A defective IL15 allele underlies the deficiency in natural killer cell activity in nonobese diabetic mice

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The nonobese diabetic (NOD) mouse strain has a genetic deficiency in natural killer (NK) cells. This defect underlies this strain’s utility in several experimental settings; in particular, it promotes engraftment of human tissue in NOD hosts during the generation of “humanized” mouse models. We have mapped the major NK-cell defect in the NOD vs. C57BL/6 (B6) strain to an inadequately expressed IL15 allele. Treatment of NOD mice with a reagent that specifically enhances interleukin (IL)-15 bioavailability normalized NK-cell numbers and activity in the absence of nonspecific stimulation. These findings raise the possibility of exploiting reagents that impact the IL-15 receptor pathway to facilitate construction of humanized mouse models on non-NOD genetic backgrounds.

We quantified two parameters in the genetic mapping studies. “NK numbers” refers to the percentage of CD49b+CD3− cells in total splenocytes and hovered around 2% in both strains (Fig. 1A). “NK activity” was measured by an in vivo assay (3, 5) that compared the survival of coinjected MHCI-positive and -negative target cells over an 18-h assay period, yielding a cytotoxicity index (Fig. 1B). Routinely, about 2- to 3-fold fewer of the MHCI-negative targets were killed in NOD than in B6g7 mice (Fig. 1C).

NOD × B6g7 F1 offspring had NK numbers indistinguishable from those of the two parental strains (Fig. 2A), whereas their NK activity was relatively high, as in B6g7 animals (Fig. 2B), indicating that this phenotype is dominant. Unexpectedly, average NK numbers in a cohort of 187 NOD × B6g7 F2 offspring were higher than in both of the parental strains or in the F1’s (Fig. 2A), suggesting that counterbalancing positive and negative influences are encoded in the NOD and B6g7 genomes. Individual F2’s showed a broad range of NK cytotoxicity indices (Fig. 2B), arguing for polygenic control of this trait. There was only a poor correlation between the NK number and activity values (Fig. 2C), meaning that at least some of the loci controlling these traits operate independently.

Genomewide single-nucleotide polymorphism (SNP) genotyping was performed on this F2 cohort and was analyzed relative to both NK numbers and activity. The data on NK numbers yielded a major peak of linkage on chr 8, with a LOD score of 7.4, well above the significance threshold of 3.3 determined by permutation testing (Fig. 2D i). The chr 8 linkage was broad, extending from 30 to 100 Mb and peaking at 65 Mb (Fig. 2D ii). The B6 haplotype in this region was correlated with lower NK-cell numbers (Fig. 2D iii). Joint LOD scores, calculated to assay epistatic influences (12), revealed interactions between the chr 8 interval and loci residing on several other chromosomes (Fig. 2E i, detailed in the legend to this figure). For example, the chr 9 locus had a strong negative impact on the chr 8 effect, the allele at chr 9 largely counteracting the B6 allele at chr 8 (Fig. 2E ii), which can easily explain why average NK numbers end up being very similar in NOD and B6g7 mice.

Intervals on chrs 1, 6, and 8 showed significant linkage to NK activity, i.e., had a LOD score >3.5 (Fig. 2F i and ii). The chr 8 interval had the strongest effect, with a directionality consistent with it being responsible for the low NK-cell activity of the NOD strain (Fig. 2F ii). The analysis of joint LOD scores revealed that the chr 1 and chr 6 regions both had an epistatic interaction with the interval on chr 8, as well as with each other (Fig. 2G i). The dampening effect of the NOD chr 8 allele on NK-cell performance was reduced when the chr 1 interval was NOD/B6 and was almost completely masked when that interval was NOD/NOD (Fig. 2G ii Left). In contrast, the negative

Results and Discussion

We chose B6.H-2fl/1 (B6g7) mice, congenic for the NOD-derived histocompatibility complex (MHC), as comparators for mapping the NOD NK-cell defect. The two mouse strains had similar fractions of CD49b+CD3− NK cells in the spleen and other lymphoid organs, as previously reported (5, 9), with similar maturation (CD27 vs. CD11b) and activation (CD25 vs. CD69) profiles (Fig. S1). However, largely reflecting the unique structure of the NK complex on chr 6 in NOD mice (10), the two strains had very different patterns of NK-cell receptor expression, e.g., of NK1.1, Ly49I, Ly6c, and NKG2A (Fig. S1).

It is well known that the nonobese diabetic (NOD) mouse strain has a genetic deficiency in natural killer (NK) cell activity (1–9). The earliest indication of such a defect was the observation that NOD splenocytes have a reduced ability to kill YAC-1 targets in vitro (1, 2). More recently, with the molecular definition of NK receptors and their ligands, coupled with their genetic localization, it became clear that NOD mice have an unusual repertoire of molecules critical for the differentiation, survival, and activity of NK cells; for example, a unique NK complex (NKC) on chromosome (chr) 6 (7, 10), or reduced levels of NKG2D, owing to enhanced expression of its ligand, Rae 1, and resulting desensitization (6, 11). However, the root of the genetic deficiency in NK cell activity has not yet been identified.

Whatever its origin, the NOD NK cell defect has important functional manifestations. First, it has long been exploited to promote engraftment of human tissues during the generation of “humanized” mouse models, explaining the popularity of the NK complex on chr 6 in NOD mice (10), the two mouse strains had major histocompatibility complex (MHC), as comparators for mapping the NOD NK-cell defect. The two mouse strains had similar fractions of CD49b+CD3− NK cells in the spleen and other lymphoid organs, as previously reported (5, 9), with similar maturation (CD27 vs. CD11b) and activation (CD25 vs. CD69) profiles (Fig. S1). However, largely reflecting the unique structure of the NK complex on chr 6 in NOD mice (10), the two strains had very different patterns of NK-cell receptor expression, e.g., of NK1.1, Ly49I, Ly6c, and NKG2A (Fig. S1).

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**Fig. 1.** "NK numbers" and "NK activity" in B6g7 vs. NOD mice. (A) Flow cytometric determination of NK numbers in 7-week-old B6g7 and NOD mice. The percent symbol (%) refers to the fraction of CD49d+ CD3+ cells in the lymphocyte gate. (B) Schema for in vivo assay of NK activity. MHCI-positive and MHCI-negative splenocytes were differentially labeled with CFSE, mixed equally, and injected into B6g7 or NOD mice. Remaining donor cells were counted 18 h later, and a cytotoxicity index was calculated. (C) Representative indices of NK activity in 7-week-old B6g7 or NOD mice.

The impact of the chr 8 allele was most evident when the chr 6 interval was present in NOD-homozygous state (Fig. 2G ii Right). Because the chr 1 and chr 6 genetic influences did not drive NK activity in the relevant direction, i.e., the B6 haplotype was associated with lower activity, we did not further pursue their localization. However, given the unique structure of the NK complex in NOD mice (10), one or more of its genes are likely candidates for the chr 6 effect. One obvious candidate for the chr 1 locus is the gene encoding Cd244 (Cd244), which is active in NK cells, or more generally the SLAM/CD2 gene cluster, of which NOD mice carry an unusual haplotype (13).

**Fig. 2.** Genetic analysis of NK cells in B6g7 vs. NOD mice. (A and B) NK numbers and in vivo activity for B6g7, NOD, and F1 mice at 7 weeks of age. (C) NK activity vs. numbers in F2 mice (n = 187). (D–F) Genetic linkage with NK numbers on activity. (i) LOD score plot (R-QTL) for genomewide linkage (n = 187). The peak LOD score on chr 8 was 7.4. The dotted line delineates significance, as calculated by a permutation test (n = 10,000; P < 0.05). (ii) A higher resolution map of individual chromosomes. (iii) Distribution of NK numbers on activity for each genotype. B/B, both alleles from B6g7; N/B, heterozygotes; N/N, both alleles from the NOD genome. Symbols to the right of each set of values indicate mean ± SEM. (E–G) Epistatic interactions influencing NK numbers and activity. (i) 2D genomewide linkage in F2 genotypes. Joint LOD scores were plotted as a heat map (12). The significant joint LOD scores of chrs 1 and 6, chrs 1 and 8, and chrs 6 and 8 were 11.88, 17.40, and 23.49, respectively. Greater than 8.4 was a significant value per permutation test (n = 10,000; P ≤ 0.05).
Each dot represents an individual mouse. (Right) A genotype schema for the congenic strains. (B) Test of stroma vs. hematopoietic cells. Marrow cells were injected into lethally irradiated NOD.CD45.2 (CD45.1) donor mice and NOD.Chr8 b/b donor mice and NOD.Chr8 b/b donors. Mixed equally and injected into lethally irradiated NOD.Chr8 b/b heterozygous recipient mice (n = 8). NK cells were distinguished by their CD45 allele and NK numbers derived from the two donors plotted. Error bars in C and D represent mean ± SD.

Fig. 3. Establishment of congenic mouse lines and bone-marrow chimeras. (A) NK cell number and activity phenotypes of B6g7, NOD, and congenic mice. (Left) NK numbers for B6g7, NOD, NOD.Chr1 b/b, NOD.Chr18 b/b, and NOD.Chr8 b/b mice. Each dot represents an individual mouse. (Right) A genotype schema for the congenic strains. (B) The different elements to be assayed in the radiation bone-marrow chimeras. Stroma are radiation resistant and hematopoietic cells radiation sensitive. (C) Test of stroma vs. hematopoietic cells. Marrow cells were injected into lethally irradiated NOD.Chr8 b/b (CD45.1) (n = 8) mice or NOD (CD45.1) (n = 8). Donor NK numbers, distinguished by their CD45.2 surface marker, were analyzed. (D) Test of NK cells vs. other hematopoietic cells. Marrow from NOD.Chr8 b/b donor mice and NOD.Chr8 b/b donor mice were mixed equally and injected into lethally irradiated NOD.Chr8 b/b heterozygous recipient mice (n = 9). NK cells were distinguished by their CD45 allele and NK numbers derived from the two donors plotted. Error bars in C and D represent mean ± SD.

Fig. 4. Fine mapping of the chr 8 influence. (A) LOD score plots of NK numbers (Upper) and NK activity (Lower) in 7-week-old recombinant mice (n = 147). A significant score for NK numbers and NK activity was estimated as 3.07 and 3.01, respectively, via a permutation test (n = 10,000; P ≤ 0.01). (B) Genotype map of F60, F63, and F65 subcongenic mice. (C) Phenotypes of NK cells from F60 (n = 6), F63 (n = 19), F65 (n = 9), NOD.Chr1 b/b (n = 12), and NOD.Chr18 b/b (n = 6) mice. Each dot represents an individual mouse. (D) Map of the relevant interval on chr 8 (adapted from the Ensembl browser; build 37) with the various genes known to be located within this interval and the relative expression in B6g7 vs. NOD splenocytes, from our microarray data. Each dot represents the NOD vs. B6 ratio for an individual probe. Probes yielding values with a false discovery rate of <0.01 are highlighted as filled dots. The two-headed arrow indicates the interval of interest, delineated by the F63 and F65 recombinant lines. (E) Probes for and profiles of candidate genes. FC, fold-change NOD vs. B6g7; FDR, false discovery rate for the corresponding FC.
Next, we constructed congenic mouse lines carrying B6-derived intervals on chr 1, chr 8, or both on the NOD genetic background (NOD.C1\textsuperscript{bb}, NOD.C8\textsuperscript{bb}, and NOD.C1\textsuperscript{bb}8\textsuperscript{bb}, respectively). Confirming the F\textsubscript{2} data, NK numbers were higher in the NOD.C8\textsuperscript{bb} and NOD.C1\textsuperscript{bb}8\textsuperscript{bb} congenic lines than in either of the parental strains (Fig. 3A Left). Also anticipated was that the NOD.C8\textsuperscript{bb} line would exhibit the relatively high NK activity characteristic of the B6g7 strain, which would be further augmented in the double-congenic line, given the epistatic interaction between the chr 1 and chr 8 intervals mentioned above (Fig. 3A Right). (The increased activity of NOD.C1\textsuperscript{bb} vis-à-vis NOD mice probably reflects epistatic interactions between loci on chr1 and other chromosomes.)

Exploiting these congenic lines, we addressed whether the NOD NK-cell defect specified by the chr 8 interval partitioned with the radio-resistant stromal-cell compartment, with the radio-sensitive hematopoietic cell compartment, or intrinsically within NK cells (Fig. 3B). First, we transferred bone-marrow cells from NOD mice carrying the CD45.2 allelic marker into lethally irradiated NOD or NOD.C8\textsuperscript{bb} hosts, both bearing the CD45.1 allele, and quantified NK-cell numbers 7 weeks later. There was no difference in the fraction of NK cells in the spleens of the two types of host (Fig. 3C), indicating that the dampening effect of the NOD chr 8 segment was not determined by the stromal, but rather by the hematopoietic cell compartment. Secondly, we performed a similar experiment, but transferred an equal mix of NOD, CD45.2 and NOD.C8\textsuperscript{bb} (CD45.1) bone-marrow cells into lethally irradiated NOD.CD45.1/CD45.2 heterozygotes. There was no difference in the fraction of donor CD45.1\textsuperscript{+}2\textsuperscript{−} and donor CD45.1\textsuperscript{+}2\textsuperscript{−} NK cells in the spleen, both being relatively low (Fig. 3D), establishing that the impact of the NOD chr 8 allele was not NK-cell intrinsic and was thus determined by other lymphoid or myeloid cells.

The congenic lines were also the starting point for finer mapping within the chr 8 interval. Crossing the NOD.C1\textsuperscript{bb} and NOD.C1\textsuperscript{bb}8\textsuperscript{bb} mice (following an intercross/backcross strategy to generate mice with a single recombinant chr 8) yielded 147 offspring with recombination events within the 65.7–110.0 Mb interval, while fixing the epistatic B6 segment on chr 1. SNP mapping on this cohort generated almost identical curves for NK numbers and activity, peaking between 76 and 91 Mb (Fig. 4A). From these, we established three subcongenic lines with recombination points within the interval (Fig. 4B). F\textsubscript{85} and F\textsubscript{80} mice had relatively low NK numbers and activity values similar to those of the NOD.C1\textsuperscript{bb} comparator, whereas F\textsubscript{80} mice had the higher numbers and activity characteristic of NOD.C1\textsuperscript{bb}8\textsuperscript{bb} and B6g7 mice (Fig. 4C). Thus, the major NK-cell defect in NOD mice maps between positions 83 and 85 Mb on chr 8. According to the Ensembl database, seven protein-coding genes reside within this interval (Fig. 4D). A microarray comparison of gene expression by splenocytes from B6g7 and NOD mice (Affymetrix M430, triplicate) revealed that the locus encoding the cytokine, IL-15, was expressed most differentially between the two strains—about 2-fold less by NOD splenocytes (Fig. 4E).

Given its well-known roles in NK-cell maturation, survival, and activity, IL-15 makes an excellent candidate for the molecule responsible for the NK-cell defect in the NOD strain. We sequenced the exons and promoter region of the \textit{Il15} genes from B6g7 and NOD mice, uncovering 18 SNPs: 8 in the promoter region, 5 in the 5′-UTR, and 5 in the 3′-UTR (Fig. 5A). As none of the SNPs alter the protein sequence, we focused on differential IL-15 expression in the two strains. RT-PCR analysis confirmed that there were reduced IL-15 transcript levels in the spleens of NOD mice and that this was also true of the two known splice variants (Fig. 5B). Expression-QTL (eQTL) analysis, using spleen RNA from the same F\textsubscript{2} offspring used for mapping the NK numbers/activity QTLs, revealed that there were cis-determined (Fig. 5C). According to RT-PCR analysis on sorted splenocyte populations, dendritic cells and macrophages showed...
the highest IL-15 transcript levels and the greatest differential between NOD and B6g7 mice (Fig. 5D), consistent with the above conclusion that the NK-cell defect was dictated by non-NK hematopoietic cells (Fig. 3B).

Soluble IL-15 has little in vivo activity on its own; rather, it is usually bound to the IL-15 receptor α (IL15Rα) chain, mostly on dendritic cells, and “presented” in trans to NK (and other) cells (14). Complexing IL-15 with IL15RαFc stabilizes it, promoting its bioavailability (15–17). To see whether “IL-15 complexes” could boost NOD NK-cell numbers and activity to B6g7 levels, we injected NOD mice with differing amounts in differing ratios every other day for 8 days, and assayed NK-cell properties. Serial injections of 25 ng IL-15 complexed with 100 ng IL-15RαFc produced the desired few-fold increase in NK numbers and activity (Fig. 6A). We also applied this regimen to the simplified (TCR transgenic) TID model, BDC2.5/NOD, of which 15–25% normally develop diabetes beginning at 15–20 weeks of age. As with the standard NOD strain, IL-15 complexes boosted NK-cell numbers in the spleens of BDC2.5/NOD mice a few-fold and also in the pancreatic lymph nodes and the islet infiltrate (Fig. 6B i). However, according to their profiles of CD69 and CD25 expression, the NK cells in complex-injected mice were not unduly activated (Fig. 6B ii). The increase in cell numbers at these doses was quite specific for the NK-cell compartment, as no augmentation in numbers of CD4+ or CD8+ cells was observed in the different organs nor of activated (CD4+25+ or CD4+44+122+) T cells, nor of dendritic cells (CD11b−CD11c+) and macrophages (CD11b+CD14+) (Fig. 6B iii). Strikingly, beginning as soon as 2 weeks after the first injection, about 30% of the BDC2.5/NOD mice administered IL-15 complexes every other day starting at 14 days of age developed diabetes, i.e., long before they normally would (Fig. 6B iv). This effect evokes previously described associations between numbers or activity of NK cells and hyperacut diabetes in mouse models (7, 8).

Thus, inadequate expression of the Il15 gene is a major contributor to the NK-cell defect in NOD mice. IL-15 regulates the maturation and survival of NK cells and also controls their activity by up-regulating expression of NK receptors and cytotoxic effector molecules (18). Whereas our genetic dissection revealed Il15 to have an important impact on both the numbers and activity of NK cells, several other loci also influenced these properties. The integration of these different effects, which could be either positive or negative and could alter numbers and activity independently, led to normal NK-cell numbers with 50–70% reduced activity in the NOD compared with the B6g7 strain. Although we did not investigate these aspects, IL-15’s known influence on other cell types (18) has the potential of explaining certain other aberrant characteristics of the NOD strain, e.g., deficiencies in the NK-T or dendritic cell compartments (19, 20).

Materials and Methods

Mice. NOD/LtdO (NOD), C57BL/6.H-2.g7 (B6g7), and BDC2.5 T cell receptor transgenic mice on the NOD and B6g7 backgrounds (BDC2.5/NOD and BDC2.5/B6g7, respectively) were bred in our specific-pathogen-free facility at Joslin Diabetes Center (protocol 99–20). F, mice were generated by intercrossing Fl mice (NOD × B6g7). NOD.C1H8, NOD.C1Hb8, and NOD.C1b8 mice were backcrossed to the NOD genetic background more than six times.

For bone-marrow chimeras, 6-week-old recipient mice were irradiated at 900 R, reconstituted with 3.10^6 cells harvested from the femur of age-matched donor mice. After 6 weeks, chimeras were tested by flow cytometry for the degree of chimerism (typically >90% of donor origin).

Analysis of Lymphoid Cell Populations. Cell suspensions were generated by mechanical disruption and stained for flow cytometry. RNA was prepared from sorted cell populations or whole spleens as described (21). CDNAs were quantified by using SYBR Green quantitative RT-PCR. For microarrays, RNA was amplified (MessageAmp aRNA, Ambion), biotin-labeled (BioArray high yield RNA transcription labeling, Enzo), purified (RNeasy minikit, QIAGEN), and hybridized to M430 2.0 chips (Affymetrix). Raw data were normalized with the RNA-seq algorithm in the GenePattern software package (22).

Fig. 6. Induction of NK cells and diabetes in NOD mice by IL-15 complexes. (A) Titration of IL-15/IL-15R complexes effect in vivo. Dilutions of IL-15 complexes, starting at 3,500 ng IL-15Fc and 750 ng IL-15, were injected every 48 h into NOD mice, from 14 days of age. Splenocytes were analyzed after eight injections. Treatment with 100 ng IL-15FcF and 25 ng IL-15 yielded the desired 2- to 3-fold increase in NK numbers (i), which translated to a similar augmentation in NK activity (ii). (B) Induction of diabetes through induction of NK activity. (i) The fraction of NK cells in spleens (SPL), pancreatic lymph nodes (PLN), or peritoneum (Panc.) of the BDC2.5/NOD mice injected with IL-15/IL-15R complexes (n = 9) or PBS (n = 9) for 2 weeks starting at 14 days of age. (ii) Expression of CD69 and CD25 activation markers on NK cells from mice after injection of IL-15/IL-15R complexes as per B. (iii) The effect of IL-15 complexes on diverse cell types. Numbers of CD49b+CD3−NK cells (NK), CD3+CD4+CD8− T cells (CD4+), CD3+CD4+CD25+ T cells (CD25+), CD3−CD4+CD8+ T cells (CD8+CD3−), CD8−CD4+CD122+ T cells (CD44+CD122+), CD11b−CD11c+ predominantly dendritic cells, DC, and CD11b+CD11c+ (predominantly macrophages, MF) in spleens of mice administered IL-15/IL-15R complexes as per B (n = 9). (iv) Diabetes incidence in BDC2.5/NOD mice treated with IL-15/IL-15R complexes every other day from 14 days to 12 weeks of age.

In Vivo Cytotoxicity Assay. B6.129− and B6g7 splenocytes were labeled with low-dose (0.5 μM) or high-dose (5 μM) carboxyfluorescein succinimidyl ester.
Genetic Mapping and eQTL. DNA was genotyped for SNP markers that distinguish B6 and NOD alleles by fluorescent PCR, for 120 SNPs covering all 19 autosomes and idd loci, average spacing of ≈19.4 Mb, and analyzed with R/qtl (data check for genotyping errors, linkage peaks identification by interval mapping). Empirical corrected P values were established by permutation (10,000). eQTL for Il15 was performed with splenic RNAs from genotyped F2 mice. Il15 expression of F2 mice was determined by RT-PCR, and the LOD score was determined for IL-15 transcript levels by R/qtl.

IL-15 Complexes and Induction of Diabetes. A total of 7 μg of murine soluble IL-15RαFc (R&D Systems) and 1.5 μg of murine IL-15 (eBioscience) were mixed and incubated in 29 μL of PBS at 37 °C for 20 min. The IL-15/IL-15RαFc complexes were diluted in PBS to 250 μL and administered every 48 h. For diabetes, 109 ng IL-15Rα chimeric protein and 23 ng IL-15 were injected intraperitoneally into BDC2.5×NOD mice every 48 h from 14 days of age. Mice were considered diabetic if a urinary glucose test was positive and blood glucose was more than 300 mg/dL in at least two consecutive measurements.

Statistical Analyses. Data from various experiments were tested for significance by the unpaired two-tailed Student’s t test. Data were expressed as means ± SD. For microarray analyses, false discovery rates (FDRs) were calculated using the GenePattern package. Significance was set at a cutoff of FDR < 0.05.

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