellite markers referenced in the Mouse Genome Database (available from www.informatics.jax.org).

Reagents. A synthetic form of α -GalCer (KRN-7000; Pharmaceutical Research Laboratory, Kirin Brewery, Tokyo) was used. Empty and α -GalCer-loaded CD1d tetramers labeled with allophycocyanin (APC) were prepared in our laboratory. Monoclonal antibodies (mAbs) were purchased from BD Pharmingen (San Diego, CA) and included fluorescein isothiocyanate (FITC)-labeled anti- $\alpha\beta$ -TCR (clone H57-597), phycoerythrin-labeled anti-CD4 (clone RM4-5), phycoerythrin-labeled anti-interleukin (IL)-4 (clone 11B11), phycoerythrin-labeled anti- γ -interferon (IFN- γ) (clone XMG1.2), and corresponding isotype control antibodies. The Fc-receptor-blocking mAb (anti-CD16/CD32, clone 24G2, culture supernatant) was added to staining mixtures to prevent nonspecific binding by labeled mAbs.

In vivo challenge with α -GalCer and cytokine production. Mice were injected with α -GalCer (1 μ g/dose, i.p. and i.v.) diluted in saline and were bled

90 min later. In some experiments, splenocytes (2.5×10^6 per ml) were prepared from treated animals and cultured without further stimulation for 90 min in RPMI 1640 Glutamax culture medium supplemented with 10% heat-inactivated FCS, 10 mmol/l HEPES, pH 7.4, 1 mmol/l sodium pyruvate, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.05 mmol/l β -mercaptoethanol. IFN- γ and IL-4 contents were measured in supernatants and in sera by sandwich enzyme-linked immunosorbent assay (ELISA).

Priming with α -GalCer-pulsed dendritic cells. Dendritic cells were grown from bone marrow cells cultured in RPMI 1640 supplemented with 200 units/ml granulocyte macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN) as previously described (22). After 10 days, dendritic cells (5×10^4 cells per well) were pulsed or not pulsed with α -GalCer (100 ng/ml) for 40 h and then injected intravenously into 6- to 7-week-old mice (5×10^4 dendritic cells per mouse). Sera were collected 24 h later and assayed for cytokine content by sandwich ELISA.

Cell preparation and flow cytometry analysis. Single-cell suspensions of splenocytes were prepared by standard techniques. Livers were perfused with PBS and pressed through a 70- μ m cell strainer, and cells were washed three times. Hepatic lymphocytes were then separated, using a 35% Percoll solution (Amersham Pharmacia Biotech, Uppsala, Sweden), and red cells were lysed in hemolysis buffer. Spleen and liver cells were then incubated with appropriate dilutions of different fluorochrome-coupled mAbs, fixed in 4% paraformaldehyde, and analyzed using a FACScalibur cytometer and CellQuest v3.3 software (BD Biosciences, Mountain View, CA). Dead cells were excluded, using forward and side scatter parameters. A minimum of 1.5×10^5 events gated from live lymphoid cells were acquired in each run. Gates on populations of interest contained at least 1×10^3 events. The absolute number of iNKT cells was calculated from the percentage of the CD1d tetramer-positive cells and the total cell number harvested from each organ.

For detection of intracellular IL-4 and IFN- γ in iNKT cells, cells were stained with APC-labeled CD1d/ α -GalCer tetramer and FITC-labeled anti- $\alpha\beta$ -TCR mAbs. Fixed cells were then permeabilized with PBS containing 1% BSA and 0.5% saponin and further stained with phycoerythrin-labeled anti-IL-4, phycoerythrin-labeled anti-IFN- γ , or control phycoerythrin-labeled isotype for 30 min at room temperature. The percentages of cytoplasmic IL-4- and IFN- γ -expressing cells were determined by cytometry.

Assessment of diabetes. The 10-week-old female mice were followed for glycosuria (Glukotests; Roche Diagnostics, Basel, Switzerland) once a week. Mice were recorded as diabetic when the glucose concentration was >500 mg/dl on three consecutive readings.

Data analysis. Data were expressed as the means \pm SE, and differences between means were evaluated by Student's *t* test or the nonparametric Wilcoxon-Mann-Whitney test. A log-rank test was used to compare incidences of type 1 diabetes. *P* values ≤ 0.05 were reported.

RESULTS

In vivo α -GalCer induced production of IFN- γ and IL-4 in congenic mice. One of the original features of iNKT cells is their capacity to promptly release large amounts of both of the prototypical T helper-1 and -2

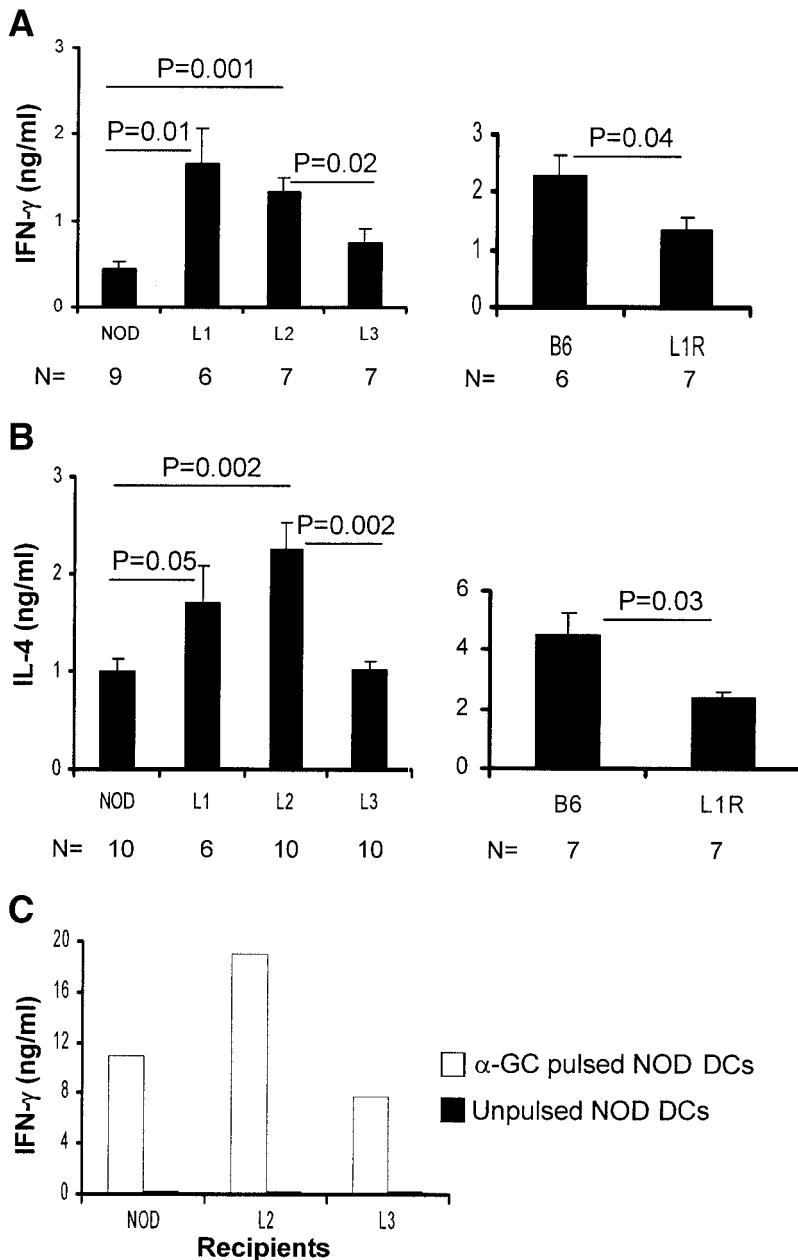


FIG. 2. Influence of distal chromosome 1 on cytokine production by in vivo stimulated iNKT cells. Serum levels (means \pm SE) of IFN- γ (A) and IL-4 (B) in NOD and NOD congenic mice (left panels) or in C57BL/6 and C57BL/6 congenic mice (right panels) were measured by ELISA 90 min after an injection of α -GalCer. The number of mice analyzed in each group is given below the horizontal axis. C: Increase in IFN- γ production stimulated by α -GalCer-pulsed dendritic cells (DCs) is influenced by the recipient genotype. Mice were injected intravenously with 5×10^4 dendritic cells prepared from NOD mice and pulsed or not pulsed with α -GalCer. Serum concentration of IFN- γ was determined by ELISA 24 h later. The data are the means of two independent experiments.

cytokines, IFN- γ and IL-4, after in vivo treatment with their cognate ligand, α -GalCer. To test an effect of the distal chromosome 1 region on iNKT cells, we injected congenic and parent mice once with the ligand and assessed their serum levels of cytokines 2 h later. As shown in Fig. 2A and B (left panels), IFN- γ and IL-4 were detected in sera within 2 h of treatment with α -GalCer at levels significantly higher in congenic L1 than in parent NOD mice (1.67 ± 0.39 vs. 0.45 ± 0.08 ng/ml for IFN- γ , $P = 0.01$, and 1.71 ± 0.38 vs. 0.99 ± 0.13 ng/ml for IL-4, $P = 0.05$). Conversely, the reciprocal L1R congenic mice showed decreased levels compared with their C57BL/6 parents (Fig. 2A and B, right panels). Similarly, freshly explanted spleen cells from α -GalCer-primed L1 mice released higher levels of the cytokines compared with NOD splenocytes (0.11 ± 0.03 vs. 0.04 ± 0.01 ng/ml for IFN- γ , $P = 0.008$, and 0.08 ± 0.02 vs. 0.02 ± 0.01 ng/ml for IL-4, $P = 0.012$) (not displayed on Fig. 2). Altogether, this first series of experiments based on congenic stocks definitively demonstrated the existence of

a locus on chromosome 1 controlling iNKT cell activity, likely closely related to that previously identified as *Nkt1* by linkage analysis (17).

We then assessed the in vivo α -GalCer response in L2 and L3 subcongenic mice to narrow down the interval of interest. Both lines were derived from L1 and harbor complementary intervals (Fig. 1). As shown in Fig. 2A and B (left panels), serum levels of cytokines were significantly higher in L2 than in NOD mice ($P = 0.001$ for IFN- γ and $P = 0.002$ for IL-4) and comparable to those of their L1 parents, whereas L3 mice responded similarly to NOD mice. Based on this observation, the *Nkt1* locus was therefore mapped to a 15-cM interval toward the distal end of the chromosome, excluding its most telomeric segment.

To investigate whether the differential response of congenic mice was caused by an alteration at the level of the presenting cells or of the responding cells, the source of dendritic cells was made constant. Thus, dendritic cells from NOD mice were prepared, pulsed or not with α -Gal-

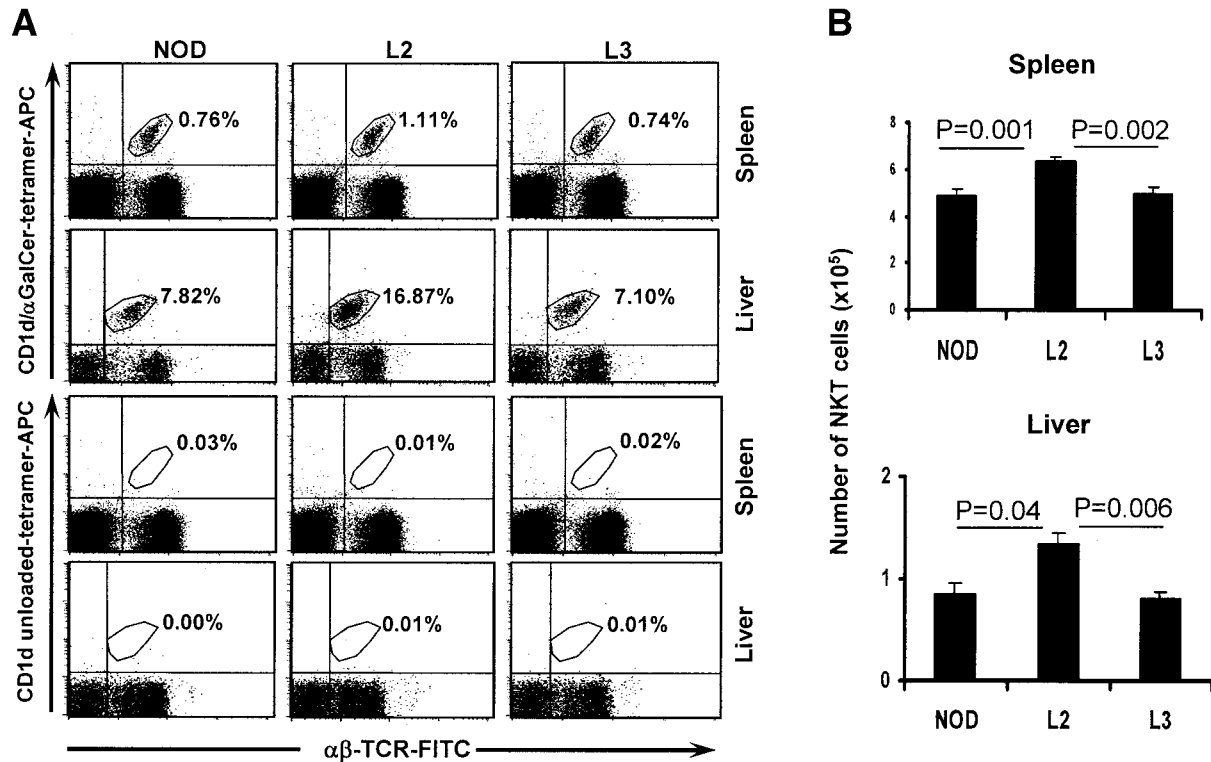


FIG. 3. Enumeration of iNKT cells in NOD, L2, and L3 mice by flow cytometry. Mononuclear cells were prepared from spleen and liver and labeled with the APC-conjugated CD1d tetramer loaded or not loaded with α -GalCer and with FITC-labeled anti- $\alpha\beta$ -TCR antibody. Double-positive cells were compared between strains. **A:** Values indicate the mean percentage of iNKT cells visualized among electronically gated live lymphocytes. Dot plots are representative of at least three experiments per strain. The specificity of α -GalCer-loaded CD1d tetramer binding was controlled with an unloaded CD1d tetramer. **B:** Absolute numbers of iNKT cells in spleen and liver of NOD, L2, and L3 congenic mice. Results represent the means \pm SE from three to eight mice.

Cer, and injected into L2, L3, or NOD mice. As illustrated in Fig. 2C, for IFN- γ production, the response of L2 mice to dendritic cells pulsed with the glycolipid was higher than that of L3 and NOD mice. This observation suggested that the *Nkt1* locus might exert its control at the level of iNKT cells directly.

Numerical and functional profiles of iNKT cells in L2 and L3 subcongenic mice. Spleen and liver iNKT cells were enumerated in NOD and in subcongenic mice by flow cytometry, using a CD1d tetramer probe. As shown in the dot plot (Fig. 3A), iNKT cells could be clearly resolved among total lymphocytes by the binding of the α -GalCer-loaded CD1d tetramer and the expression of an intermediate level of $\alpha\beta$ -TCR. Relative frequencies of iNKT cells are known to vary widely with tissues. In both the spleen and in the liver, L2 mice showed a significant increase in the proportions and in the absolute numbers of iNKT cells compared with NOD or L3 mice (Fig. 3B). By comparison with the parent strains, the numbers of iNKT cells in L2 mice ($1.33 \times 10^5 \pm 0.12 \times 10^5$ in the liver and $6.3 \times 10^5 \pm 0.2 \times 10^5$ in the spleen) were intermediate between those of NOD ($9.4 \times 10^4 \pm 2.2 \times 10^4$ and $4.9 \times 10^5 \pm 2.4 \times 10^4$) and C57BL/6 parent mice ($2.5 \times 10^5 \pm 2.2 \times 10^4$ and $7.3 \times 10^5 \pm 7 \times 10^4$).

The CD4 marker was reported to delineate two functionally distinct subsets of iNKT cells (23,24). A detailed analysis in the liver indicated that both the proportions and the absolute numbers of CD4⁺, but not CD4⁻, iNKT cells were significantly influenced by the L2 interval (Fig. 4).

Next, we assessed cytokine expression at the single-cell level in iNKT cells. Mice were treated in vivo by α -GalCer,

and the magnitude of IL-4 and IFN- γ expression in iNKT cells was determined 2 h after stimulation by intracellular fluorescence staining. A representative experiment is depicted in Fig. 5. Liver iNKT cells from L2 mice had an improved expression of IL-4 and IFN- γ compared with NOD mice. In contrast, L3 mice behaved as NOD mice (data not shown). These findings indicate that the L2 segment alters both the number and the cytokine production of iNKT cells.

Numerical profiles of iNKT cells in L6 and L7 subcongenic mice. To refine the localization of the *Nkt1* locus, we derived two additional subcongenic strains: L6 from L2, and L7 from L1 (Fig. 1). Both of these new strains lack the proximal part of the interval, most notably that including the *Fcgr2* locus, whereas they share 8.7 cM of the distal end of the L2 segment. As shown in Fig. 6, the absolute numbers of liver iNKT cells in L6 and L7 mice were higher than those of NOD mice and similar to those of L2 mice. This observation situates the *Nkt1* locus in the L6 segment, between the NR1i3 and D1Mit458 markers.

The C57BL/6 allele at the *Nkt1* locus does not affect diabetes incidence in NOD mice. Given the well-established immunoregulatory role of iNKT cells, it was important to investigate the effect of the *Nkt1* locus on diabetes susceptibility in NOD mice. As shown in Fig. 7, the course of spontaneous diabetes was very similar in the L2 and the control NOD mice housed at same time and in the same conditions. Up to 60% of L2 mice were diabetic at 30 weeks of age (Fig. 7). Also, the inflammatory infiltrate of the islets of Langerhans was not different in NOD and L2 mice (not shown).

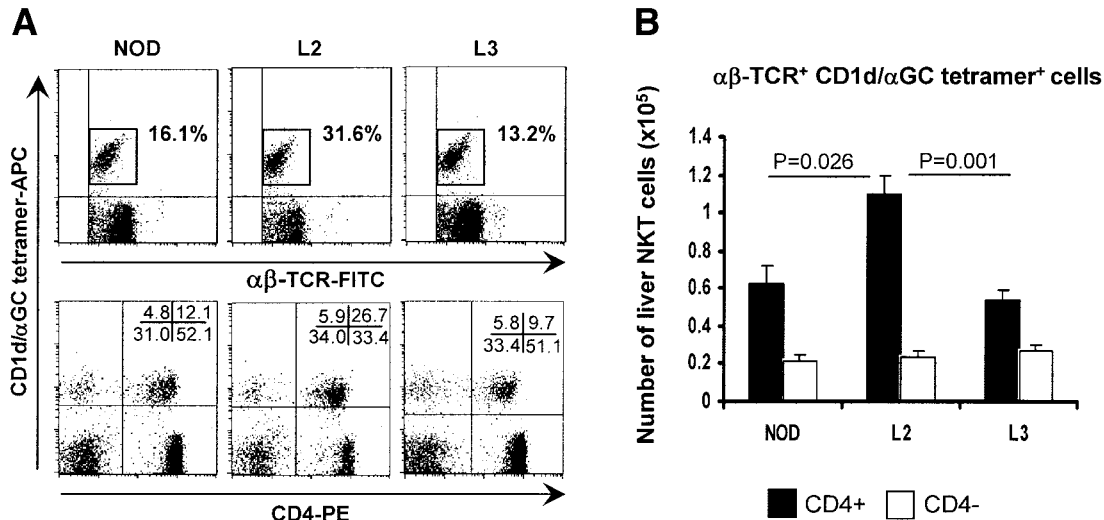


FIG. 4. Distribution of iNKT cells according to CD4 expression. Mononuclear cells were recovered from liver of NOD, L2, and L3 subcongenic mice and stained with APC-conjugated CD1d/α-GalCer tetramer, FITC-labeled anti-αβ-TCR, and phycoerythrin (PE)-labeled anti-CD4 mAbs. **A: Upper panels:** representative dot plots of iNKT cells among αβ-TCR-gated cells. The numbers indicate the proportion of iNKT cells in the windows. **Lower panels:** CD1d/α-GalCer tetramer binding depending on CD4 expression among αβ-TCR-positive cells. The numbers indicate the proportion of cells in each quadrant. **B:** Increased number of CD4-positive iNKT cells in L2 subcongenic mice compared with wild-type NOD and L3 subcongenic mice. Data represent the means ± SE of three to five experiments.

DISCUSSION

The genetic basis of numerical and functional variations of iNKT cells among mouse strains has been the matter of several recent investigations. Three studies exploited available congenic strains and therefore investigated pre-defined genetic segments (18,19,25). These strains had

been developed to determine the genetic basis of quantitative antibody response or of susceptibility to autoimmune diseases, including type 1 diabetes and systemic lupus erythematosus. In contrast, two other studies were aimed at a genomewide screen of loci influencing iNKT cells (17,26). Altogether, these studies indicated that the number and function of iNKT cells behave as complex traits, having a strain-dependent polygenic and heterogeneous genetic control. The most notable loci mapped to chromosomes 1, 2, 4, 6, 11, and 18.

The genome scan conducted by Esteban et al. (17) was particularly relevant to the current study because it involved the NOD and C57BL/6J strains and identified two main loci. The strongest of these loci, *Nkt1*, was located on distal chromosome 1 in a region not reported to influence diabetes susceptibility in the NOD strain but that nonetheless was already known for its role in controlling serum levels of IgG (20) and production of anti-nuclear autoantibodies in NOD mice (21). Using mice congenic for this interval, which had been previously derived in our laboratory, we were able to obtain definitive proof of the

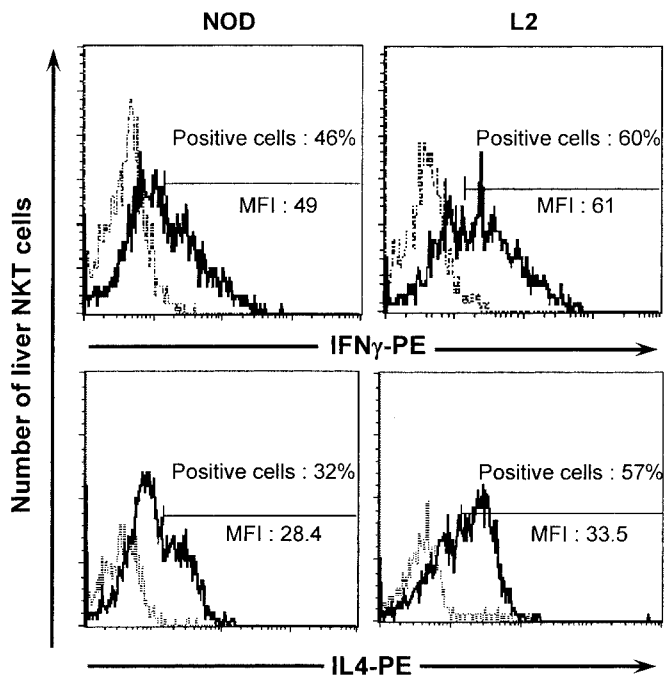


FIG. 5. Increased expression of IFN-γ and IL-4 in liver iNKT cells in L2 mice. Activated liver iNKT cells were harvested from wild-type NOD and L2 subcongenic mice 2 h after administration of α-GalCer and were examined by flow cytometry for production of IFN-γ (**upper panels**) and IL-4 (**lower panels**). Solid lines represent the labeling for cytokines and the dotted lines the background staining with phycoerythrin (PE)-labeled control Ig of the same isotype. The percentage of positive cells and the mean fluorescence intensity (MFI) for IFN-γ and IL-4 staining are given. The histograms are representative of three independent experiments.

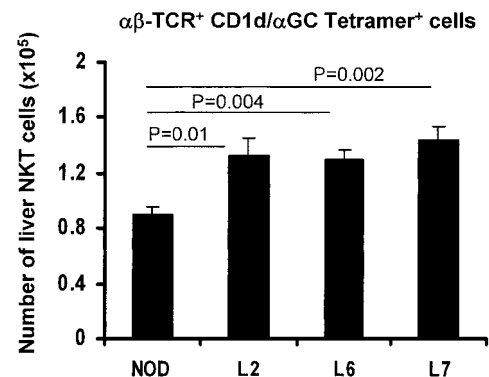


FIG. 6. Increased number of iNKT cells in the liver of L6 and L7 subcongenic mice. Liver mononuclear cells were prepared and labeled as described in the legend of Fig. 3. Data represent absolute numbers of iNKT cells (means ± SE from four to five mice).

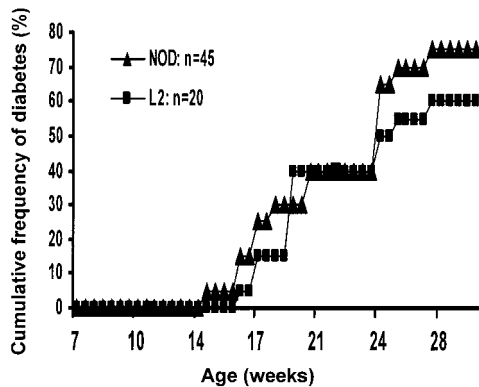


FIG. 7. Cumulative incidence of spontaneous diabetes in L2 congenic and control NOD female mice. Two other experiments using L1 mice yielded similar results (not shown).

existence of *Nkt1*. These congenic mice also provided us with a powerful tool to further investigate the effect of this region on both iNKT cell activity and diabetes susceptibility. We found that the locus affects both the number of iNKT cells and the rapid burst of cytokines induced by α -GalCer, explaining at least half of the difference between the NOD and C57BL/6 strains. It was detected by transferring the C57BL/6 allele onto the NOD background and vice versa, indicative of its strong significance. Interestingly, it concerned preferentially the CD4⁺ subset of iNKT cells. This finding is consistent with this subset being the major producer of cytokines on activation with α -GalCer (27,28 and A.H., unpublished results). Whether the locus is expressed intrinsically in iNKT cells remains to be investigated. However, our experiment showing that the genotype of iNKT cells responding to fixed-genotype stimulatory dendritic cells influences their response strongly is consistent with this possibility.

Using subcongenic strains, we narrowed down the *Nkt1* interval to a segment of 8.7 cM between markers NR1i3 and D1Mit458. Several genes of immunological importance map within the new interval. They most notably include the signaling lymphocytic activation molecule (SLAM)/CD2 family cluster (CD244, CD229, CS1, CD48, CD84, and Ly108), which are major candidates to explain the loss of self-tolerance in NZM2410 lupus mice, corresponding to the *Sle1* locus (29,30). The NOD strain appears to share the same haplotype in this region as NZW mice, the donor strain of the susceptibility allele in NZM2410 mice. The SLAM/CD2 family members have been shown to regulate important aspects of lymphocyte function, such as proliferation, cytokine secretion, and cytotoxicity (31). Their intracellular domain binds to an adaptor molecule, SLAM-associated protein, and this interaction was shown to be critical for T-cell signaling (32). Moreover, recent studies showed that SLAM-associated protein is a crucial regulator of iNKT cell ontogeny in both humans and mice (33–35). However, which SLAM member(s) is expressed and interacts with SLAM-associated protein in NKT cells is currently not known.

It should be also stressed that the SLAM/CD2 gene cluster extends over just 400 Kb, a minor portion of the current *Nkt1* interval, which contains numerous other potential candidates. Therefore, additional subcongenic strains with smaller intervals will be necessary before a detailed investigation of the polymorphism and expression of candidates can be undertaken.

Experiments of several types point to a relationship

between type 1 diabetes susceptibility and iNKT cells, at least in mice. Findings obtained with transgenic mice and after pharmacological treatments clearly indicate a strong impact of iNKT cells on the course of type 1 diabetes (11–13,15,16). In addition, analysis of iNKT cells in NOD mice congenic with a protective interval, including *Idd6* and *Idd9.1*, favors a role for iNKT cells in spontaneous diabetes because these protective intervals modify the number and the function of iNKT cells (18,19). Conversely, the NOR strain shows depressed number and function of iNKT cells similar to NOD mice (18), even though the *Idd13* locus, which contributes to diabetes resistance in NOR mice and maps on chromosome 2 (36), overlaps with the *Nkt2* locus and significantly attenuates iNKT cell activity when transferred onto the C57BL/6 background (18). Therefore, our present finding that the *Nkt1* locus does not affect the course of spontaneous diabetes in NOD.C57BL/6-*Nkt1* (L2 or L6) mice is not entirely unexpected.

Several explanations should be considered to account for such complexity. First, IL-4 plays a significant role in the protection against diabetes afforded by iNKT cells after activation by α -GalCer (37), and it was also put forward as the mediator of protection against diabetes in V α 14J α 81 transgenic mice (11). In these model systems, however, large amounts of IL-4 are made artificially available. In contrast, in NOD congenic mice, the improvement of IL-4 production is physiological and limited in magnitude, and it is probably not sufficient to alter the course of diabetes significantly. Other genetic factors might then be necessary and interact with *Nkt1* to restore the defect of iNKT cells beyond a certain threshold required for a significant effect on the course of diabetes. Importantly, such interactions might influence the outcome of autoimmunity in both directions and be either beneficial or detrimental to the host. This concern was recently highlighted in systemic lupus erythematosus, where iNKT cells can play both a protective and a pathogenic role in a manner that is genetically determined (38). The development of mice congenic for two or more loci should therefore shed more light on the relationship with type 1 diabetes susceptibility and iNKT cell functions.

Second, other mechanisms might be involved as well. They might depend on different cytokines or even be independent of cytokines, such as cell-cell contacts (39). Such mechanisms have not been yet investigated in genetic studies. Altogether, these observations indicate that the genetic models provide a powerful tool to dissect the complexity of iNKT cell activation mechanisms and their role in pathophysiology. Their study should also help to better anticipate the outcome of pharmacological activation of iNKT cells.

The distal chromosome 1 region is of broad significance in the immune response, as suggested by its involvement in several animal models of systemic lupus (29,40,41) and in anti-nuclear antibody production in NOD mice (21). This region has also been mapped to susceptibility to collagen-induced arthritis (42) and it was linked to murine cerebral malaria (43). Finally, the human equivalent synteny, which lies on distal chromosome 1q, was linked to type 1 diabetes susceptibility (44,45). This region, therefore, seems to be an important one to investigate the genetic basis of autoimmune disorders, notably including type 1 diabetes.

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REFERENCES

- Castano L, Eisenbarth GS: Type-I diabetes: a chronic autoimmune disease of human, mouse, and rat. *Annu Rev Immunol* 8:647–679, 1990
- Delovitch TL, Singh B: The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. *Immunity* 7:727–738, 1997
- Bendelac A, Rivera MN, Park SH, Roark JH: Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu Rev Immunol* 15:535–562, 1997
- Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo E, Koseki H, Taniguchi M: CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 278:1626–1629, 1997
- Zhou D, Mattner J, Cantu C 3rd, Schrantz N, Yin N, Gao Y, Sagiv Y, Hudspeth K, Wu YP, Yamashita T, Teneberg S, Wang D, Proia RL, Lavery SB, Savage PB, Teyton L, Bendelac A: Lysosomal glycosphingolipid recognition by NKT cells. *Science* 306:1786–1789, 2004
- Kikutani H, Makino S: The murine autoimmune diabetes model: NOD and related strains. *Adv Immunol* 51:285–322, 1992
- Gombert JM, Herbelin A, Tancrede-Bohin E, Dy M, Carnaud C, Bach JF: Early quantitative and functional deficiency of NK1+ -like thymocytes in the NOD mouse. *Eur J Immunol* 26:2989–2998, 1996
- Hammond KJ, Pellicci DG, Poulton LD, Naidenko OV, Scalzo AA, Baxter AG, Godfrey DI: CD1d-restricted NKT cells: an interstrain comparison. *J Immunol* 167:1164–1173, 2001
- Poulton LD, Smyth MJ, Hawke CG, Silveira P, Shepherd D, Naidenko OV, Godfrey DI, Baxter AG: Cytometric and functional analyses of NK and NKT cell deficiencies in NOD mice. *Int Immunol* 13:887–896, 2001
- Baxter AG, Kinder SJ, Hammond KJ, Scollay R, Godfrey DI: Association between alphabetaTCR+CD4-CD8- T-cell deficiency and IDDM in NOD/Lt mice. *Diabetes* 46:572–582, 1997
- Lehuen A, Lantz O, Beaudoin L, Laloux V, Carnaud C, Bendelac A, Bach JF, Monteiro RC: Overexpression of natural killer T cells protects Valpha14-Jalpha281 transgenic nonobese diabetic mice against diabetes. *J Exp Med* 188:1831–1839, 1998
- Falcone M, Facciotti F, Ghidoli N, Monti P, Olivieri S, Zaccagnino L, Bonifacio E, Casorati G, Sanvito F, Sarvetnick N: Up-regulation of CD1d expression restores the immunoregulatory function of NKT cells and prevents autoimmune diabetes in nonobese diabetic mice. *J Immunol* 172:5908–5916, 2004
- Wang B, Geng YB, Wang CR: CD1-restricted NK T cells protect nonobese diabetic mice from developing diabetes. *J Exp Med* 194:313–320, 2001
- Shi FD, Flodstrom M, Balasa B, Kim SH, Van Gunst K, Strominger JL, Wilson SB, Sarvetnick N: Germ line deletion of the CD1 locus exacerbates diabetes in the NOD mouse. *Proc Natl Acad Sci U S A* 98:6777–6782, 2001
- Hong S, Wilson MT, Serizawa I, Wu L, Singh N, Naidenko OV, Miura T, Haba T, Scherer DC, Wei J, Kronenberg M, Koezuka Y, Van Kaer L: The natural killer T-cell ligand alpha-galactosylceramide prevents autoimmune diabetes in non-obese diabetic mice. *Nat Med* 7:1052–1056, 2001
- Sharif S, Arreaza GA, Zucker P, Mi QS, Sondhi J, Naidenko OV, Kronenberg M, Koezuka Y, Delovitch TL, Gombert JM, Leite-De-Moraes M, Gouarin C, Zhu R, Hameg A, Nakayama T, Taniguchi M, Lepault F, Lehuen A, Bach JF, Herbelin A: Activation of natural killer T cells by alpha-galactosylceramide treatment prevents the onset and recurrence of autoimmune type 1 diabetes. *Nat Med* 7:1057–1062, 2001
- Esteban LM, Tsoutsman T, Jordan MA, Roach D, Poulton LD, Brooks A, Naidenko OV, Sidobre S, Godfrey DI, Baxter AG: Genetic control of NKT cell numbers maps to major diabetes and lupus loci. *J Immunol* 171:2873–2878, 2003
- Matsuki N, Stanic AK, Embers ME, Van Kaer L, Morel L, Joyce S: Genetic dissection of V alpha 14J alpha 18 natural T cell number and function in autoimmune-prone mice. *J Immunol* 170:5429–5437, 2003
- Carnaud C, Gombert J, Donnars O, Garchon H, Herbelin A: Protection against diabetes and improved NK/NKT cell performance in NOD.NK1.1 mice congenic at the NK complex. *J Immunol* 166:2404–2411, 2001
- Luan JJ, Monteiro RC, Sautes C, Fluteau G, Eloy L, Fridman WH, Bach JF, Garchon HJ: Defective Fc gamma RII gene expression in macrophages of NOD mice: genetic linkage with up-regulation of IgG1 and IgG2b in serum. *J Immunol* 157:4707–4716, 1996
- Jordan MA, Silveira PA, Shepherd DP, Chu C, Kinder SJ, Chen J, Palmisano LJ, Poulton LD, Baxter AG: Linkage analysis of systemic lupus erythematosus induced in diabetes-prone nonobese diabetic mice by *Mycobacterium bovis*. *J Immunol* 165:1673–1684, 2000
- Fujii S, Shimizu K, Kronenberg M, Steinman RM: Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat Immunol* 3:867–874, 2002
- Bendelac A, Killeen N, Littman DR, Schwartz RH: A subset of CD4+ thymocytes selected by MHC class I molecules. *Science* 263:1774–1778, 1994
- Vicari A, de Moraes Mdo C, Gombert JM, Dy M, Penit C, Papiernik M, Herbelin A: Interleukin 7 induces preferential expansion of V beta 8.2+CD4-8- and V beta 8.2+CD4+8- murine thymocytes positively selected by class I molecules. *J Exp Med* 180:653–661, 1994
- Araujo LM, Puel A, Gouarin C, Hameg A, Mevel JC, Koezuka Y, Bach JF, Mouton D, Herbelin A: NKT lymphocyte ontogeny and function are impaired in low antibody-producer Biozzi mice: gene mapping in the interval-specific congenic strains raised for immunomodulatory genes. *Int Immunol* 12:1613–1622, 2000
- Zhang F, Liang Z, Matsuki N, Van Kaer L, Joyce S, Wakeland EK, Aune TM: A murine locus on chromosome 18 controls NKT cell homeostasis and Th cell differentiation. *J Immunol* 171:4613–4620, 2003
- Gumperz JE, Miyake S, Yamamura T, Brenner MB: Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J Exp Med* 195:625–636, 2002
- Lee PT, Benlagha K, Teyton L, Bendelac A: Distinct functional lineages of human V(alpha)24 natural killer T cells. *J Exp Med* 195:637–641, 2002
- Morel L, Blenman KR, Croker BP, Wakeland EK: The major murine systemic lupus erythematosus susceptibility locus, Sle1, is a cluster of functionally related genes. *Proc Natl Acad Sci U S A* 98:1787–1792, 2001
- Wandstrat AE, Nguyen C, Limaye N, Chan AY, Subramanian S, Tian XH, Yim YS, Pertsemliadis A, Garner HR Jr, Morel L, Wakeland EK: Association of extensive polymorphisms in the SLAM/CD2 gene cluster with murine lupus. *Immunity* 21:769–780, 2004
- Engel P, Eck MJ, Terhorst C: The SAP and SLAM families in immune responses and X-linked lymphoproliferative disease. *Nat Rev Immunol* 3:813–821, 2003
- Howie D, Okamoto S, Rietdijk S, Clarke K, Wang N, Gullo C, Bruggeman JP, Manning S, Coyle AJ, Greenfield E, Kuchroo V, Terhorst C: The role of SAP in murine CD150 (SLAM)-mediated T-cell proliferation and interferon gamma production. *Blood* 100:2899–2907, 2002
- Chung B, Aoukaty A, Dutz J, Terhorst C, Tan R: Signaling lymphocytic activation molecule-associated protein controls NKT cell functions. *J Immunol* 174:3153–3157, 2005
- Nichols KE, Hom J, Gong SY, Ganguly A, Ma CS, Cannons JL, Tangye SG, Schwartzberg PL, Koretzky GA, Stein PL: Regulation of NKT cell development by SAP, the protein defective in XLP. *Nat Med* 11:340–345, 2005
- Pasquier B, Yin L, Fondaneche MC, Relouzat F, Bloch-Queyrat C, Lambert N, Fischer A, de Saint-Basile G, Latour S: Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked lymphoproliferative syndrome gene product. *J Exp Med* 201:695–701, 2005
- Serreze DV, Prochazka M, Reifsnnyder PC, Bridgett MM, Leiter EH: Use of recombinant congenic and congenic strains of NOD mice to identify a new insulin-dependent diabetes resistance gene. *J Exp Med* 180:1553–1558, 1994
- Mi QS, Ly D, Zucker P, McGarry M, Delovitch TL: Interleukin-4 but not interleukin-10 protects against spontaneous and recurrent type 1 diabetes by activated CD1d-restricted invariant natural killer T-cells. *Diabetes* 53:1303–1310, 2004
- Singh AK, Yang JQ, Parekh VV, Wei J, Wang CR, Joyce S, Singh RR, Van Kaer L: The natural killer T cell ligand alpha-galactosylceramide prevents or promotes pristane-induced lupus in mice. *Eur J Immunol* 35:1143–1154, 2005

39. Novak J, Beaudoin L, Griseri T, Lehuen A: Inhibition of T cell differentiation into effectors by NKT cells requires cell contacts. *J Immunol* 174:1954–1961, 2005
40. Kono DH, Burlingame RW, Owens DG, Kuramochi A, Balderas RS, Balomenos D, Theofilopoulos AN: Lupus susceptibility loci in New Zealand mice. *Proc Natl Acad Sci U S A* 91:10168–10172, 1994
41. Vyse TJ, Rozzo SJ, Drake CG, Izui S, Kotzin BL: Control of multiple autoantibodies linked with a lupus nephritis susceptibility locus in New Zealand black mice. *J Immunol* 158:5566–5574, 1997
42. Johansson AC, Sundler M, Kjellen P, Johannesson M, Cook A, Lindqvist AK, Nakken B, Bolstad AI, Jonsson R, Alarcon-Riquelme M, Holmdahl R: Genetic control of collagen-induced arthritis in a cross with NOD and C57BL/10 mice is dependent on gene regions encoding complement factor 5 and FcγRIIb and is not associated with loci controlling diabetes. *Eur J Immunol* 31:1847–1856, 2001
43. Bagot S, Campino S, Penha-Goncalves C, Pied S, Cazenave PA, Holmberg D: Identification of two cerebral malaria resistance loci using an inbred wild-derived mouse strain. *Proc Natl Acad Sci U S A* 99:9919–9923, 2002
44. Concannon P, Gogolin-Ewens KJ, Hinds DA, Wapelhorst B, Morrison VA, Stirling B, Mitra M, Farmer J, Williams SR, Cox NJ, Bell GI, Risch N, Spielman RS: A second-generation screen of the human genome for susceptibility to insulin-dependent diabetes mellitus. *Nat Genet* 19:292–296, 1998
45. Ewens KG, Johnson LN, Wapelhorst B, O'Brien K, Gutin S, Morrison VA, Street C, Gregory SG, Spielman RS, Concannon P: Linkage and association with type 1 diabetes on chromosome 1q42. *Diabetes* 51:3318–3325, 2002