Original Article

Altered Natural Killer Cells in Type 1 Diabetic Patients

Melanie Rodacki,[1] Britta Svoren,[2] Vincent Butty,[1] Whitney Besse,[1] Lori Laffel,[2] Christophe Benoist,[3] and Diane Mathis[3]

Evidence from animal models suggests that natural killer (NK) cells can be important players in the development of type 1 diabetes, although data in humans are still sparse. We studied the frequency and activation state of blood NK cells at different stages of human type 1 diabetes, and whether genetic or phenotypic NK cell peculiarities could be associated with an early onset of diabetes. The onset period is marked by a slight reduction in blood NK cells, but these are unusually activated in some patients (γ-interferon expression). This activation status does not correlate, however, with a particularly young age at onset. In contrast, NK cells in patients with long-standing type 1 diabetes had a markedly lower expression of p30/p46 NK-activating receptor molecules compared with those of control subjects. A slightly decreased expression of NKG2D in all type 1 diabetic patients relative to control subjects was observed, independent of the duration of disease, parallel to prior observations in the NOD mouse. Finally, type 1 diabetic patients had an increased frequency of KIR gene haplotypes that include the activating KIR2DS3 gene, with a genetic interaction between the KIR and HLA complexes. The reduced activation of NK cells in individuals with long-standing type 1 diabetes would seem to be a consequence rather than a cause, but other peculiarities may relate to type 1 diabetes pathogenesis. *Diabetes* 56: 177–185, 2007

Type 1 diabetes follows an immunologically mediated destruction of the pancreatic β-cells (1). Autoreactive T-cells have a critical role in this process (2), but the precise mechanisms involved in the appearance of insulitis and its progression to a destructive lesion remain elusive. Given the multiplicity of possible interactions between the cells in the immune system, one can assume that many of its components could influence this immune attack, either directly or indirectly. The presence of multiple controlling factors seems reasonable when we consider how heterogeneous disease progression can be, ranging from a rapid destruction with a very early onset of type 1 diabetes to a slow process with a late and often insidious clinical presentation (3).

Natural killer (NK) cells are potentially involved in this complex process, given their ability to kill target cells and to interact with antigen-presenting cells and T-cells (4–6). Indeed, NK cells can lyse islet cells in vitro (7,8). Because NK cells are a major source of γ-interferon, a key proinflammatory cytokine, a plausible hypothesis is that their pathophysiological impact on diabetes is to modulate the aggressiveness of the immune attack and the rate of progression from insulitis to overt diabetes. On the other hand, there have been conflicting reports on their direct role in vivo in animal models. A pathogenic role of NK cells in islet destruction was initially suggested from antibody depletion studies (9,10), but the specificity of the reagents was later questioned (11). A pathogenic role for these cells has more recently been supported in several contexts: in a model of coxsackievirus-induced autoimmunity, where NK cells were important for disease progression (12), and in a T-cell receptor transgenic model, where a higher frequency of NK cells was observed in conditions of aggressive insulitis (resulting from genetic variation or costimulatory blockade), a direct pathogenic role being supported by antibody blockade (13). On the other hand, it has been suggested that NK cells may play a role in the protection afforded by complete Freund's adjuvant in NOD mice (14).

In addition, there are indications that the NOD mouse has an unusual genetic composition in the genomic regions that condition NK activity. Ogasawara et al. (15) reported that the NKG2D receptor is underexpressed in NOD NK cells because of the overexpression of its Rae-1 ligand, and many of the NK inhibitory receptors are differentially expressed in NOD relative to control C57BL/6 mice (13). Indeed, these genetic particularities contribute to an overall low activity of NK cells in NOD mice (16,17). This defect might, at first, appear in contradiction with a positive role of NK cells in promoting rapid and aggressive diabetes until one realizes that NOD mice have been selected over generations for a susceptibility to breakdown of T-cell tolerance, but at the same time, for a slow progression to diabetes, compatible with reproduction and continuity of the line. Overall, a delicate balance in the frequency and function of NK cells may be necessary to avoid type 1 diabetes, and possibly other autoimmune conditions (6).

Data on NK cells in human type 1 diabetes remain sparse. In the 1980s, a few groups investigated the number and activity of NK cells in individuals with type 1 diabetes, often with conflicting results (18–23). However, small

From the [1]Joslin Diabetes Center, Boston, Massachusetts; the [2]Division of Endocrinology, Children's Hospital Boston, Boston, Massachusetts; and the [3]Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts.

Address correspondence and reprint requests to Christophe Benoist or Diane Mathis, Harvard Medical School, One Joslin Place, Boston, MA 02215. E-mail : cbdm@joslin.harvard.edu.

Received for publication 12 April 2006 and accepted in revised form 12 October 2006.

Additional information can be found in an online appendix at http://diabetes.diabetesjournals.org.

DKA, diabetic ketoacidosis; DPT-1, Diabetes Prevention Trial of Type 1 Diabetes; IFN-γ, γ-interferon; MFI, mean fluorescence intensity; NCR, natural killer–activating receptor; NK, natural killer; SNP, single-nucleotide polymorphism; TNF-α, tumor necrosis factor-α.

DOI: 10.2337/db06-0493

TABLE 1
Characteristics of the study group

	Recent-onset group	Long-standing diabetes group	Control group
n	50	83	64
Mean age (years)	9.43 ± 4.60	17.77 ± 8.50	15.81 ± 8.90
Mean age at onset (years)	9.43 ± 4.60	7.61 ± 6.40	N/A
Mean duration of diabetes (years)	N/A	10.05 ± 5.90	N/A
F/M (*n*)	25/25	41/42	38/26
Caucasians (%)	92	91.6	81.3
Mean A1C	10.42 ± 1.90	8.34 ± 1.40	4.96 ± 0.43
Subjects with other autoimmune diseases (%)	0	21.6 (18/83)*	0
Subjects with diabetic nephropathy (%)	0	13.25 (11/83)†	N/A
Overweight subjects (%)‡	12.2	43.7	29.7
Recent diabetic ketoacidosis	26 (13/50)	0	N/A

Data are the means ± SE or % (M/F), unless otherwise indicated. *Hashimoto's thyroiditis (8 cases), celiac disease (4), juvenile rheumatoid arthritis (3), vitiligo (3), alopecia (2), and hypoparathyroidism (1); †micro- and macroalbuminuria in 8 and 3 case subjects, respectively (renal function intact in all); ‡defined as BMI >85th percentile for individuals younger than 20 years and >27 kg/m^2 ≥20 years of age.

study groups were often involved, the antibodies used to identify NK cells were limited and lacked specificity, and NK cell function was assessed by cytotoxicity on tumor targets, an incomplete reflection of the entire NK pool. Some studies suggested a numeric deficiency of NK cells in the peripheral blood of patients with type 1 diabetes (18,21) or a functional abnormality (18,24). In some of these cases, there were permanent numeric and/or functional deficits (20,23), whereas in others, transitory abnormalities were associated with the stage of disease (18,24).

The aim of this study was to investigate possible abnormalities in the frequency or activation state of NK cells in the peripheral blood of patients with type 1 diabetes, especially around the onset of diabetes. Given the role of NK cells in determining the aggressiveness of insulitis in animal models, we hypothesized that NK cells might appear more activated around the time of diabetes onset, or that patients presenting with type 1 diabetes at a very young age might have NK cell characteristics different from those of patients diagnosed during adolescence.

RESEARCH DESIGN AND METHODS

A total of 197 individuals enrolled in the Diabetes in the Adolescents and the Very Young (DAVY) study were tested in this cross-sectional analysis. Diagnosis of type 1 diabetes was based on clinical presentation according to World Health Organization criteria. Informed consent was obtained from all subjects. The protocol was approved by the Joslin Diabetes Center and Children's Hospital Boston institutional review boards (CHS 02-15 and 04-09-125).

Flow cytometry. Immunostaining was performed in 100-μl aliquots of fresh peripheral blood collected in sodium citrate tubes with isotype-matched negative control monoclonal antibodies (reagents are listed in supplemental Table S1, which can be found in the online appendix [available at http://diabetes.diabetesjournals.org]). Samples were analyzed using a MoFlo with Summit 3.0 software (Cytomation). The cutoff for CD56 positivity was based on isotype plus streptavidin control staining. A minor population of CD3$^-$CD16$^-$ cells with very low CD56 staining intensity, also expressing NKp46/NKp30 and NKG2D, was identified in most cases and was included in the NK cell counts. The mean fluorescence intensities (MFIs) for markers was corrected for background (based on isotype control staining for NKp30/46 or unstained cells for NKG2D). All data analyses were performed in a blinded fashion.

RNA preparation, amplification, and labeling. Peripheral blood lymphocytes (2–4 × 10^6) were obtained by density gradient centrifugation in CPT tubes (Becton Dickinson), and NK cells were positively selected by magnetic sorting with anti-CD56–conjugated beads. The selected cells (2–4 × 10^5, >85% CD56$^+$) were lysed in 1 ml of TRIzol for RNA isolation, and 1–4 μg RNA was reverse-transcribed. γ-Interferon (IFN-γ), tumor necrosis factor-α (TNF-α), and perforin mRNA levels were measured (using β-actin as a standard) by quantitative real-time RT-PCR (probes listed in supplemental Table S2). $2^{\Delta\Delta CT}$ calculations were used to estimate relative expression levels.

DNA genotyping. Genotyping for single-nucleotide polymorphisms (SNPs) in type 1 diabetes susceptibility loci or KIR genes was performed by fluorogenic PCR (primers and probes in supplemental Table S2).

Statistical analysis. We used χ^2 and Mann-Whitney *U* tests to compare variables between groups. Spearman's correlation and Kruskal-Wallis tests were used for association (all two-tailed, $\alpha = 0.05$). Linkage disequilibrium among alleles was assessed by Mattiuz's Δ formula, which is based on 2 × 2 tables of the presence/absence counts for the alleles of interest (25).

RESULTS

Characteristics of the study groups. The entire study population consisted 197 individuals: 50 with recent-onset type 1 diabetes, 83 with long-standing type 1 diabetes, and 64 control subjects apparently free of autoimmune or metabolic diseases (control group). Patients in the recent-onset group were tested within the 1st month after diagnosis; long-standing disease was defined as type 1 diabetes of duration >1 year. Subjects with clinical evidence of infection or acute diabetic ketoacidosis (DKA) were excluded from the analysis. To test whether NK cell parameters might affect the age of onset, we weighted the recruitment for two main subgroups of age at onset: *1*) diagnosis before 6 years of age (n = 19 and 40 in recent-onset and long-standing groups, respectively) and *2*) diagnosis between 10 and 15 years of age (n = 23 and 24). Care was taken to standardize the acquisition and processing of blood samples.

The main clinical and epidemiological characteristics of the study group are shown in Table 1. Although long-standing and control groups were age-matched, recent-onset patients were younger on average. As expected, recent-onset patients showed higher A1C levels than those with established disease. The proportion of overweight individuals was highest among patients with long-standing type 1 diabetes than in other individuals. The long-standing group was also the only one to include a proportion of patients with other concomitant autoimmune diseases (18 of 83) and/or diabetic nephropathy (11 of 83, with normal serum creatinine in all cases). These differences were tested as covariates during the analyses reported below, but they had no specific influence.

We also characterized these individuals by genotyping for a panel of genetic susceptibility alleles in established or candidate type 1 diabetes susceptibility loci as well as at

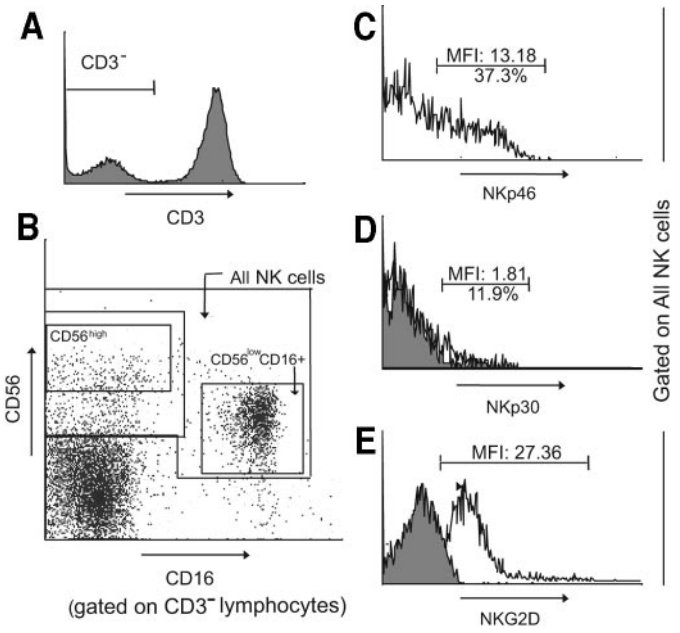


FIG. 1. NK cell flow cytometry. A representative analysis of blood NK cells, defined from the CD3$^-$ lymphocyte fraction (*A*), and further distinguished with CD16 and CD56 markers (*B*). *C–E*: Expression of NK markers NKp46, NKp30, and NKG2D on whole NK cells.

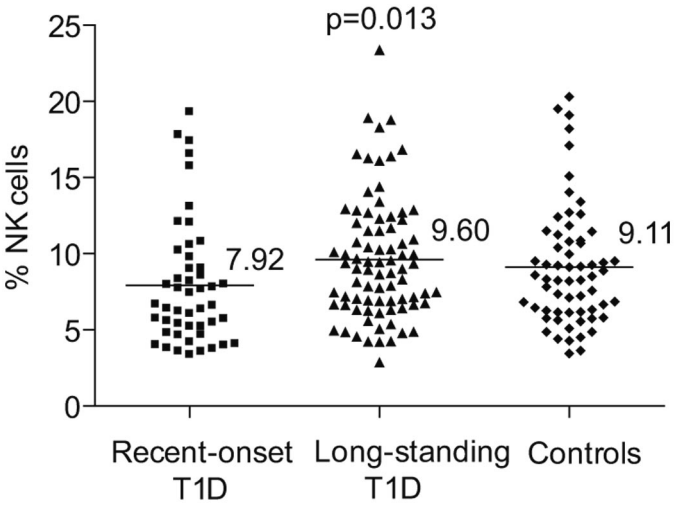


FIG. 2. NK cell frequency in blood. The proportion (%) of total NK cells among blood lymphocytes was quantified as shown in Fig. 1. Each dot represents a different individual; the *P* value shown corresponds to a Kruskall-Wallis test for three groups together (*P* = 0.006 and *P* = 0.031 for the difference in population mean in the recent-onset group relative to the two other groups). T1D, type 1 diabetes.

HLA genes. As expected, the HLA loci showed a strong association, in particular for the DQB1*0302 and DQB1*0201 alleles (and DRB1*03 and DRB1*04, with which the DQB1 alleles are in linkage disequilibrium), as did the R620W SNP at the *PTPN22* locus (supplemental Table S3).

NK cells through type 1 diabetes progression. We first investigated possible differences in the frequency and activation state of NK cells in patients with recent-onset type 1 diabetes versus individuals with long-standing disease or unaffected control subjects. If NK cells are involved in the establishment of type 1 diabetes, recently diagnosed patients might have more, or more active, circulating NK cells. This hypothesis was tested by flow cytometric analysis of peripheral blood, where NK cells were detected as scatter-gated lymphocytes, CD3$^-$, CD56$^+$, and CD16^{+or-} (Fig. 1*A* and *B*). Two NK cell subsets can be recognized in the blood (peripheral blood). The vast majority express moderate levels of CD56 and high CD16 (CD56^{low}/CD16$^+$ NK cells), whereas a minor fraction is CD56^{bright} and lacks expression of CD16. The CD56^{low}/CD16$^+$ subset has strong cytotoxic potential, whereas the CD56^{bright} subset has a greater ability to produce cytokines. These subsets were distinguished as shown in Fig. 1*B*, and a total NK cell count was also recorded. Counting gates were based on control staining with irrelevant antibodies.

A small but distinct reduction in NK cell frequency was found in patients with recent-onset diabetes in comparison with the other groups (Fig. 2). Age-matched comparisons were similar, indicating that the differences were not attributable to the average younger age of the recent-onset patients (not shown). A tendency toward an inverse correlation between A1C and percent NK cells was observed in recent-onset patients ($R = -0.417$, $P = 0.007$), but the deficit in NK cells was not merely a consequence of the loss of glucose control because there was no correlation between A1C and percent NK cells in long-standing patients (not shown). No significant difference was observed between the three groups in the repartition of the CD56^{high}/CD16$^-$ and CD56^{low}/CD16$^+$ NK cell subsets. No association was found between NK cell frequency and the duration of symptoms preceding diagnosis of diabetes, the frequency of DKA, or any of the other clinical characteristics (supplemental Table S4). These observations suggest that the onset of type 1 diabetes correlates with a slight reduction in circulating NK cells and that this reduction is more marked when there is a greater metabolic impairment.

The status of NK cells was then determined. Surface expression of the NK-activating receptors (NCRs) NKp46 and NKp30 (also known as NCR1 and NCR3), known to correlate with NK cell cytotoxic capacity (26,27), was quantitated by flow cytometry (expression of the acute activation markers CD69 and CD44 was also tested in the first 50 samples, but levels little different from background were observed in blood NK cells, and these markers were not investigated further). In addition, we used quantitative real-time PCR (RT-PCR) to measure in purified NK cells the mRNA levels for IFN-γ, TNF-α, and perforin, key effector molecules of activated NK cells. Several points emerged (Fig. 3), as described below.

First, NK cells in the recent-onset group showed the widest scatter of IFN-γ expression levels, with values in five individuals that were unmatched in any of the long-standing or control individuals (highlighted in Fig. 3*A*). Interestingly, these elevated IFN-γ values were not associated with particularly high NK cell numbers or expression of the NKp46 marker. Careful analysis of the clinical and biological data did not reveal any features characteristic of this standout group of patients (e.g., no evident difference in age, age of diabetes onset, duration of prodromic symptomatology before diagnosis of DKA, prior or concomitant infections, therapeutic regimen, or balance of NK cell subsets).

Second, NK cells from long-standing patients, although comparable in number with those from the other groups, displayed fewer hallmarks of activity than NK cells from control subjects or recent-onset individuals. This differ-

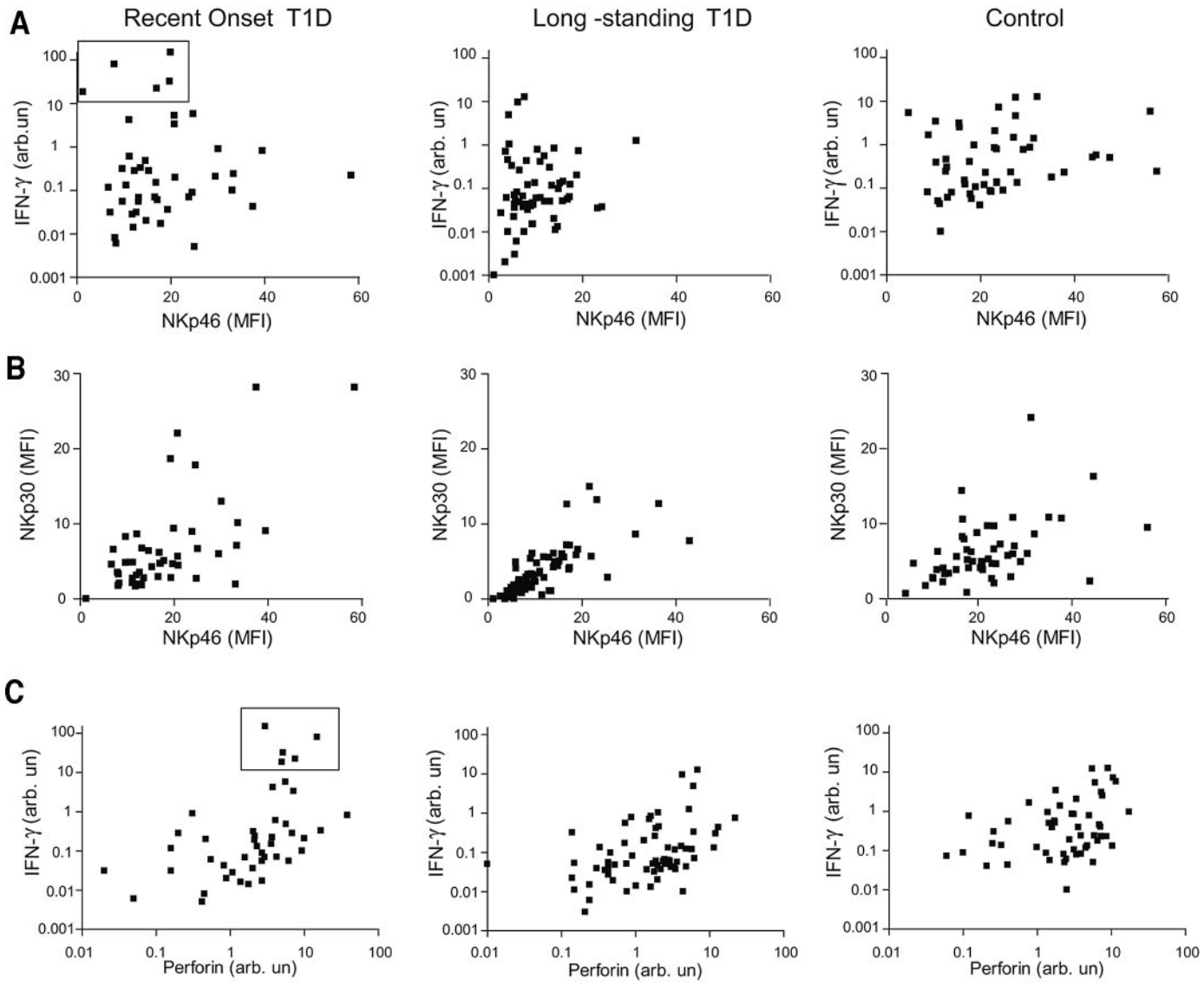


FIG. 3. Activation status of NK cells. The activation status of blood NK cells was determined from the expression of surface activation receptors NKp36 and NKp30 (expressed as MFI) and from RT-PCR analysis of IFN-γ and perforin mRNA in RNA prepared from purified NK cells (as arbitrary units relative to the housekeeping gene standards). Each dot represents a different individual. *A*: p46 versus IFN-γ. *B*: p46 versus p30. *C*: Perforin versus IFN-γ. $P < 0.001$ for the lower surface NKp46 and IFN-γ RNA expression in patients in the long-standing type 1 diabetes group compared with either recent-onset or control groups; the recent-onset patients whose NK cells showed high IFN-γ expression are boxed. Note the strong correlation between the expression of NKp30 and NKp46 receptors ($r = 0.7$, $P < 0.001$) and between IFN-γ and perforin RNA ($r = 0.5$, $P < 0.001$). arb. un, arbitrary units; T1D, type 1 diabetes.

ence was particularly clear for the NKp46 activation receptor (mean MFI 11.7 ± 7.6 for long-standing vs. 18.3 ± 10.5 and 21.9 ± 11.5 for recent-onset and control groups, respectively; $P < 0.0001$) (Fig. 3*A*). The lower expression of surface activating receptors in the long-standing group also applied to NKp30 (3.7 ± 3.2 vs. 6.8 ± 6.4 and 7.1 ± 7.5, $P < 0.001$) (Fig. 3*B*). The differences in p46 and p30 expression were equally true of the $CD56^{bright}CD16^{-}$ and $CD56^{low}CD16^{+}$ NK cell populations (data not shown). A divergence in IFN-γ expression was also readily detectable (median 0.056 in long-standing vs. 0.15 and 0.3 in recent-onset and control groups, respectively; $P < 0.001$). Long-standing patients also showed reduced mRNA levels for perforin, a molecule directly involved in the lysis of target cells by NK cells, although the reduction was more subtle in this case (median 1.8 in long-standing vs. 2.7 and 3.05 in recent-onset and control groups, respectively; $P = 0.023$) (Fig. 3*C*). TNF-α expression was similar in all three groups ($P = 0.65$). No correlation was found between the expression of any of these markers and the duration of diabetes, indicating that the reduction in NK cell activation appeared within a year or so of disease onset. No significant differences were found between the recent-onset and control groups for any of the variables. All of the above-cited results were maintained when stratified by age; thus, the difference in age between the recent-onset and long-standing groups did not seem to interfere with the results.

Lastly, we assessed whether the differences in NK cell activation receptors could be explained by any clinical characteristic, such as a diabetic complication or obesity. Even though overweight individuals were more frequent in the long-standing group, no relationship was found between NK cell status and the BMI percentile, the incidence of diabetic nephropathy, or other concomitant autoimmune diseases (supplemental Table S3).

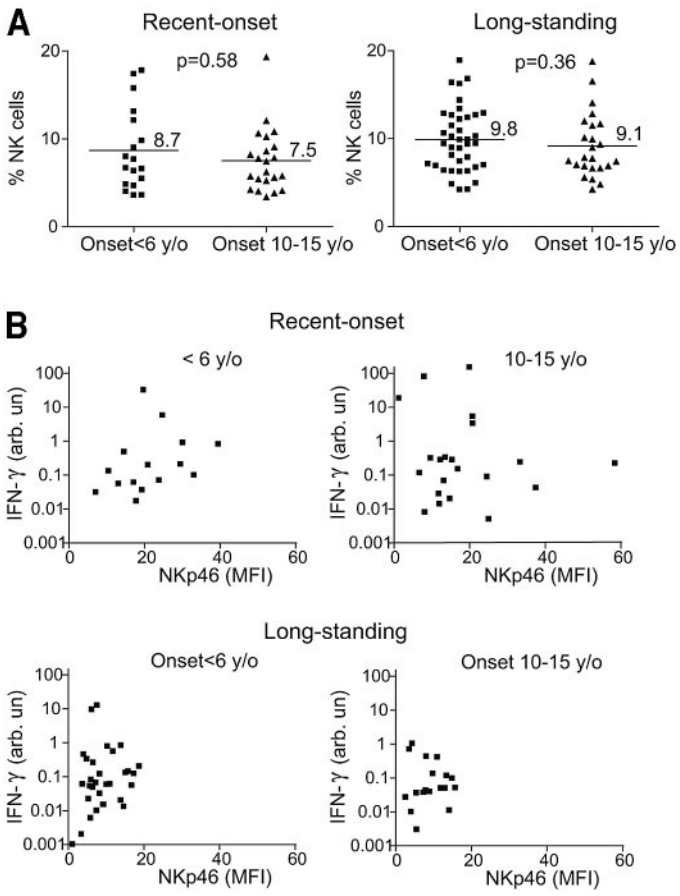


FIG. 4. NK cells do not correlate with the age of onset. The proportion of total NK cells (*A*, measured as for Fig. 2) and activation status (*B*, displayed as for Fig. 3) were distinguished according to age of onset. y/o, years of age.

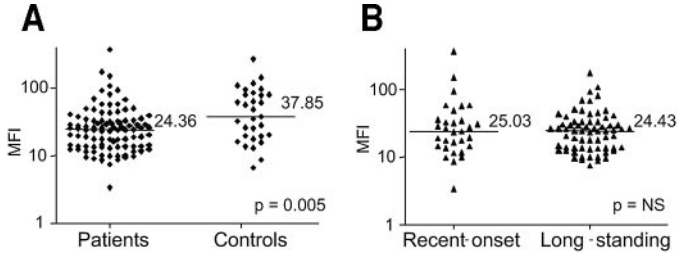


FIG. 5. Flow cytometric expression of the NKG2D marker. *A* and *B*: Surface expression of the NKG2D surface receptor measured by flow cytometry. Each dot represents a different individual.

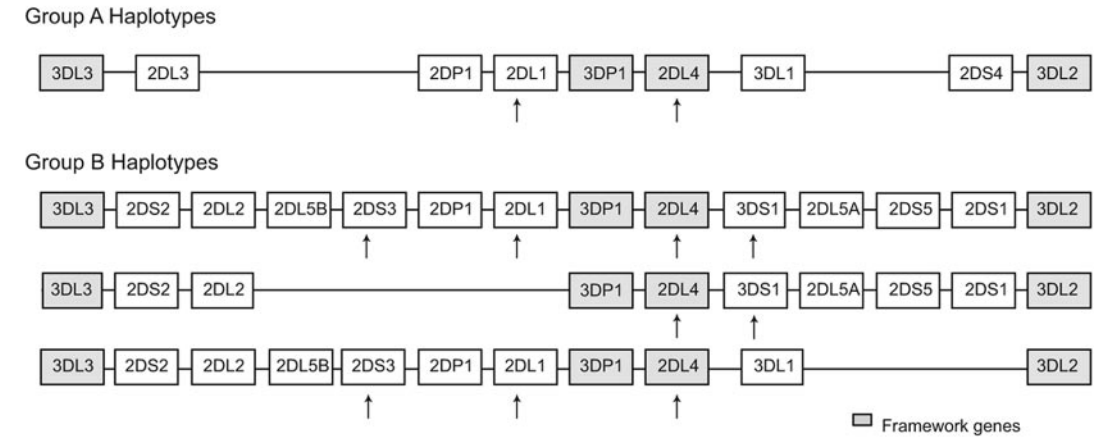
NK cell parameters versus age at onset of type 1 diabetes. Evidence from studies on animal models suggested that high numbers of NK cells might lead to faster progression from insulitis to overt type 1 diabetes. Thus, we asked whether the same might apply in humans, in particular whether type 1 diabetic patients with very early disease onset might be characterized by a higher NK cell activity. To address this issue, we compared the frequency and activation of NK cells in very early onset individuals (<6 years of age) and in patients diagnosed during adolescence (10–15 years of age). No difference in NK cell number was found between the two age-at-onset subgroups, either when all diabetic patients were considered as a whole, or when the recent-onset or long-standing groups were considered individually (Fig. 4*A* and supplemental Table S4). Likewise, NK cell status did not differ between the two subgroups, with a similar surface expression of the activating receptors NKp46 and NKp30 and similar levels of IFN-γ and perforin RNAs (Fig. 4*B* and data not shown). Thus, peripheral blood NK cell number and status did not seem to be associated with the appearance of type 1 diabetes at a very young age (<6 years of age).

Generic differences between type 1 diabetic patients and control subjects. Finally, we found that differences in certain NK cell parameters generally differentiated type 1 diabetic patients from unaffected control subjects, irrespective of disease stage or age of onset. Ogasawara et al. (15) have demonstrated that activated NK cells of NOD mice display low levels of the activating receptor NKG2D (also known as Klrk1); this difference was traced to an induction of NKG2D ligands in NOD cells, leading to receptor modulation. To determine whether this phenomenon also occurred in humans, we compared the surface expression of NKG2D in type 1 diabetic patients and control subjects. We did observe a lower expression of NKG2D in patients relative to control subjects ($P = 0.005$) (Fig. 5). In contrast to the differences in the NKp30 and NKp46 markers, the NKG2D divergence was independent of disease duration (Fig. 5*B*). We tested for an association between haplotypes at the *Klrk1* locus and these expression parameters, using SNP combinations described by Hayashi et al. (NKG2D.4 and NKG2D.11) (28). The genotypes observed fit the expected distribution, but there was no sign of association between these genotypes or haplotypes and type 1 diabetes status, or with NKG2D levels (supplemental Table S.3), suggesting that the modulation of NKG2D might be an indirect effect, as in the NOD mouse (15).

NK cell effector function is controlled by the balance of cell surface receptors of the KIR family, many of which map together in a large complex on chromosome 19 (29). KIR genes all share a great degree of sequence similarity, but they differ in the presence of activating or inhibitory signaling motifs in their intracytoplasmic tails. Genes of the L type carry ITIMs (immunoreceptor tyrosine-based inhibition motifs) that contribute to inhibitory signaling, whereas S-type genes can pair with the activating adapter DAP12. There is extensive polymorphism among KIR haplotypes, which differ by the number and organization of the KIR genes (30). Some genes are present in all haplotypes (so-called framework genes), whereas others are only found in a subset of haplotypes. Because the very close similarity between KIR genes greatly complicates the analysis, the exact composition of KIR haplotypes in humans remains incompletely charted to date. Two large classes have been defined, the A and B haplotypes (30). Genes encoding inhibitory receptors predominate in the A class, whereas two or more activating KIR genes are present in the B class. To begin to assess the distribution of KIR haplotypes in the subtypes of patients, we assayed the presence or absence of a few representative genes: KIR2DL1, KIR2DS2, KIR2DS3, and KIR3DS1, using a novel fluorogenic (TaqMan) real-time PCR assay. 2DL1 is present in both classes of haplotypes; 2DS2, 2DS3, and 3DS1 are characteristic of B haplotypes. We also developed assays for two SNPs in exons of KIR2DL4, a polymorphic framework gene shared by A and B haplotypes.

As shown in Fig. 6, little difference in the distribution of KIR genes was found between patients and control subjects for these markers, with the exception of the 2DS3 gene, an activating KIR gene present in B haplotypes,

A



B

Marker	Assay	SNP ID	Cases	Controls	OR (95% CI)	p value
KIR2DL4ex5	SNP	rs1051455				
0/1/2			3/ 25/ 98	2/ 21/ 40		
Freq. Allele 1			87.7%	80.2%	1.76 (0.99 - 3.14)	0.05
KIR2DL4ex7	SNP	rs649216				
0/1/2			36/ 42/ 37	17/ 16/ 21		
Freq. Allele 1			53.7%	50.4%	-	ns
KIR2DL1	P/A		119/122 (97.5)	62/64 (96.9)	-	ns
KIR2DS2	P/A		87/119 (73.1)	32/64 (50.0)	1.74 (0.95 - 3.20)	ns
KIR2DS3	P/A		45/124 (36.6)	13/61 (21.3)	2.10 (1.07 - 4.15)	0.03
KIR3DS1	P/A		32/111 (29.6)	20/49 (39.5)	-	ns

FIG. 6. Genotypes and haplotypes in the KIR complex. *A*: Structure of representative haplotypes of the A and B groups, which vary in gene composition. Each box represents a gene; shaded boxes represent framework genes present in all haplotypes. *B*: Distribution of genotypes for the two SNPs in the 2DL4 gene and for the presence/absence of the 2DL2 (inhibitory) and 2DS2, 2DS3, and 3DS1 (activating) genes, as determined from analysis of blood DNA. The genotype frequencies are shown in case and control subjects, as well as the odds ratio (OR) and *P* value when significant. ID, identification; P/A, presence/absence.

which was found in 36.6% of cases but only 21% of control subjects ($P < 0.03$, uncorrected for multiple testing). 2DS2 was also more common in case than in control subjects, although the difference failed to reach significance (63.6 and 50% positive, respectively; $P < 0.09$), similar to a previous report (31). Case subjects were also more likely to bear two or more activating receptors among 2DS3, 2DS2, and 3DS1 (41.5 and 29.1%, respectively; $P = 0.10$). No association was observed with the 2DL4 SNP. We attempted to fit the genotypes according to the haplotype classification of Hsu et al. (32), and, although this analysis confirmed the overrepresentation of B haplotypes among case subjects, no finer description could be reached because of the difficulty of unambiguously assigning haplotype structure from presence/absence genotypes.

To evaluate the significance of the suggestive association of 2DS3 with diabetes, we genotyped an extended set of samples from 427 type 1 diabetic case subjects and 209 control subjects (samples collected within a larger type 1 diabetes candidate gene study) (W.B. and M. Roy, unpublished observations). In this cohort, the same trend was observed but did not reach significance (29 and 24% $2DS3^+$ individuals among case and control subjects, respectively; $P = 0.25$).

We then tested for combinatorial association between KIR genes and some known diabetes susceptibility loci. No interaction was observed with the *PTPN22* R620W or *INS* −23 SNPs (not shown), but the presence of 2DS3 was strongly skewed according to HLA haplotype. As illustrated in Table 2, $2DS3^+$ individuals carried the DR3 allele less often than expected from the single-allele frequencies (43.8 vs. 58.7%), whereas the majority of $DR3^+$ individuals were $2DS3^-$ (Mattiuz' $\Delta = -0.06$, $P = 0.009$). A bias was also observed in the control subjects, suggesting that coupling of DR3 and 2DS3 might generally be disfavored (30,33). A similar pattern held true between 2DS3 and DQB1*02, probably because of the strong linkage disequilibrium between DR and DQ. On the other hand, there was a clear positive association between 2DS3 and both DRB1*04 and DQB1*0302 in case but not control subjects, perhaps suggesting a causal relationship with disease susceptibility. To evaluate the generality of the 2DS3-HLA association, we analyzed the distribution of 2DS3 in DNA samples from 672 subjects enrolled in the Diabetes Prevention Trial of Type 1 Diabetes (DPT-1) (35). The positive association between 2DS3 and DQB1*0302 was also observed among 247 case subjects that progressed to diabetes ($\Delta = 0.03$), but the negative association between 2DS3 and DQB1*02 was not reproduced.

TABLE 2
KIR–HLA class II combinations in patients with type 1 diabetes and control subjects

Proportion positive for HLA allele	Case subjects					Control subjects				
	Expected	2DS3$^+$	2DS3$^-$	Δ	*P*	Expected	2DS3$^+$	2DS3$^-$	Δ	*P*
DRB1*03 (DR3)	58.7	43.8	67.9	−0.060	0.009	29.5	7.7	35.4	−0.032	0.08
DRB1*04 (DR4)	63.5	79.2	53.8	0.059	0.005	27.9	30.8	27.1	0.004	1
DQB1*02	68.3	54.2	76.9	−0.065	0.01	39.3	38.5	39.6	−0.001	1
DQB1*0302	57.9	70.8	50.0	0.046	0.026	16.1	23.1	14.3	0.009	0.42

Data are %, unless otherwise indicated.

DISCUSSION

The initial goal of this study was to test whether, as in the mouse models, greater numbers and a higher activity of NK cells were associated with particularly early onset in human patients. This proved not to be the case, but we did uncover a number of NK cell particularities in individuals with type 1 diabetes throughout its course, consistent with the notion that NK cells do play a role in type 1 diabetes progression.

We found no direct relationship between the age of onset and NK cell parameters: no difference in NK numbers, in their subset distribution, in their phenotypic profile, or in the distribution of KIR genes. This lack of correlation was true whether analyzing the patients as a whole or within the recent-onset and long-standing subgroups. However, an important caveat is that this study, per force, only addressed the NK compartment in blood; it remains possible that important differences occur in the pancreas or draining lymph nodes, differences not reflected in the blood. Indeed, the mouse experiments that prompted the current human study focused on NK cell changes in the infiltrate invading the pancreas.

On the other hand, consistent with a role for NK cells in promoting β-cell destruction, the onset phase did appear to be marked by NK cell perturbations. Numbers of NK cells were decreased, modestly but significantly. Activation phenotypes were more diverse than those observed in control subjects or patients with established type 1 diabetes, with particularly strong IFN-γ expression in a subset of individuals. No other characteristics (age, age of onset, or genetic haplotypes) appeared to distinguish these high–IFN-γ individuals. These observations will need to be confirmed in larger number of patients, but they raise the possibility that perturbations of the NK cell pool may accompany the onset phase of type 1 diabetes.

Unexpectedly, patients with long-standing type 1 diabetes exhibited a reduced activation of NK cells, relative to control subjects or recent-onset patients, that was manifested by reduced expression of the cell surface markers NKp30/p46 and of IFN-γ and perforin mRNA. These results are compatible with the decreased lytic activity identified by Lorini et al. (24) in NK cells of patients with long-standing type 1 diabetes. This study also found decreased NK activity in patients with recently diagnosed disease; the difference in results between their and our study is likely attributable to the criteria used because Lorini et al. considered recent-onset patients to be those with up to 1 year of disease duration.

The reduced activation of NK cells solely in long-standing type 1 diabetic patients suggests that reduction is a consequence, rather than a cause, of disease. Prolonged hyperglycemia or its consequences could be an underlying mechanism. Although we did not find a correlation between A1C and NK cell status, serial measurements of A1C were not available to definitely exclude this possibility. One explanation might be impaired secretion of cytokines modulating NK cell function. Interestingly, it was previously suggested that individuals with type 1 diabetes exhibit a deficient production of interleukin-2, a potent NK cell stimulator (35,36). On the other hand, serum levels of interleukin-15 and -12, other molecules implicated in NK cell physiology, were found to be elevated in patients with type 1 diabetes (37,38). Another potential explanation would be an alteration in the systemic level of ligands that engage activating receptors on NK cells, reducing their expression. A decrease in the synthesis and an increase in the degradation of heparin sulfate proteoglycans in individuals with long-standing diabetes has been reported (39); these molecules are ligands for both NKp46 and NKp30 (40).

This apparent effect of long-term diabetes may have clinical consequences. Protection against infections and the destruction of neoplastic cells are the most important functions of NK cells. Interestingly, diabetes has been proposed as a risk factor for both infectious diseases and various types of neoplasias (41–44). These links may certainly have complex determinism, but the current results suggest that dysfunction of NK cells might be involved.

Another interesting finding was the reduced expression of the activating receptor NKG2D in the NK cells of patients with type 1 diabetes, irrespective of disease duration. Ogasawara et al. (15) have demonstrated a reduction of NKG2D expression in NK cells from NOD mice. Might this shared deficit reflect perturbations in NKG2D ligands in diabetes-prone individuals of both species?

The analysis of a few KIR genes also led to suggestive distinctions between type 1 diabetic patients and control subjects. In general, activating KIR genes borne by B haplotypes were enriched in patients, in particular KIR2DS3 and KIR2DS2, reminiscent of two prior reports concerning 2DS2 (31,45), where a KIR-HLA interaction was also detected. Such complex associations with KIR/HLA combinations have also been suggested for viral or antitumor responses, or in other autoimmune conditions (rev. in 30,33). Because KIR products interact primarily with major histocompatibility complex class I molecules, it is likely that the associations of 2DS3 with DRβ and DQβ alleles reflect an association between KIR and major histocompatibility complex class I alleles. Similarly, the 2DS2 and 2DS3 genes belong to an intricate patchwork of KIR haplotypes, and it is likely that any apparent association of a KIR gene with an HLA allele reflects complex interactions with several KIR genes, both inhibitory and activating (30,33). Interestingly, some of the same trends were observed in control subjects, suggesting that the underrepresented KIR/HLA combinations may be disfavored independently of diabetes, perhaps through influences of HLA and KIR alleles on feto-maternal interactions

or antiviral responses (46,47). On the other hand, the KIR/HLA association was only partially reproduced in the DPT-1 cohort.

In summary, this study identified several alterations in the NK cell compartment of patients with type 1 diabetes, some involving all type 1 diabetic patients, others more specifically linked to the onset of disease or to long-term dysglycemia. It will be important to confirm these observations with more extensive genetic analysis and independent groups of diabetic patients.

ACKNOWLEDGMENTS

The work was funded by grants from the Charles H. Hood Foundation and the Stewart Trust and by Joslin Diabetes Center's NIDDK (National Institute of Diabetes and Digestive and Kidney Diseases)-supported DERK (Diabetes and Endocrinology Research Center) cores. M.R. was supported by a fellowship from the American Diabetes Association and B.S. by the National Institutes of Health (5T32DK063702)

We thank Julie Griffiths and Justine Bode for excellent patient recruitment; Natasha Asinovsky, Joyce LaVecchio, Girijesh Buruzula, Chris Campbell, and Adriana Ortiz-Lopez for help with antibodies, flow cytometry, genotyping and RT-PCR; and the Diabetes and Diabetes in the Adolescents and the Very Young (DAVY) groups for discussions.

REFERENCES

1. Kelly MA, Rayner ML, Mijovic CH, Barnett AH: Molecular aspects of type 1 diabetes. *Mol Pathol* 56:1–10, 2003
2. Eisenbarth GS: Type 1 diabetes mellitus: a chronic autoimmune disease. *New Engl J Med* 1360–1368, 1986
3. Leslie RD, Delli Castelli M: Age-dependent influences on the origins of autoimmune diabetes: evidence and implications. *Diabetes* 53:3033–3040, 2004
4. Colucci F, Caligiuri MA, Di Santo JP: What does it take to make a natural killer? *Nat Rev Immunol* 3:413–425, 2003
5. Baxter AG, Smyth MJ: The role of NK cells in autoimmune disease. *Autoimmunity* 35:1–14, 2002
6. Johansson S, Berg L, Hall H, Hoglund P: NK cells: elusive players in autoimmunity. *Trends Immunol* 2005
7. MacKay P, Jacobson J, Rabinovitch A: Spontaneous diabetes mellitus in the Bio-Breeding/Worcester rat: evidence in vitro for natural killer cell lysis of islet cells. *J Clin Invest* 77:916–924, 1986
8. Nakamura N, Woda BA, Tafuri A, Greiner DL, Reynolds CW, Ortaldo J, Chick W, Handler ES, Mordes JP, Rossini AA: Intrinsic cytotoxicity of natural killer cells to pancreatic islets in vitro. *Diabetes* 39:836–843, 1990
9. Maruyama T, Watanabe K, Takei I, Kasuga A, Shimada A, Yanagawa T, Kasatani T, Suzuki Y, Kataoka K, Saruta, et al.: Anti-asialo GM1 antibody suppression of cyclophosphamide-induced diabetes in NOD mice. *Diabetes Res* 17:37–41, 1991
10. Jacobson JD, Markmann JF, Brayman KL, Barker CF, Naji A: Prevention of recurrent autoimmune diabetes in BB rats by anti-asialo-GM1 antibody. *Diabetes* 37:838–841, 1988
11. Ellerman K, Wrobleski M, Rabinovitch A, Like A: Natural killer cell depletion and diabetes mellitus in the BB/Wor rat (revisited). *Diabetologia* 36:596–601, 1993
12. Flodstrom M, Maday A, Balakrishna D, Cleary MM, Yoshimura A, Sarvetnick N: Target cell defense prevents the development of diabetes after viral infection. *Nat Immunol* 3:373–382, 2002
13. Poirot L, Benoist C, Mathis D: Natural killer cells distinguish innocuous and destructive forms of pancreatic islet autoimmunity. *Proc Natl Acad Sci U S A.* 101:8102–8107, 2004
14. Lee IF, Qin H, Trudeau J, Dutz J, Tan R: Regulation of autoimmune diabetes by complete Freund's adjuvant is mediated by NK cells. *J Immunol* 172:937–942, 2004
15. Ogasawara K, Hamerman JA, Hsin H, Chikuma S, Bour-Jordan H, Chen T, Pertel T, Carnaud C, Bluestone JA, Lanier LL: Impairment of NK cell function by NKG2D modulation in NOD mice. *Immunity* 18:41–51, 2003
16. Poulton LD, Smyth MJ, Hawke CG, Silveira P, Shepherd D, Naidenko DI, Baxter AG: Cytometric and functional analyses of NK and NKT cell deficiency in NOD mice. *Int Immunol* 13:887–896, 2001
17. Johansson SE, Hall H, Bjorklund J, Hoglund P: Broadly impaired NK cell function in non-obese diabetic mice is partially restored by NK cell activation in vivo and by IL-12/IL-18 in vitro. *Int Immunol* 16:1–11, 2004
18. Negishi K, Waldeck N, Chandy G, Buckingham B, Kershnar A, Fisher L, Gupta S, Charles MA: Natural killer cell and islet killer cell activities in type 1 (insulin-dependent) diabetes. *Diabetologia* 29:352–357, 1986
19. Nair MP, Lewis EW, Schwartz SA: Immunoregulatory dysfunctions in type I diabetes: natural and antibody-dependent cellular cytotoxic activities. *J Clin Immunol* 6:363–372, 1986
20. Hussain MJ, Alviggi L, Millward BA, Leslie RD, Pyke DA, Vergani D: Evidence that the reduced number of natural killer cells in type 1 (insulin-dependent) diabetes may be genetically determined. *Diabetologia* 30:907–911, 1987
21. Herold KC, Huen A, Gould L, Traisman H, Rubenstein AH: Alterations in lymphocyte subpopulations in type 1 (insulin-dependent) diabetes mellitus: exploration of possible mechanisms and relationships to autoimmune phenomena. *Diabetologia* 27 (Suppl.):102–105, 1984
22. Scheinin T, Maenpaa J, Kontiainen S: Immune responses to insulin and lymphocyte subclasses at diagnosis of insulin-dependent diabetes and one year later. *Immunobiology* 180:431–440, 1990
23. Wilson RG, Anderson J, Shenton BK, White MD, Taylor RM, Proud G: Natural killer cells in insulin dependent diabetes mellitus. *Br Med J* (*Clin Res Ed*) 293:244, 1986
24. Lorini R, Moretta A, Valtorta A, d'Annunzio G, Cortona L, Vitali L, Bozzola M, Severi F: Cytotoxic activity in children with insulin-dependent diabetes mellitus. *Diabetes Res Clin Pract* 23:37–42, 1994
25. Mattiuz PL, Ihde D, Piazza A, Ceppellini R, Bodmer W: New approaches to the population genetic and segregation analysis of the HL-A system. In *Histocompatibility Testing*. Terasaki PI, Ed. Copenhagen, Munksgaard, 1970
26. Sivori S, Pende D, Bottino C, Marcenaro E, Pessino A, Biassoni R, Moretta L, Moretta A: NKp46 is the major triggering receptor involved in the natural cytotoxicity of fresh or cultured human NK cells: correlation between surface density of NKp46 and natural cytotoxicity against autologous, allogeneic or xenogeneic target cells. *Eur J Immunol* 29:1656–1666, 1999
27. Moretta L, Moretta A: Unravelling natural killer cell function: triggering and inhibitory human NK receptors. *EMBO J* 23:255–259, 2004
28. Hayashi T, Imai K, Morishita Y, Hayashi I, Kusunoki Y, Nakachi K: Identification of the NKG2D haplotypes associated with natural cytotoxic activity of peripheral blood lymphocytes and cancer immunosurveillance. *Cancer Res* 66:563–570, 2006
29. Lanier LL: NK cell recognition. *Annu Rev Immunol* 23:225–274, 2005
30. Parham P: MHC class I molecules and KIRs in human history, health and survival. *Nat Rev Immunol* 5:201–214, 2005
31. van der Slik AR, Koeleman BP, Verduijn W, Bruining GJ, Roep BO, Giphart MJ: KIR in type 1 diabetes: disparate distribution of activating and inhibitory natural killer cell receptors in patients versus HLA-matched control subjects. *Diabetes* 52:2639–2642, 2003
32. Hsu KC, Liu XR, Selvakumar A, Mickelson E, O'Reilly RJ, Dupont B: Killer Ig-like receptor haplotype analysis by gene content: evidence for genomic diversity with a minimum of six basic framework haplotypes, each with multiple subsets. *J Immunol* 169:5118–5129, 2002
33. Rajagopalan S, Long EO: Understanding how combinations of HLA and KIR genes influence disease. *J Exp Med* 201:1025–1029, 2005
34. Diabetes Prevention Trial Type 1 Diabetes Study Group: Effects of insulin in relative of patients with type 1 diabetes mellitus. *N Engl J Med* 346:1685–1691, 2002
35. Kaye WA, Adri MN, Soeldner JS, Rabinowe SL, Kaldany A, Kahn CR, Bistrian B, Srikanta S, Ganda OP, Eisenbarth GS: Acquired defect in interleukin-2 production in patients with type I diabetes mellitus. *N Engl J Med* 315:920–924, 1986
36. Tomoda T, Kurashige T, Taniguchi T: Imbalance of the interleukin 2 system in children with IDDM. *Diabetologia* 37:476–482, 1994
37. Kuczynski S, Winiarska H, Abramczyk M, Szczawinska K, Wieruszs-Wysocka B, Dworacka M: IL-15 is elevated in serum patients with type 1 diabetes mellitus. *Diabetes Res Clin Pract* 69:231–236, 2005
38. Winkler G, Dworak O, Salamon F, Salamon D, Speer G, Cseh K: Increased interleukin-12 plasma concentrations in both, insulin-dependent and non-insulin-dependent diabetes mellitus. *Diabetologia* 41:488, 1998
39. Conde-Knape K: Heparan sulfate proteoglycans in experimental models of diabetes: a role for perlecan in diabetes complications. *Diabetes Metab Res Rev* 17:412–421, 2001
40. Bloushtain N, Qimron U, Bar-Ilan A, Hershkovitz O, Gazit R, Fima E, Korc M, Vlodavsky I, Bovin NV, Porgador A: Membrane-associated heparan sulfate proteoglycans are involved in the recognition of cellular targets by NKp30 and NKp46. *J Immunol* 173:2392–2401, 2004

41. Muller LM, Gorter KJ, Hak E, Goudzwaard WL, Schellevis FG, Hoepelman AI, Rutten GE: Increased risk of common infections in patients with type 1 and type 2 diabetes mellitus. *Clin Infect Dis* 41:281–288, 2005
42. Swerdlow AJ, Laing SP, Qiao Z, Slater SD, Burden AC, Botha JL, Waugh NR, Morris AD, Gatling W, Gale EA, Patterson CC, Keen H: Cancer incidence and mortality in patients with insulin-treated diabetes: a UK cohort study. *Br J Cancer* 92:2070–2075, 2005
43. Zendehdel K, Nyren O, Ostenson CG, Adami HO, Ekbom A, Ye W: Cancer incidence in patients with type 1 diabetes mellitus: a population-based cohort study in Sweden. *J Natl Cancer Inst* 95:1797–1800, 2003
44. Joshi N, Caputo GM, Weitekamp MR, Karchmer AW: Infections in patients with diabetes mellitus. *N Engl J Med* 341:1906–1912, 1999
45. Nikitina-Zake L, Rajalingham R, Rumba I, Sanjeevi CB: Killer cell immunoglobulin-like receptor genes in Latvian patients with type 1 diabetes mellitus and healthy controls. *Ann N Y Acad Sci* 1037:161–169, 2004
46. Hiby SE, Walker JJ, O'shaughnessy KM, Redman CW, Carrington M, Trowsdale J, Moffett A: Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. *J Exp Med* 200:957–965, 2004
47. Desrosiers MP, Kielczewska A, Loredo-Osti JC, Adam SG, Makrigiannis AP, Lemieux S, Pham T, Lodoen MB, Morgan K, Lanier LL, Vidal SM: Epistasis between mouse Klra and major histocompatibility complex class I loci is associated with a new mechanism of natural killer cell-mediated innate resistance to cytomegalovirus infection. *Nat Genet* 37:593–599, 2005