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*J Immunol* 2010; 184:2272-2280; Prepublished online 3 February 2010;
doi: 10.4049/jimmunol.0804358
http://www.jimmunol.org/content/184/5/2272

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/02/01/jimmunol.0804358.DC1

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Distinct Phenotype and Function of NK Cells in the Pancreas of Nonobese Diabetic Mice

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Little is known about target organ-infiltrating NK cells in type 1 diabetes and other autoimmune diseases. In this study, we identified NK cells with a unique phenotype in the pancreas of NOD mice. Pancreatic NK cells, localized to the endocrine and exocrine parts, were present before T cells during disease development and did not require T cells for their infiltration. Furthermore, NK cells, or NK cell precursors, from the spleen could traffic to the pancreas, where they displayed the pancreatic phenotype. Pancreatic NK cells from other mouse strains shared phenotypic characteristics with pancreatic NK cells from NOD mice, but displayed less surface killer cell lectin-like receptor G1, a marker for mature NK cells that have undergone proliferation, and also did not proliferate to the same extent.

A subset of NOD mouse pancreatic NK cells produced IFN-γ spontaneously, suggesting ongoing effector responses. However, most NOD mouse pancreatic NK cells were hyporesponsive compared with spleen NK cells, as reflected by diminished cytokine secretion and a lower capacity to degranulate. Interestingly, such hyporesponsiveness was not seen in pancreatic NK cells from the non-autoimmune strain C57BL/6, suggesting that this feature is not a general property of pancreatic NK cells. Based on our data, we propose that NK cells are sentinel cells in a normal pancreas. We further speculate that during inflammation, pancreatic NK cells initially mediate proinflammatory effector functions, potentially contributing to organ-specific autoimmunity, but later become hyporesponsive because of exhaustion or regulation. The Journal of Immunology, 2010, 184: 2272–2280.

NK cells are part of the innate immune system and are involved in the elimination of viruses and tumors and the rejection of bone marrow transplants. The possible role for NK cells in autoimmune diseases is less studied. NK cells are both cytotoxic cells and producers of many types of cytokines (1). Thus, NK cells could contribute to organ-specific autoimmunity both by direct destruction of tissue in the target organ (2–4) and by providing an early source of cytokines affecting the adaptive components of the autoimmune response (1, 5, 6).

NOD mice develop the organ-specific autoimmune disease type 1 diabetes as a result of defective immune regulation and destruction of the pancreatic β cells (7, 8). We and others have previously shown a broadly impaired function of the NK cells from the spleen of NOD mice (9, 10), which may be reversible under conditions of NK cell activation by cytokines (10). Studies on the role of NK cells in murine type 1 diabetes have given conflicting results. For example, NK cells were responsible for protection against diabetes after treatment of NOD mice with CFA (11). In contrast, NK cells accelerated disease in BDC2.5/NOD mice given either cyclophosphamide or anti–CTLA-4 Abs (12), and in transgenic NOD mice expressing IFN-β in the β cells (13). Conflicting results on the role of NK cells have been noted also in other autoimmune models (14–17).

To better understand the divergent results on NK cells in diabetes, a more detailed characterization of the phenotype and function of NOD mouse NK cells will be necessary. Growing evidence supports the existence and importance of particular tissue niches for NK cells in vivo, and functionally distinct NK subsets are being defined and attributed to certain body compartments (18–23). It is therefore important to investigate the phenotypic and functional properties of NK cells in the circulation and at the site of pathogenesis—in the autoimmune target organ itself, the pancreas. Therefore, the aim of this study was to explore the properties of pancreatic NK cells in the NOD mouse model for type 1 diabetes. To do this, we developed a protocol that enabled us to retrieve and purify NK cells from the pancreas, a task that poses particular difficulties because of the risk of contamination from the blood or the closely located pancreatic lymph nodes (PLNs) (24). On the basis of our results, we discuss the possible role of organ-infiltrating NK cells as sentinel cells under healthy conditions and as immune regulators in autoimmune disease development.

Materials and Methods

Mice

NOD/Lt (denoted NOD throughout this study) mice, NOD mice lacking the rag-2 gene needed for generation of T and B cells (NOD RAG-2−/−; denoted NOD RAG) or NOD mice lacking both the rag-2 gene and the il-2 receptor γ-chain (Il2rg−/−; denoted Cyt) gene needed for generation of NK cells (NOD RAG-2−/−Il2rg−/−; denoted NOD RAG Cyt), and C57BL/6 (B6), BALB/c and C3H mice were kept and bred at the animal facility at the Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden. Because NOD females have a higher diabetes incidence than males (80–90% in our cohort), only female mice were used, except one male recipient in one transfer experiment and two male diabetic NOD mice in the BrdU incorporation experiments. All experiments were performed according to animal ethics guidelines and approvals.
Dissection and lymphocyte preparation for flow cytometry

The mice were perfused via the ascending aorta with PBS for 5 min, to minimize contamination by leukocytes from the blood. The pancreas was dissected from the PLNs and cut into pieces. Pancreases and PLN were digested into single-cell suspensions by incubation with RPMI 1640 containing 4 mg/10 ml collagenase P (Roche, Indianapolis, IN) for 30 min at 37°C, and then washed with 14 ml RPMI 1640 supplemented with 10% FCS. Single-cell suspension of spleen cells was prepared by mechanical homogenization, followed by depletion of erythrocytes using 0.83% NH₄Cl or dH₂O. We verified that the expression pattern of the markers on spleen NK cells did not notably change after collagenase treatment (data not shown). For functional experiments, collagenase was used to prepare lymphocytes from all organs.

Reagents and methods for flow cytometry

For flow cytometry, we used Abs to BrdU, CD3 (145-2C11), CD25 (7D4), CD27 (LG.3A1D), CD62L (MEL-14), CD69 (HL2F3), CD107a (ID48), CD127 (A10), eBioscience, San Diego, CA), CD49b (DX5), GM-CSF (MII-22E9; eBioscience), killer cell lectin-like receptor G1 (KLRG1) (MAFA/2F1), IFN-γ, IL-2Rβ (SH4), Ly49D (4E5), Ly49G2 (LGL-1/4D11), Mac-1 (M1/70), NKG2A (20D5; AbD, Serotec, Oxford, U.K.), NKG2D (CX5; eBioscience), NKp46/NCR1 (R&D Systems, Minneapolis, MN), programmed death-1 (PD-1) (J43; eBioscience). Unless stated differently, Abs were obtained from BD Biosciences (San Diego, CA). As a marker for viability, 5 μl 7AAD (BD Biosciences) was added per 200 μl sample. Cells were stained with Abs for 30–40 min at 4°C and analyzed on a Becton Dickinson FACS calibur flow cytometer using CellQuest software and a FACS aria flow cytometer using FACS Diva software (BD Biosciences, San Diego, CA).

Functional measurement by CD107a expression and intracellular IFN-γ and GM-CSF

The lymphocyte fraction was isolated using a density gradient separation (Lympholyte-M; Cederlane Laboratories, Ontario, CA); 0.2–0.5 million lymphocytes were incubated at 37°C with anti-CD107a Ab and monensin for 4 h, either directly ex vivo, in the presence of 50 μg/ml PMA (Sigma P-8139, Sigma-Aldrich, St. Louis, MO) and 1 μg/ml ionomycin (Sigma 10634), or only with NK cells in the spleen and the PLN. For flow cytometry, we used Abs to BrdU, CD3 (145-2C11), CD25 (7D4), CD49b (DX5), GM-CSF (MII-22E9; eBioscience), killer cell lectin-like receptor G1 (KLRG1) (MAFA/2F1), IFN-γ, IL-2Rβ (SH4), Ly49D (4E5), Ly49G2 (LGL-1/4D11), Mac-1 (M1/70), NKG2A (20D5; AbD, Serotec, Oxford, U.K.), NKG2D (CX5; eBioscience), NKp46/NCR1 (R&D Systems, Minneapolis, MN), programmed death-1 (PD-1) (J43; eBioscience). Unless stated differently, Abs were obtained from BD Biosciences (San Diego, CA). As a marker for viability, 5 μl 7AAD (BD Biosciences) was added per 200 μl sample. Cells were stained with Abs for 30–40 min at 4°C and analyzed on a Becton Dickinson FACS calibur flow cytometer using CellQuest software and a FACSpita flow cytometer using FACS Diva software (BD Biosciences, San Diego, CA).

Immunofluorescence

For immunofluorescence, five 4–5-wk-old and nine 8–10-wk-old female NOD SCID/e mice were anesthetized and perfused transcardially with 10 ml Tyrode’s Ca²⁺-free solution (37°C) containing 10% sucrose. The tissue was frozen, cryostat-sectioned (Microm, Wiesbaden, Germany) to 14 μm thickness, and thaw-mounted onto glass slides. Conventional immunofluorescence was used to visualize pancreatic islets using polyclonal antisera raised in guinea pig against insulin (1:1000 to 1:4000), from Dr. S. Efendic) and T cells using an Ab raised in Syrian hamster against CD3 (1:100; BD Biosciences). The tyramide signal amplification method (Perkin–Elmer, Waltham, MA) was used to visualize tyramide enhanced peroxidase (TMB) using a polyclonal Ab raised in goat against mouse NKp46 (1:4000; R&D Systems), as previously described (26). The following secondary Abs were used: goat anti-rabbit IgG HRP for NKp46, goat anti guinea pig IgG Alexa 594 or Alexa 633 for insulin and goat anti-Syrian hamster IgG-AMCA (Jackson ImmunoResearch, West Grove, PA), or Alexa 546 for CD3. Unless stated differently, secondary Abs were obtained from Invitrogen (Carlsbad, CA).

Quantifications of longitudinal pancreatic sections from five 4–5-wk-old and five 9–10-wk-old mice were performed on a Zeiss Axios Imager M1 Fluorescence microscope (Carl Zeiss, Jena, Germany). The localization of NK cells in the pancreas was analyzed on three pancreatic sections from each mouse, sampled throughout the tissue at regular intervals of a distance of ~150 μm. NKp46-immunopositive cells were classified for location as either inside an islet, directly peri-insular, or outside of the islets, in the exocrine tissue. The number of islets with NK cells localize both to the endocrine and exocrine pancreas and accumulate before T cells in the islet infiltrate

We developed an isolation protocol for pancreatic NK cells, including perfusion of mice with PBS and dissection under microscopy, to assure minimal contamination from blood or PLN NK cells. To visualize NK cells using flow cytometry, Abs to the NK cell markers NKp46 and IL-2Rβ were combined with a marker for dead cells (viaprobe, 7AAD) and T cells (CD3). 7AAD CD3 IL-2Rβ NKp46 NK cells were easily detected in the pancreas of prediabetic NOD mice aged 8–10 wk (Fig. 1A).

Proliferation measured by BrdU incorporation

Mice were injected with 200 μl BrdU i.p. (BD Biosciences) in the provided concentration every 12 h over 2 d. Lymphocytes were prepared as described above and stained with Abs for surface markers, then permeabilized, treated with DNase, and stained with anti-BrdU Ab from the BD Pharmingen BrdU Flow Kit according to the manufacturer’s protocol.

Transfer of spleen cells

Spleen cells from NOD mice aged 9–10 wk were prepared sterile as described above, and 20–60 million cells were injected i.v. into 7–14 wk-old NOD RAG Cγ mouse. Two weeks after transfer, lymphocytes were prepared for flow cytometric analysis as described above.

Statistical analysis

Unless stated otherwise, data are presented as mean ± SD. Statistical analyses of the data were performed using Student t test or one way ANOVA with Tukey’s multiple comparison test, as appropriate. *p < 0.05; **p < 0.01; ***p < 0.001.

Results

NK cells localize both to the endocrine and exocrine pancreas and accumulate before T cells in the islet infiltrate

We investigated the relationship between T cells and NK cells in the progression of disease, we next analyzed the number of islets with NK cell and/or T cell infiltrate as defined with an anti CD3 Ab. In 4–5 wk-old mice, almost all NK cells were located in the exocrine pancreas, whereas the fraction of NK cells in the endocrine pancreas had increased at 9–10 wk-old mice (Fig. 1C). Further supporting a progressive development of the cellular infiltrate over time, we found that younger mice had fewer NK cells in each pancreas section compared with the 9–10 wk-old mice (Supplemental Fig. 1A). In addition, the number of NK cells per infiltrated islet (insulitis or peri-insulitis) increased significantly between 4–5 and 9–10 wk-old mice (Supplemental Fig. 1B). To investigate the relationship between T cells and NK cells in the endocrine disease, we next analyzed the number of islets with NK cell and/or T cell infiltrate as defined with an anti CD3 Ab. In 4–5 wk-old mice, infiltrating NK cells could be seen in a fraction of islets, but there were no detectable T cells (Fig. 1D, 1F). In the prediabetic 9–10 wk-old mice, however, islets either had a mixed NK/T cell infiltrate or contained only NK cells (Fig. 1E, 1G). We consistently failed to observe any islets containing T cells but lacking NK cells. We conclude that NK cells infiltrated the islets of Langerhans at an early age in the NOD mouse, and this process does not require a previously established T cell infiltrate.

Pancreatic NK cells show a distinct phenotype compared with NK cells in the spleen and the PLN

After having established the presence of NK cells in the pancreas of NOD mice, we characterized them further using flow cytometry. Compared to spleen NK cells, pancreatic NK cells had a higher expression of the activation markers CD69 and CD25 and showed downregulation of the lymph node homing marker CD62L (Fig. 2A, 2B). Furthermore, more pancreatic NK cells than spleen NK cells expressed KLRG1, a receptor that is upregulated on NK cells after activation and proliferation (e.g., in MCMV infection) (27–29).
Pancreatic NK cells also had a moderately increased expression of the receptor PD-1. The activating receptor NKG2D was either uniformly expressed or downregulated on a fraction of pancreatic NK cells relative to spleen NK cells (Fig. 2B). Pancreatic and splenic NK cells had a similar low expression of the α subunit of the IL-7 receptor (CD127), a marker associated with NK cells in the thymus and in lymph nodes (22). A large fraction of NK cells in the PLN expressed CD127. Like spleen NK cells, the PLN contained fewer KLRG1-positive NK cells. For other markers, such as CD69, PLN NK cells had an expression level intermediate relative to that in the pancreas and the spleen. PD-1 and CD62L were more strongly expressed on PLN NK cells.

The expression pattern of CD27 and Mac-1 define different stages of NK cell maturation associated with distinct functions (20). Spleen and pancreatic NK cells had a similar expression pattern of CD27 and Mac-1, corresponding to a more mature phenotype, whereas lymph node NK cells had a more immature phenotype (Mac-1low) compared with splenic NK cells, as reported previously (20) (Fig. 2C). We conclude that pancreatic NK cells appear fully mature, but display a phenotype distinct from that in the spleen and the PLN, with a profile consistent with either activation or exhaustion.

**Increased proliferation of pancreatic NK cells compared with those in the spleen and PLN, and compared with T cells**

One of the receptors that were overexpressed on pancreatic NK cells was KLRG1, a marker associated with NK cells that have undergone proliferation (27–29). Correspondingly, assessment of BrdU incorporation in vivo showed a higher proliferation rate in pancreatic NK cells compared with spleen and PLN NK cells (Fig. 3A), which indicates that pancreatic NK cells expand at this site. BrdU incorporation was even higher in pancreatic NK cells than in pancreatic T cells (Fig. 3B). Surprisingly, there was no obvious correlation between the expression of KLRG1 and CD69 on pancreatic NK cells and the acquisition of BrdU; both markers were similarly expressed on BrdU-positive and negative NK cells (Fig. 3C). The expression of CD69 was, however, strongly associated with BrdU incorporation in T cells, suggesting a link between these two phenotypes, specifically in T cells. In contrast to KLRG1 and CD69, the expression of PD-1 was higher on BrdU-positive cells, both in T and NK cells, suggesting that PD-1 was expressed on cells that had undergone proliferation during the last 2 d (Fig. 3C).

**Activated and proliferating NK cells are present in the early prediabetic infiltrate**

The magnitude of the pancreatic NK cell infiltrate, as well as its distribution and relation to T cells differed between 4–5 and 9–10-wk-old prediabetic NOD mice (Fig. 1B–G), suggesting a critical transition phase in the autoimmune pathogenesis between these time points. To investigate whether this transition was associated with NK cell activation, we performed a flow cytometry-based kinetic analysis of CD69 expression and BrdU incorporation of NK cells in NOD mice 2.5–9 wk old and in diabetic mice. CD69 was absent on both spleen and pancreatic NK cells at 2.5 wk of age. From 4 wk onward, CD69 was upregulated on NK cells from the pancreas but not from the spleen (Fig. 4A), implying activation of NK cells in the pancreas long before disease onset and before the presence of T cells in the islets (Fig. 1). A 2-d pulse with BrdU in mice of different ages resulted in a high percentage of BrdU-positive NK cells in the pancreas of 2.5-wk-old mice, suggesting that activation at 4 wk of age is preceded by a high rate of proliferation. The BrdU incorporation was less in the pancreas of 6–10-wk old mice and in the diabetic mice (Fig. 4B), but was still higher than in the spleen (data not shown).
Pancreatic NK cells from NOD mice are hyporesponsive upon stimulation, but display higher spontaneous ex vivo IFN-γ production

NK cells from the spleen of NOD mice and the blood of diabetic humans are known to be impaired in function (3, 10, 30). Given their phenotype and high proliferation rate, we speculated that pancreatic NK cells would instead display an enhanced responsiveness, perhaps reflecting a pathogenic role in diabetes pathogenesis. In contrast, we found that pancreatic NK cells from NOD mice showed an even poorer capacity to produce IFN-γ and degranulate, as measured by CD107a expression (31), than spleen NK cells. This was seen when they were stimulated using Abs against specific activating receptors or generally using PMA and ionomycin as stimulating agents (Fig. 5A–F). The response was reflected by a lower number of responding NK cells, as well as lower amounts of IFN-γ and CD107a per NK cell, as shown by the lower MFI levels (Fig. 5A–B). Interestingly, despite the general picture of poor responsiveness, we noticed that a fraction of nonstimulated ex vivo pancreatic NK cells stained positive for CD107a and intracellular IFN-γ compared with spleen NK cells (Fig. 5C–F). This was particularly evident for IFN-γ (Fig. 5G).

The lower response in these effector functions could reflect hyporesponsiveness, but could also be a consequence of NK cell subset specialization. The notion of hyporesponsiveness would be strengthened if the functions could be reverted after NK cell activation. To test this, we stimulated lymphocytes from the pancreas or spleen with IL-15, which promotes proliferation of NK cells, and IL-18, which is a potent inducer of IFN-γ production. Overnight stimulation with IL-15 and IL-18 restored the capacity of NOD mouse pancreatic NK cells to produce IFN-γ (Fig. 5H), demonstrating that the lower IFN-γ response was indeed consistent with hyporesponsiveness. CD107a was not induced even in control spleen cells by these cytokines (data not shown). GM-CSF is another NK cell cytokine that has been implicated in the pathogenesis...
of diabetes in NOD mice (32). GM-CSF was also produced in lower levels by the NOD mouse pancreatic NK cells compared with spleen NK cells after PMA and ionomycin stimulation (Supplemental Fig. 2A). Stimulation with IL-15 and IL-18 was rather inefficient in inducing GM-CSF in NOD mouse NK cells. However, we noted that the induction was higher in spleen NK cells than in pancreatic NK cells (Supplemental Fig. 2B), suggesting that the low production of GM-CSF by pancreatic NK cells was not restored by IL-15 and IL-18. Our data indicate that pancreatic NK cells are hyporesponsive relative to spleen NK cells regarding some effector functions, but leave open the possibility that other parts of their effector profile results from subset specialization rather than anergy.

Intrigued by the distinct functional properties of the NOD mouse pancreatic NK cells, we asked whether pancreatic NK cells from other mouse strains would show similar functional properties. To test this, we isolated NK cells from the pancreas of C57BL/6 (B6) mice, a strain with no known pancreas-associated pathology and with high resistance to autoimmunity. Interestingly, pancreatic NK cells from B6 mice were responding in a similar fashion as spleen NK cells in response to PMA and ionomycin, both in terms of IFN-γ production and degranulation (Fig. 5I).

Pancreatic NK cells in mouse strains not prone to diabetes development have a similar phenotype as in the NOD mouse but proliferate less

The fact that we could isolate pancreatic NK cells from normal B6 mice was surprising, and we decided to explore their characteristics further in B6 mice and in two other mouse strains, BALB/c and C3H, both lacking known pathology of the pancreas and differing in MHC genotype. The pancreases of all strains contained NK cells, in percentages equivalent or even increased of what we had observed in the NOD mouse (Fig. 6A). The phenotypes were largely similar, with two notable differences. First, the NOD strain contained more KLRG1-positive cells in the pancreas than in the
spleen, whereas the opposite relationship appeared to be the case in the other strains (Fig. 6 C). Second, the NK2D receptor was uniformly expressed on pancreas and spleen NK cells in all strains except in B6, in which the levels on pancreatic NK cells were lower in all mice (Fig. 6 B), and in NOD in which it was downregulated in some mice (Fig. 2). BrdU incorporation experiments showed a significantly higher ongoing proliferation of pancreatic NK cells from the NOD mouse compared with the other mouse strains (Fig. 6D), providing a distinctive feature of pancreatic NK cells from a strain with an autoimmune inflammation in the pancreas.

NK cell migration to the pancreas: origin and dependency on other lymphocytes

We next asked whether the entry of NK cells into the pancreas depended on a previous T cell infiltrate. Pancreases from NOD RAG mice, lacking T and B cells, contained NK cells (Fig. 7A), demonstrating that NK cell homing to the pancreas was T and B cell-independent. Pancreatic NK cells from NOD RAG mice expressed even higher levels of KLRG1 than NOD mice pancreatic NK cells, possibly reflecting a higher proliferation rate as a consequence of an environment without competing lymphocytes (Fig. 7A).

The differences in phenotype between spleen and pancreatic NK cells in NOD mice could be due to tissue-specific NK cell development pathways, or result from local factors at each tissue site conveying unique phenotypes on a generic NK cell lineage. If spleen NK cells would home to the pancreas after adoptive transfer and change their phenotype in the new tissue, then this would support the second scenario. Indeed, when spleen cells were transferred to NOD mice lacking T, B, and NK cells (NOD RAG Cy mice), six of nine transferred mice contained NK cells in the pancreas 2 wk after transfer (Fig. 7B, 7C). Pancreatic NK cells in the transferred NOD RAG Cy mice expressed higher levels of KLRG1, CD69, and PD-1 than spleen NK cells before transfer, supporting the hypothesis that the local microenvironment in the pancreas can regulate the NK cell phenotype (Fig. 7D). Particularly, KLRG1 levels on NK cells were upregulated also in the spleen of the recipient mice, probably as the result of homeostatic proliferation in the lymphopenic environment (28). Nevertheless, the expression levels of KLRG1, CD69, and PD-1 were all higher in the NK cells in the pancreas, suggesting additional stimulation at that site (Fig. 7D). Thus, NK cell infiltration into the pancreas does not require prior tissue destruction mediated by adaptive immunity, and the spleen contains NK cells or NK cell progenitors that can migrate to the pancreas and exhibit a KLRG1hi, CD69hi, and PD-1hi phenotype.
Discussion
Although many details of the complex pathophysiology of type 1 diabetes and other autoimmune disorders have been clarified, several aspects are still unknown, such as the role of NK cells. In this study we present, for the first time, a detailed characterization of the NK cells in the pancreas during disease progression in NOD mice.

NK cells infiltrated both the endocrine and exocrine parts of the NOD mouse pancreas already at an early age. In young mice, infiltration of NK cells was less prominent, and only few NK cells were localized within the islets of Langerhans or peri-insular. In 9–10-wk-old NOD mice, most islets had both NK cell and T cell infiltration. Interestingly, irrespective of age, we were never able to detect islets with only T cells and no NK cells. This finding suggests that NK cells localize to islets before T cells, inviting the speculation that T cell infiltration may be promoted by NK cells. A regulatory role for NK cells in islet inflammation could be mediated by the direct production of T cell recruiting or activating factors or in a more indirect way by means of conditioning the islet environment by producing factors toxic for β cells (e.g., TNF and IFN-α). An early T cell-independent homing of NK cells to the pancreas is also supported by our results showing the presence of NK cells in NOD RAG mice.

We found differences in the phenotypic profile of pancreatic and splenic NK cells. Pancreatic NK cells showed signs of activation, expressing high levels of CD69, low levels of CD62L and augmented levels of CD25 (33). The CD69 expression may be secondary to the general inflammation, as CD69 expression can be upregulated through the influence of IFN-α/β (34). The NKG2D receptor, which recognizes ligands expressed on stressed cells (35, 36), was sometimes downregulated on a large fraction of pancreatic NK cells. One of the ligands for NKG2D, Rae-1, is expressed in the NOD mouse pancreas, and its expression increases with age (2). It is possible that the NKG2D receptor is downregulated upon interactions with Rae-1 on β cells or on other cells in the infiltrate, similar to what has been described after interactions between NK cells and tumor cells (37). Alternatively, NKG2D downregulation could be a result of the induced expression of Rae-1 on the NK cells themselves, as has been suggested (38). It was of interest that NKG2D was consistently downregulated also in the pancreas of B6 mice. This finding shows that NKG2D downregulation in the pancreas is not always associated with diabetes development. However, a productive interaction between the NKG2D receptors with ligands in the NOD pancreas could still be of pathologic importance for diabetes in this strain.

The expression of PD-1 has mainly been studied in the context of T cells, where it has a role as inhibitory receptor (39). PD-1 was upregulated both on pancreatic NK cells and T cells and was associated with proliferation in both subsets. The ligand for PD-1, PD-L1, is also expressed on cells from inflamed islets from prediabetic NOD mice (40), and a polymorphism in the PD-1 gene has been linked to susceptibility of type 1 diabetes in humans (41). Interestingly, blocking of PD-1 or one of its ligands, PD-L1, accelerated diabetes in prediabetic NOD mice, usually attributed to PD-1 expression on T cells (40). Our data on PD-1 expression on pancreatic NK cells suggest the possible involvement of NK cells in this system.

KLRG1 is a receptor expressed on phenotypically mature NK cells and NK cells that have undergone homeostatic or infection-induced proliferation (27–29, 42, 43); it is also a molecular signature of CD8+ T cells exposed to repetitive and persistent Ag stimulation (44). We speculate that the high KLRG1 expression on pancreatic NK cells is a consequence of proliferation in response to specific stimulation or to more nonspecific cues delivered by a chronic inflammation. Several cytokines that are known activators of NK cells have been associated with inflammation in the pancreatic islets of NOD mice, such as IL-1β, IFN-γ, IL-15, IL-12, IL-18, and TNF (45). Upregulation of KLRG1 was indeed seen in parallel with higher proliferation rate of pancreatic NK cells in the NOD mouse, compared with NK cells from the spleen and the PLN, as well as pancreatic NK cells in the other investigated mouse strains. There was no direct correlation between KLRG1 and proliferation at the single cell level, indicating that they are not synchronously regulated. The kinetic differences, with high BrdU-incorporation in young NOD mice, still relatively high in 6–10-wk-old mice and lower in the diabetic mice, was interesting. We speculate that the high rate of proliferation in very young mice depends on homeostatic proliferation, whereas division at older ages results from an ongoing chronic inflammation.

A large fraction of NK cells in lymph nodes are thymus-derived and phenotypically characterized by their expression of CD127 (IL-7Rα) (22). These NK cells share some characteristics with pancreatic NK cells, such as lower Ly49 receptor expression (Supplemental Fig. 3), but differ in expression of CD127 compared with pancreatic NK cells. The expression of Ly49 receptors on pancreatic NK cells (Supplemental Fig. 3) is important in relation to a report, suggesting that NK cells in the synovial fluid from inflamed joints of rheumatoid arthritis patients primarily expressed CD94 and not killer immunoglobulin receptors, the human Ly49 counterparts (46). Another distinct NK cell subset resides in the uterus. These NK cells show a dense granularity in the cytoplasm (19), but are also poor in forming immune synapses and are not cytotoxic (47). Like NOD mouse pancreatic NK cells, uterine NK cells show profound functional deficiencies. Uterine NK cells fall in two distinct subpopulations in terms of phenotype, function, and tissue localization (23). The major subpopulation (the Nkp46+NK1.1⁺Dx5⁺ subset) shares some of the phenotypic particularities of pancreatic NK cells, such as high expression levels of CD69 and KLRG1. Distinct differences have been described between hematopoietic organs and the lung in the NK cell expression of the maturation markers CD27 and Mac-1 (20). Our results did not reveal a large difference in the CD27/Mac-1 pattern between NK cells from the pancreas and those from the spleen, implying that pancreatic NK cells may be different compared with the cells from other solid organs.

The phenotypic changes are particularly interesting in light of the hyporesponsiveness we observed in pancreatic NK cells compared with spleen NK cells. Many of the markers with increased expression on pancreatic NK cells have been identified in exhausted CD8+ T cells, in particular PD-1, KLRG1, and CD69 (48). Although hypothetical, the presence of these markers on pancreatic NK cells may signify exhaustion of these cells, possibly as a result of overstimulation by chronic exposure to ligands (49) and inflammatory mediators. To reconcile this idea with our finding that a fraction of NK cells in the pancreas showed spontaneous IFN-γ producing capacity, one may envision a scenario in which newly arrived NK cells become activated and start to produce IFN-γ. During this phase, they may participate in disease pathogenesis. However, subsequent to chronic stimulation, the activating input may become too strong, leading to exhaustion and hyporesponsiveness in the NK cell population.

Another possibility is that the hyporesponsiveness of NOD mouse pancreatic NK cells is not caused by exhaustion, but by downregulation of effector functions by other immune cells (50, 51). In this scenario, NK cells are actively prevented from mediating effector functions, e.g., by pancreas-residing regulatory T cells (50). If this regulation fails, NK cells would regain function locally and contribute to disease. Further work will be needed to distinguish these mutually nonexclusive models.
Regardless of the mechanisms for poor IFN-γ production, it was of interest that the level of intracellular IFN-γ in NOD mouse pancreatic NK cells was augmented after overnight stimulation with cytokines. Hence, NOD pancreatic NK cells were capable of responding strongly with some effector functions, supporting their potential immunoregulatory role in inflammatory insulitis (45). The observation that IL-15 plus IL-18 stimulation did not increase GM-CSF production in the same NK cells adds further complexity to the biology of NK cell hyporesponsiveness in the pancreas. This finding suggests the possibility that pancreatic NK cells represents a distinct subpopulation of NK cells with a unique phenotypic profile, which altogether lack the possibility of producing GM-CSF in large amounts.

NK cells were also found in the pancreas of other mouse strains not prone to develop diabetes. However, the properties of these NK cells differed from NOD mouse pancreatic NK cells. Most importantly, B6 pancreatic NK cells were not hyporesponsive in response to PMA and ionomycin to the same extent as pancreatic NK cells from the NOD mouse. NK cells from the pancreas of NOD mice also had a higher expression of KLRG1 and a higher BrdU incorporation than any of the other investigated mouse strains. Collectively, these findings suggest that poor effector functions in the NK cells are not caused by the pancreatic environment per se, but may be caused by active mechanisms such as the ones discussed previously, unique to the pancreatic environment in the NOD mouse. Furthermore, the presence of NK cells in the non-diabetes–prone mouse strains suggests that NK cell trafficking to the pancreas and possibly other organs represent a general route of NK cell migration, not restricted to mice with the predisposition to develop autoimmune diabetes. Pancreatic NK cells would thus act as sentinel cells, surveying the organ for signs of damage or cellular stress. Such a system for NK cells is highly speculative, but not unlikely given their expression of receptors for danger signals such as stress-induced molecules known to be expressed on pancreatic tissue (2). The finding that a large portion of the NK cells in the NOD mouse pancreas is located outside of the islets of Langerhans also supports the notion of a complex role for NK cells in the pancreas. An important future task will be to further explore such a potential sentinel role for NK cells in local immune surveillance, as well as elucidate the differences between organ-specific immune infiltration in the healthy state and in autoimmunity. New knowledge in this area will be of great importance, because prevention of homing or local proliferation of pathogenic NK cells could offer a potential therapeutic strategy.

Acknowledgments
We thank the staff in the animal facility at the Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet for help with experiments and caring for the mice and Björn Öufelt for help with image preparations.

Disclosures
The authors have no financial conflicts of interest.

References


