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Protection Against Diabetes and Improved NK/NKT Cell Performance in NOD.NK1.1 Mice Congenic at the NK Complex

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The NK.1 cell surface receptor, which belongs to the NKR-P1 gene cluster, has been bred onto nonobese diabetic (NOD) mice for two purposes. The first was to tag NK and NKT cells for easier experimental identification of those subsets and better analysis of their implication in type 1 diabetes. The second was to produce a congenic strain carrying Idd6, a susceptibility locus that has been repeatedly mapped in the vicinity of the NKR-P1 gene cluster and the NK complex, to explore the impact of this locus upon autoimmune diabetes. NOD.NK1.1 mice express the NK1.1 marker selectively on the surface of their NK and NKT cell subsets. In addition, the mice manifest reduced disease incidence and improved NK and NKT cell performance, as compared with wild-type NOD mice. The association of those two features in the same congenic strain constitutes a strong argument in favor of Idd6 being associated to the NK complex. This could explain at the same time the multiple alterations of innate immunity reported in NOD mice and the fact that disease onset can be readily modified by boosting the innate immune system of the mouse. The Journal of Immunology, 2001, 166: 2404–2411.

Interactions between innate and cognate immunity appear to play a key role in the elicitation and regulation of numerous immunological responses (1). In that respect, NK cells occupy a strategic position, half-way between adaptive and non-adaptive immunity (2). They possess a TCR/CD3 complex that allows them to sense their antigenic environment with an apparent predilection for lipids, glycolipids, or highly hydrophobic peptides essentially presented on CD1 determinants (3, 4). At the same time, they are equipped with NK cell receptors and react to antigenic stimuli with kinetics reminiscent of NK cells and of innate effector cells in general (5, 6). Although their physiological role is far from fully understood, NKT cells have been implicated in a wide spectrum of immune conditions, including some that were classically attributed to NK cells such as early protection against infectious agents (7–9) and rejection of tumor metastases following elicitation by IL-12 (10). They have also been implicated in the regulation of autoimmune conditions and particularly of human and mouse type 1 diabetes. Both experimental data in nonobese diabetic (NOD)3 mice (11, 12) and BioBreeding (BB) rats (13) and mouse type 1 diabetes. Both experimental data in nonobese diabetic (NOD)3 mice (11, 12) and BioBreeding (BB) rats (13) and clinical observations on twin patients (14) associate the dysfunction of NKT cells to disease susceptibility and, conversely, the increase of NKT activity to protection. NKT cells seem to curb the virulence of anti-islet T lymphocytes through the burst of anti-inflammatory (IL-4, IL-10) (15, 16) or resolutive (IFN-γ) lymphokines (17).

Besides a numerical and functional deficit of NKT cells (12), NOD mice present multiple defects of innate immunity, including poor NK function, which explains the remarkable permissiveness NOD.SCID (18) and NOD.RAG1−/− mice (19) to xenografts. Whether such defects contribute to the autoimmune profile of the strain is still not clear, but indirect arguments suggest that this might be the case. For instance, most microbiological or pharmacological agents that boost innate immune defenses such as viruses (20), bacillus Calmette-Guérin (21), poly(I:C) (21–23), or IL-12 (24) also exert an influence upon the onset of type 1 diabetes in NOD mice. In many cases they delay or totally prevent the disease, in a few situations they may accelerate the process, but they are never neutral. Another indirect argument comes from genetic studies. A region of disease susceptibility, referred to as Idd6, has been recurrently mapped on the distal portion of chromosome 6 (25) in an interval that overlaps the NK complex (NKC) (26). This 2-Mb segment contains all the critical genes involved in the physiology and the regulation of NK and NKT cells, including the Nkrp1 gene cluster, the set of inhibitory and activating Ly49 genes (27), Cd69, Cd94 (28), and a number of functional genes providing NK-mediated innate resistance against mouse CMV (Cmv1) (29), ecromelia poxvirus (Rmp1) (30), and Chinese hamster ovary xenotransplants Chok (28).

A few years ago, we started backcrossing onto NOD mice the cell surface marker NK1.1, a receptor of the NKR-P1 family expressed in the C57BL/6 (B6) but not in the NOD strain. There were two main purposes to this breeding. One was to tag NK and NKT cells with NK1.1, which is specifically recognized by the mAb PK136 (31). The second was to construct a NOD strain congenic for the Idd6 genetic interval, to explore the impact of this locus upon the pathogenesis of type 1 diabetes and the functional defects of innate immunity in this strain.

The NOD.NK1.1 congenic strain obtained after 10 backcrosses has already been successfully used for discriminating FcγRs on...
NK cells and macrophages of NOD mice (32) and for identifying NKT cells in V\textsubscript{\textalpha}14\textalpha}281-transgenic NOD mice (16). Here we report data directly connected to the objectives for which this congenic strain was initially produced, namely 1) the expression of the NK1.1 marker on NK and NKT cells, 2) the diabetic profile of the congenic strain was initially produced, namely 1) the expression of the NK1.1 marker on NK and NKT cells, 2) the diabetic profile of the congenic strain was initially produced, namely 1) the expression of the NK1.1 marker on NK and NKT cells, 2) the diabetic profile of the congenic strain was initially produced, namely 1) the expression of the NK1.1 marker on NK and NKT cells, 2) the diabetic profile of the congenic strain was initially produced, namely 1) the expression of the NK1.1 marker on NK and NKT cells, 2) the diabetic profile of the congenic strain was initially produced, namely 1) the expression of the NK1.1 marker on NK and NKT cells, 2) the diabetic profile of the congenic strain was initially produced, namely 1) the expression of the NK1.1 marker on NK and NKT cells, 2) the diabetic profile of the congenic strain was initially produced, namely 1) the expression of the NK1.1 marker on NK and NKT cells, 2) the diabetic profile of the congenic strain was initially produced, namely 1) the expression of the NK1.1 marker on NK and NKT cells, 2) the diabetic profile of the congenic strain was initially produced, namely 1) the expression of the NK1.1 marker on NK and NKT cells, 2) the diabetic profile of the congenic strain was initially produced, namely 1) the expression of the NK1.1 marker on NK and NKT cells, 2) the diabetic profile of the congenic strain was initially produced, namely 1) the expression of the NK1.1 marker on NK and NKT cells, 2) the diabetic profile of the congenic strain was initially produced, namely 1) the expression of the NK1.1 marker on NK and NKT cells, 2) the diabetic profile of the congenic strain was initially produced, namely 1) the expression of the NK1.1 marker on NK and NKT cells, 2) the diabetic profile of the congenic strain was initially produced, namely 1) the expression of the NK1.1 marker on NK and NKT cells, 2) the diabetic profile of the congenic strain was initially produced, namely 1) the expression of the NK1.1 marker on NK and NKT cells, 2) the diabetic profile of the congenic strain was initially produced, namely 1) the expression of the NK1.1 marker on NK and NKT cells, 2) the diabetic profile of the congenic strain was initially produced, namely 1) the expression of the NK1.1 marker on NK and NKT cells, 2) the diabetic profile of the congenic strain was initially produced, namely 1) the expression of the NK1.1 marker on NK and NKT cells, 2) the diabetic profile of the congenic strain was initially produced, namely 1) the expression of the NK1.1 marker on NK and NKT cells, 2) the diabetic profile of the congenic strain was initially produce...
some stage of NKT cell ontogeny. Further studies on the thymic differentiation pathways of NKT cells will be necessary for clearing this issue.

NKT cells could be also visualized in total spleen, using the same triple staining combination (Fig. 2A). Here, however, NKT cells represented <1% of the total population—0.48% and 0.80%, respectively, in NOD.NK1.1 and B6 spleen samples—and were less homogeneously clustered on the NK1.1 by TCRαβ dot-plot. Conventional NK cells (NK1.1^-TCR^+) are also clearly visible.

An overexpression of Vβ8 gene segments could be easily demonstrated in the population gated in the upper right quadrant, as compared with mainstream T cells (histograms in Fig. 2B). Splenic NKT cells presented also an overactivated phenotype as demonstrated by the high percentage of CD44^+ cells. There was indeed an increased percentage of CD44^+ cells among NOD.NK1.1 NKT cells (in the order of 60%), yet when compared with B6 NKT cells, the percentage of the latter was systematically higher in every experiment done (in the order of 90%), suggesting a possible defect of activation of NOD NKT cells that might account for the functional deficit described in NOD mice.

Conventional NK cells were reanalyzed per se by double staining with PK136 and DX5 mAbs, the latter staining indiscriminately the vast majority of NK cells. CD3^-T lymphocytes were gated out using a third color. As can be seen from Fig. 3, the distribution of NK cells with respect to the two classical NK markers is quasi-identical in NOD.NK1.1 and B6 mice. In both samples, double-positive NK1.1/DX5 cells are predominant and constitute an homogeneous, well-resolved population amounting to ~3% of the splenocytes. Interestingly, in contrast to NKT cells, there was no obvious numerical deficit in NOD.NK1.1 mice. If
anything, conventional NK cells seemed slightly more abundant in some NOD.NK1.1 spleens as compared with B6 (see Fig. 2A and 3).

**Diabetes prevalence and related autoimmune manifestations in NOD.NK1.1 congenic mice**

Cumulative incidence was followed over a 9-mo period, in a cohort of ∼300 males and 300 females. The mice were the progeny of a large intercross between BC 10 mice. They were individually genotyped at the boundaries of the foreign B6 segment, D6 Mit254 upstream and D6 Mit14 downstream. Mice that had recombed within this interval were excluded from the analyses. The global results are presented in Fig. 4A for females and 4B for males. Differences between congenic and wild-type mice are clearly observed. Time of onset is not affected, but incidence is lowered in BB (homozygous B6) compared with NN (homozygous NOD) mice. The differences are statistically significant by log rank test, well below 0.01%, and can be demonstrated both in females and males, suggesting that the protection afforded by the B6 alleles (or the lost susceptibility) is not linked to the sexual dimorphism of the strain. The fact that we compared littermates, bred under the same conditions and housed in common cages, genotyping being done after weaning, made unlikely an implication of environmental factors.

Protection is genetically dominant (or susceptibility recessive) as suggested by the still significant differences between homozygous NN and heterozygous BN littermates, but not between BN and BB littermates.

However, when histology was examined at 10–12 wk, i.e., at a preclinical stage, there was no significant difference in insulitis

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**FIGURE 2.** NKT cells in the spleen. A, Total splenocytes were stained with anti-TCRαβ-APC, PK136-FITC, and anti-Vβ8-PE or anti-CD44-PE. B, Histogram overlay comparing Vβ8 usage in R1 (mainstream T cells) vs R2 (NKT cells). C, Histogram overlay comparing CD44 percentages in R1 vs R2 regions.
NK and NKT cell functions in NOD.NK1.1 congenic mice

As mentioned above, NKT cells were not numerically restored in NOD.NK1.1 mice, neither in the thymus nor in the spleen, when compared with the same subsets in B6 mice. We also measured the capacity of NKT cells from NOD.NK1.1 mice to release explosively cytokines and of NK cells to lyse YAC-1 target cells in a standard chromium assay and found that neither functions were restored to the levels achieved by the same subsets in B6 mice (data not shown). Thus, as reported for SJL mice (37), the introduction of a B6 NKC was not sufficient to reconstitute the functional deficit of innate immunity in NOD congenic mice.

Yet, because we had found significant differences in disease susceptibility between NOD.NK1.1 and wild-type NOD islets (data not shown). In both strains, the proportion of intact, peri-, and intrainfiltrated islets was identical.

The two strains showed absolutely identical susceptibility to disease acceleration by CY at 7 wk of age (data not shown). Thus the protection afforded by the B6 alleles from distal chromosome 6 is sex independent and does not seem to affect the early stages of the disease such as islet infiltration or precocious effectors still under control. B6 genes rather seem to regulate the late developments of autoimmune diabetes, at a time when effector T cells are probably expanding and accumulating around or within the islets.

Discussion

As already observed in a previous work on the phenotyping of NKT cells in Vα14Jα281-transgenic NOD mice (16), NK1.1 expression is not inhibited in the NOD context. Here we confirm that the marker is spontaneously expressed on thymic NKT cells whose ontogeny has not been transgenically enforced by the canonical α-chain rearrangement. Furthermore, we show that NK1.1 expression coincides with the subset of mature thymocytes that overexpresses Vβ8, that is predominantly CD44+, and that has an intermediate display of TCR. In the periphery, too, the T cells that stain with PK136 mAb respond to the definition of NKT cells: preponderant usage of Vβ8, CD44 positivity, and intermediate TCR display. We know also that the subset contains both CD4+ and double-negative T cells (data not shown). The numerical deficit in thymic NKT cells reported by the group of Baxter and our own group (11, 12), and hypothesized as being one of the defects responsible for deleterious autoimmune in NOD mice, is confirmed with the NK1.1 marker and can also be observed within the small population of splenic NKT cells.

Spleen phenotyping shows in addition that the PK136 mAb properly identifies the majority of NOD NK cells that coexpress DX5. Thus, the NOD.NK1.1 congenic mouse fulfills one of its initial purposes, namely to provide an experimental tag for NK and NKT cell subsets. These latter populations will be more easily followed in inflamed tissues, more easily deleted in vivo by treatment with PK136, and more easily sorted for functional studies or adoptive transfers. Yet, if NK1.1 labels practically all NK cells, it does not do so for NKT cells. NKT cells are heterogeneous in many respects (39), including CD4/CD8 double-negative surface expression (40) and NK1.1 expression (41). It is thus so far not clear whether NK1.1 positivity defines a distinct lineage as suggested by differences in Vα repertoire and CD1d expression, or simply reflects a stage of maturation or of activability in the life of an NKT cell, as suggested by the lability of the marker upon in

FIGURE 3. Conventional NK cells in the spleen. Total splenocytes depleted of red cells, and triple stained with anti-CD3-APC to gate out T cells, PK136-FITC, and DX5-PE, is shown.
FIGURE 5. NK and NKT cell performances in NOD. NK1.1 vs NOD wild-type mice. A, NK cytotoxicity measured in LU2/1 × 10^6 effector cells. Each point represents an individual spleen from a mouse boosted 16 h before the assay with poly(I: C). wt, Wild type. B, NKT-mediated release of IL-4, 2 h after challenge with 4 μg α-GC. Each point represents the serum of an individual mouse. C, NKT-mediated release of IFN-γ. Same conditions of challenge as above. Each point represents the serum of an individual mouse.

vitro culture contrasting with the stability of NK1.1 on conventional NK cells (42). In any event, the practical consequence is that the deletion of CD3ε NK1.1+ cells does not ensure total elimination of NKT cells, and, reciprocally, the infusion of CD3ε NK1.1+ cells may only partially reflect all the facets of NKT cell physiology. There is a similar ambiguity with regard to the DX5 marker, which labels only a fraction of NKT cells. We are presently examining this issue in the CD4ε NKT subset of I-Aε-old mice (43). Preliminary data suggest that NK1.1+ cells do more readily release IFN-γ following α-GC boost than DX5− or DX5+/PK136− cells (C.C., personal data).

The finding of augmented resistance to disease in congenic NOD.NK1.1 mice, both homo- and heterozygous for B6 genes, underlines once more the presence of susceptibility genes contributed by the NOD alleles. The distal portion of chromosome 6 has repeatedly emerged as a region of influence upon type 1 diabetes, in several independent outcross-backcrosses or outcross F3 crosses, with foreign mouse strains (44–48) or PWK subspecies (49). If all the incriminated intervals encircle the NKC or are compatible with it, within the limits of precision of the method, it does not mean that all these crosses identify the same genes. For instance in the outcrosses with NON and PKW mice, the foreign alleles increase susceptibility rather than afford protection and, as far as PKW is concerned, can modify CY-induced diabetes. Our observations are different, and unless one assumes some complex epistatic effects the simplest explanation is that the B6 genes introgressed in the NOD.NK1.1 congenic strain are not the same as those revealed in the PKW or NON outcross. More relevant to our present data are the two crosses reported by Todd and colleagues (44, 45) and the one by Penha-Gonçalves et al. (47), which attribute disease resistance to the foreign alleles—B10 and B6, respectively—and show maximal linkage scores around the Prp gene cluster (D6 Mit 13), which is a major physical anchor for NKC, at 0.3 cM of Cmv1 and 0.5 cM of the Ly49 cluster (26). Todd et al. were unable at the time of their study to associate Idd6 to putative candidate gene(s) or to a partial phenotype. Penha-Gonçalves, in contrast, associated the locus with thymocyte resistance to steroid apoptosis, a trait that could explain the emergence of autoimmune by allowing more self-reactive clones to escape negative selection. Again, no candidate gene could be proposed at that time, but now it is clear that the presence 5–10 cM upstream of Tnfr1, coding for the p55 TNFR1, might have been a good candidate. Tnfr1 comaps with D6 Mit 254, our most upstream marker on the congenic NOD.NK1.1. In view of the immense impact of TNF and TNFRs on NOD disease (50–52), it is obviously an important candidate that will be necessarily considered in our further backcrosses aimed at reducing the B6 genetic interval upstream of the NKC.

As a candidate for Idd6, the NKC is certainly an attractive gene cluster providing a link between innate immunity and type 1 diabetes. The facts that the NKC is at the center of our introgressed region, that both NK and NKT cell responses are modified by the introgression, and that, simultaneously, disease incidence is reduced constitute a good set of evidence in support of this hypothesis, even if time- and labor-consuming breeding will be necessary to further restrict the genetic interval down to the NKC itself.

An inherited defect in the NKC could explain the multiple dysfunctions of innate immunity affecting the NOD strain. We have recently shown that NKT, NK, and B cells constitute a functional network that is rapidly set into motion following the initial activation of NKT cells (53). NKT cells also promptly transactivate dendritic cells (54). Thus a defect originally located on NKT or dendritic cells do more...
NKT cell performances that have been identified in the present study.

Finally, how relevant are those findings to human type 1 diabetes? Both the clustering of human NK genes in a unique complex (28) and the conservation of an NKT cell subset (58) support a possible generalization from mouse to human. So far, no susceptibility gene has been identified in the syntenic region carrying the NKC, on chromosome 12p (59), but NK defects have been observed and associated to a genetic origin in diabetic patients (60, 61) and NKT cells have been shown to be deficient in identical twins discordant for type 1 diabetes (14, 62). It is thus an area worth exploration with the hope that the identification of NK or NKT cell-related genetic differences among individuals may have an important impact on various medical domains including infectious immunity, antitumor immunity, and autoimmune diseases.

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