Natural Killer Cells From Children With Type 1 Diabetes Have Defects in NKG2D-Dependent Function and Signaling

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OBJECTIVE—Natural killer (NK) cells from NOD mice have numeric and functional abnormalities, and restoration of NK cell function prevents autoimmune diabetes in NOD mice. However, little is known about the number and function of NK cells in humans affected by type 1 diabetes. Therefore, we evaluated the phenotype and function of NK cells in a large cohort of type 1 diabetic children.

RESEARCH DESIGN AND METHODS—Peripheral blood mononuclear blood cells were obtained from subjects whose duration of disease was between 6 months and 2 years. NK cells were characterized by flow cytometry, enzyme-linked immunosorbent spot assays, and cytotoxicity assays. Signaling through the activating NK cell receptor, NKG2D, was assessed by immunoblotting and reverse-phase phosphoprotein lysate microarray.

RESULTS—NK cells from type 1 diabetic subjects were present at reduced cell numbers compared with age-matched, nondiabetic control subjects and had diminished responses to the cytokines interleukin (IL)-2 and IL-15. Analysis before and after IL-2 stimulation revealed that unlike NK cells from nondiabetic control subjects, NK cells from type 1 diabetic subjects failed to downregulate the NKG2D ligands, major histocompatibility complex class I–related chains A and B, upon activation. Moreover, type 1 diabetic NK cells also exhibited decreased NKG2D-dependent cytotoxicity and interferon-γ secretion. Finally, type 1 diabetic NK cells showed clear defects in NKG2D-mediated activation of the phosphoinositide 3-kinase–AKT pathway.

CONCLUSIONS—These results are the first to demonstrate that type 1 diabetic subjects have aberrant signaling through the NKG2D receptor and suggest that NK cell dysfunction contributes to the autoimmune pathogenesis of type 1 diabetes. *Diabetes* **60:857–866, 2011**

Type 1 diabetes is a multifactorial autoimmune disease characterized by T-cell destruction of insulin-producing β-cells and the eventual loss of glucose homeostasis (1). Although both genetic and environmental factors contribute to the breakdown of

immunological self-tolerance, and many of the hallmarks of disease in humans are recapitulated in the NOD mouse (2), the precise mechanisms driving pathogenesis remain unclear. Current evidence suggests that natural killer (NK) cells may be both important regulators and inducers of autoimmune diseases (3–8), and several reports (9–13) have documented that NK cells in NOD mice are impaired compared with those in healthy mice. Although investigations of human subjects with type 1 diabetes have described NK cell alterations, these studies have been limited in size, and the mechanisms underlying the phenotype have not been identified (14–20).

NK cells are well known to have critical roles against viral, bacterial, and parasitic pathogens through the direct killing of infected cells and the production of proinflammatory cytokines such as interferon (IFN)-γ and tumor necrosis factor-α (21). A balance of signals received through a diverse array of activating and inhibitory surface receptors determines whether NK cells evoke their potent effector functions toward a target (22). Some activating receptors are known to bind foreign viral proteins, whereas others recognize self-proteins that are induced upon cellular stress (23). A prominent activating receptor involved in the recognition of stressed, infected, or transformed cells is the C-type lectin NKG2D (24). Signaling by NKG2D is mediated through its association with the transmembrane adaptor protein DNAX-activating protein of 10 kDa (DAP10). Although the NKG2D-DAP10–signaling complex is unusual because it lacks an immunoreceptor tyrosine–based activation motif, DAP10 does contain a "YxxM" motif that functions to recruit the p85 subunit of phosphoinositide3-kinase (PI3K) upon tyrosine phosphorylation (25,26).

Recent work (27) has shown that NOD NK cells exhibit decreased NKG2D-dependent functioning and that this deficit may contribute to disease in this murine model. Activated NOD NK cells, but not C57BL/6 NK cells, were found to maintain NKG2D ligand expression, resulting in the downmodulation of the NKG2D receptor through a mechanism dependent on the "YxxM" motif of DAP10 (27). Reduced NKG2D expression on NOD NK cells was mirrored by decreased cytotoxic and cytokine-secreting functions (27). Notably, we have previously shown that administration of complete Freund adjuvant (CFA) to NOD mice causes NK cells to downregulate NKG2D ligand expression and that the phenomenon is correlated with increased NKG2D receptor expression and heightened NK cell functions (28,29). In addition, NK cells rejuvenated by CFA treatment were able to protect NOD SCID (severe combined immune deficiency) mice from the development of autoimmune diabetes following the adoptive transfer of these hosts with diabetogenic splenocytes (28,29). Collectively, these findings suggest that the chronic exposure

of NOD NK cells to NKG2D ligands results in their desensitization and also that augmentation of NK cell function protects NOD mice from disease.

Given the important regulatory role of NK cells in diabetes of the NOD mouse, we sought to determine whether numeric or functional deficits also are present among human type 1 diabetic NK cells. Here, we report that NK cells from children with type 1 diabetes constitute a significantly reduced fraction of peripheral mononuclear cells relative to age-matched nondiabetic control subjects and that these NK cells are poorly responsive to interleukin (IL)-2/IL-15 stimulation. Analogous to findings in the NOD mouse (27–29), dysregulated expression of the NKG2D ligands on activated type 1 diabetic NK cells is present and associated with both impaired NKG2D-mediated effector function and signaling. These results suggest that NK cell dysfunction and aberrant NKG2D signaling may be a consequence of, or contribute to, the pathogenesis of type 1 diabetes.

RESEARCH DESIGN AND METHODS

Subject recruitment, sample collection, and complete blood-cell counts. The University of British Columbia Clinical Research Ethics Board (certificate nos. H07-01707 and H03-70046) approved the collection of blood, and informed consent was received from nondiabetic control and type 1 diabetic subjects. Complete blood-cell counts were performed on fresh blood using a Sysmex XE-2100 automated multiparameter blood-cell counter at the Children's and Women's Health Centre of British Columbia.

Antibodies and flow cytometry. Cells were pretreated with anti-CD16 (3G8; Biolegend) antibody (Ab) to block nonspecific binding to Fc receptors prior to samples being stained with the indicated markers. Abs specific for CD3 (HIT3a), CD4 (RPA-T4), CD8 (HIT8a), CD19 (HIB19), CD25 (2A3), 2B4 (2-69), LAIR-1 (DX26), NKB1 (DX9), CD94 (HP-3D9), CD56 (B159), CD122 (Mik-b2), and CD132 (AG184) were purchased from BD Biosciences. Abs recognizing IL-15Rα (eBioJM7A4; eBioscience), CD16 (CB16, eBioscience), NKG2D (FAB139P; R&D Systems), major histocompatibility complex class I–related chains A and B (MICA/B) clone 159207 (R&D Systems), and MICA/B clone 6D4 (Biolegend) were acquired from the indicated sources. Samples were analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences).

Purification and in vitro culture of human NK cells. NK cells were isolated from peripheral blood using a human NK cell enrichment kit (StemCell Technologies), and typical isolation resulted in ≥96% purity, as determined by staining with anti-CD3 and anti-CD56 Abs (data not shown). Purified NK cells were expanded in RPMI-1640 medium containing 10% AB human serum, 1 mmol/L nonessential amino acids, 5×10^5 2-ME, 1000 units/mL human rIL-2 (BD Biosciences), and 50 units/mL rIL-15 (eBioscience). NK cells were expanded in vitro for 5–7 days prior to their use in cytotoxicity and enzyme-linked immunosorbent spot (ELISpot) assays.

Cytotoxicity assays. Target cell lines K562, Raji, and Daudi were acquired from the American Type Culture Collection. One million target cells were labeled by incubating cells with 100 µCi ^{51}Cr for 90 min at 37°C, washing them three times with PBS, and seeding them at 10^4 cells/well in round-bottom 96-well plates. Various numbers of effectors were added to each well, and plates were centrifuged at 500 rpm for 2 min and incubated at 37°C. After 4 h incubation, 100-µL volumes of supernatant were collected and the amount of ^{51}Cr released was measured using a γ counter. For NKG2D stimulation, NK cells were treated with 10 µg/mL anti-NKG2D (MAB139; R&D Systems) Abs for 20 min at 37°C. After stimulation, cells were washed twice with complete RPMI medium prior to their use as effectors in cytotoxic T-lymphocyte (CTL) assays.

ELISpot assays. The preparation of NK cell effectors was carried out identically as that performed for the CTL assays. ELISpots were performed in 96-well flat-bottom MAIP S4510 plates (Millipore) using a human IFN-γ ELISpot kit from Mabtech. Immunospot plates were coated with 15 µg/mL capture anti–human IFN-γ mAbs (clone 1-D1 K) by overnight incubation at 4°C. A total of 5,000 of the indicated target cells were mixed with 50,000 or 25,000 lymphokine-activated killer (LAK) cells (10:1 or 5:1 effector:target ratios) and cultured for 24 h. Captured cytokines were detected with biotinylated anti–IFN-γ mAbs (clone 7-B6-1), and spots were developed using streptavidin-alkaline phosphatase and counted with Bioreader-4000 (Bio-Sys).

Cell-signaling studies. IL-2/IL-15–cultured NK cells (10×10^6 cells/mL) were stimulated with 10 µg/mL of anti-NKG2D Ab (MAB139; R&D Systems) for 15 min at 37°C. Cells were lysed in ice-cold lysis buffer (20 mmol/L Tris-HCl, pH 8.2; 100 mmol/L NaCl; and 10 mmol/L EDTA) containing a protease inhibitor cocktail (Sigma). NKG2D was immunoprecipitated using anti-NKG2D Abs (1D11; eBioscience) and a combination of protein G-Sepharose and anti-mouse IgG-agarose (Santa Cruz Biotechnology). Cell lysates and immunoprecipitates were analyzed by blotting with either anti-PI3K (06-496; Upstate Biotechnology) or anti-NKG2D (1D11; eBioscience) Abs. Anti-mouse IgG Abs coupled to horseradish peroxidase (BioRad) and electrochemiluminescence (Pierce Biotechnology) were used to detect membrane-bound anti-PI3K and anti-NKG2D Abs.

Lysate preparation, microarray production, data acquisition, and array analyses. NK cells, expanded in complete medium containing 1,000 units/mL IL-2 and IL-15 (BD Biosciences) for 10 days, were serum starved for 4 h. For NKG2D-signaling studies, NK cells (10^7 cells/mL) were incubated with 10 µg/mL of anti-NKG2D mAbs (R&D Systems) for 10 min on ice, washed twice with PBS, and incubated for 1 min or 5 min in 37°C warmed PBS containing affinity-purified rabbit anti-mouse IgG F(ab')$_2$ (Jackson Immunoresearch Laboratories). The fabrication and processing of lysate arrays has previously been discussed in detail (30). Slides were probed with anti–P(Y)-p85 PI3K (no. 3821), P(T308)-AKT (no. 9275), P(S473)-AKT (no. 9271), and P(S473)-AKT (no. 4058; mAbs) primary Abs from Cell Signaling Technology. The processed slides were scanned using a GenePix 4000A microarray scanner (Molecular Devices) and analyzed with GenePix Pro 6.0 software (Molecular Devices). For each sample printed in triplicate, the background-subtracted median fluorescence intensities were averaged and the intensity fold-change compared with the unstimulated sample calculated as a ratio of background-subtracted median fluorescence intensities for each time point versus the background-subtracted median fluorescence intensities of the unstimulated sample. The log base 2 values of these ratios were depicted in heatmap format using TIGR MultiExperiment Viewer software, and data were expressed as the means ± SD (31).

Statistical analyses. A Student t test was used to calculate statistical significance where indicated, and a single-factor ANOVA was used for multigroup comparison. Prism software (GraphPad Software) was used to create graphs and provided assistance with statistical tests.

RESULTS

NK cells from type 1 diabetic subjects are present at reduced frequencies and respond poorly to IL-2 and IL-15. To address whether numerical or functional NK cell defects are present in human type 1 diabetic subjects, we analyzed peripheral blood mononuclear cells (PBMCs) from subjects with established type 1 diabetes (>0.5 years and <2 years; mean age 9.3 ± 4.5 years; mean type 1 diabetes duration 1.4 ± 0.5 years) and age-matched nondiabetic control subjects (mean age 10.7 ± 4.0 years) using great care to follow standardized and consistent processing of blood samples and experimental conditions (Table 1). We rationalized that if NK cell dysfunction was an intrinsic property of the type 1 diabetes immune system, long-standing measurable defects would still be present in subjects after establishment of disease. We also limited our subjects to those whose onset of diabetes was no greater than 2 years in order to minimize the potential effects of chronic hyperglycemia on lymphocyte number and function. Frequencies of NK (CD3$^-$CD56$^+$), NKT (CD3$^+$CD56$^+$), CD4 T (CD3$^+$CD56$^-$CD4$^+$), CD8 T (CD3$^+$CD56$^-$CD8$^+$), and B-cell (CD3$^-$CD19$^+$) subsets among PBMCs were assessed using standard flow cytometric techniques (Fig. 1A and B). In contrast to the similar proportions of CD4 T-, CD8 T-, and B-cells in the peripheral blood of type 1 diabetic subjects relative to control subjects, the NK cell fraction in type 1 diabetic subjects was markedly reduced (~37%) relative to nondiabetic age-matched control subjects (control subjects: 6.58 ± 2.93% vs. type 1 diabetic subjects: 4.18 ± 1.66%; $P < 0.0005$). To ascertain whether type 1 diabetic subjects exhibit a decrease in absolute NK cell numbers, complete blood-cell counts were performed on fresh blood samples from type 1 diabetic and nondiabetic subjects (Fig. 1C). Total lymphocyte numbers were found to be modestly reduced in type 1 diabetic subjects relative to control subjects, although these numbers both fell within the normal

TABLE 1
Characteristics of the type 1 diabetic subjects and nondiabetic control groups from British Columbia's Children's Hospital are presented

	Type 1 diabetic subjects	Nondiabetic control subjects
n	116	145
Female/male subjects	49/67	90/55
Mean age (years)	9.3 ± 4.5	10.7 ± 4.0
Mean age of onset (years)	8.9 ± 4.2	N/A
Mean duration of type 1 diabetes (years)	1.4 ± 0.5	N/A
Mean A1C (%)	7.8 ± 1.2	N/A

Data are means ± SD. All type 1 diabetic subjects were being treated with insulin and did not show evidence of other autoimmune diseases. Age-matched subjects with no autoimmune or metabolic diseases were used as nondiabetic control subjects.

range for age at our institution (type 1 diabetic subjects: $2.21 \pm 0.74 \times 10^9$/L, $n = 11$ vs. control subjects: $2.96 \pm 0.74 \times 10^9$/L, $n = 10$; $P < 0.02$), and absolute NK cell numbers per blood volume were decreased approximately twofold in type 1 diabetic subjects relative to control subjects (type 1 diabetic subjects: $0.92 \pm 0.37 \times 10^8$/L vs. control subjects: $1.94 \pm 0.86 \times 10^8$/L; $P < 0.0001$).

The critical roles of the cytokines IL-2 and IL-15 in NK cell homeostasis (32,33) led us to hypothesize that a lack of responsiveness by type 1 diabetic NK cells to IL-2 and IL-15 could underlie their decreased representation. To address this question, purified NK cells from type 1 diabetic and age-matched control subjects were labeled with the mitotic tracker carboxyfluorescein succinimidyl ester (CFSE), as described previously (34), and cultured in vitro either in media alone or with addition of IL-2 and IL-15 (Fig. 2A). After 1 week, measurements of cellular proliferation indicated that very few type 1 diabetic NK cells had proliferated. In contrast, significant numbers of control NK cells had undergone one or more cell divisions. The vast majority of NK cells, whether type 1 diabetic or control derived, failed to proliferate in the absence of exogenous cytokines, demonstrating that cell division was

dependent upon cytokine stimulation (data not shown). To determine whether the lack of proliferation by type 1 diabetic NK cells was associated with a decreased cellular recovery, equivalent numbers of control and type 1 diabetic NK cells were placed into culture with IL-2 and IL-15 (Fig. 2B). One week later, cell counts of cultures revealed that the yield from wells containing type 1 diabetic NK cells was decreased twofold relative to the control group. These findings indicate that reduced frequencies of NK cells in type 1 diabetic subjects are correlated with poor responsiveness to IL-2 and IL-15.

To address whether poor IL-2/IL-15 responsiveness by type 1 diabetic NK cells is a result of insufficient cytokine receptor expression, we compared levels of IL-2 and IL-15 receptor subunits (Fig. 2C). Flow cytometric analyses revealed that type 1 diabetic NK cells expressed modestly reduced levels, as determined by comparison of mean fluorescence intensity (MFI) values, of IL-2Rβ/IL-15Rβ (CD122) and IL-2Rγ/IL-15Rγ (CD132 or common-γ chain) relative to control (CD122: type 1 diabetic = 65.3 ± 5.8 vs. control = 75.2 ± 12.5; CD132: type 1 diabetic = 26.1 ± 5.9 vs. control = 29.8 ± 2.7). CD122 and CD132 interact with CD25 to form the high-affinity IL-2 receptor, whereas these two subunits are thought to bind IL-15 through *trans*-presentation by IL-15Rα chain on an accessory cell (35). Regardless of the NK cell origin, we were unable to detect significant expression of either of the unique subunits of these two cytokine receptors, IL-2Rα (CD25) and IL-15Rα (data not shown). These results indicate that the hyporesponsiveness of type 1 diabetic NK cells to IL-2/IL-15 stimulation is not a result of a lack of cytokine receptor expression.

Activated type 1 diabetic NK cells fail to downregulate the NKG2D ligands, MICA/B. To investigate the surface phenotype of type 1 diabetic NK cells and potential causes of their dysfunction, we analyzed the expression of different NK cell markers on cells directly ex vivo and after in vitro activation with IL-2/IL-15 (Fig. 3A). Type 1 diabetic NK cells were found to express normal levels of 2B4, CD94, LAIR, and NKB-1 directly ex vivo, as judged by percent-positive and MFI values (Fig. 3A and data not shown, respectively). Type 1 diabetic and control NK cells induced the expression of the C-type lectin CD94 and extinguished
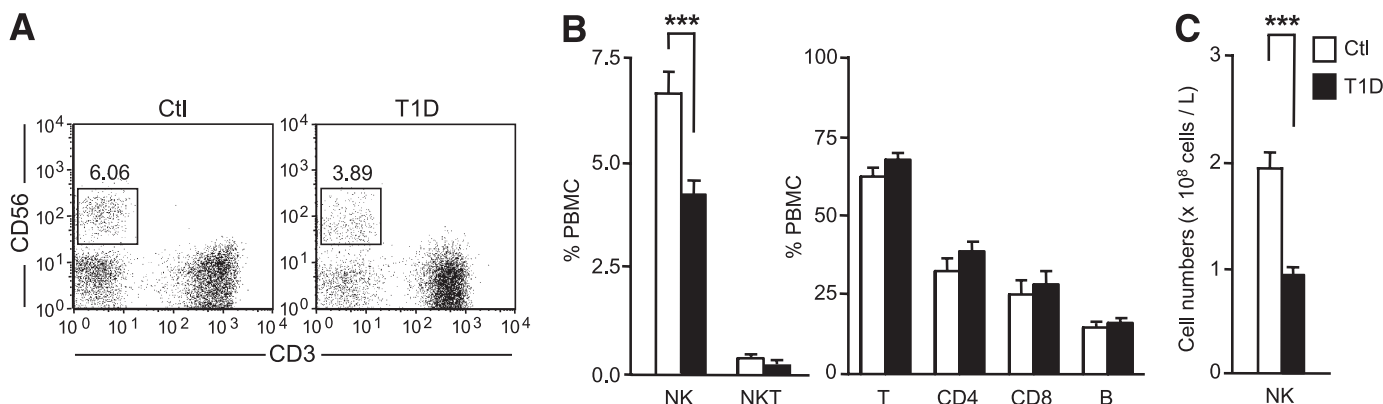
FIG. 1. NK cells from PBMCs of type 1 diabetic (T1D) subjects are present at reduced cell frequencies and numbers. *A*: Flow cytometric analyses of nondiabetic control (Ctl) and type 1 diabetic PBMCs using Abs against CD3 and CD56 markers. *B*: The frequencies of NK cells (Ctl: $n = 36$; T1D: $n = 22$) and other lymphocyte subsets (Ctl: $n = 11$; T1D: $n = 14$) among PBMCs obtained from type 1 diabetic subjects (■) and age-matched, nondiabetic control subjects (□) were determined by flow cytometry: NK cells (CD3⁻CD56⁺), B-cells (CD3⁻CD19⁺), T-cells (CD3⁺CD19⁻), CD4 T-cells (CD4⁺CD19⁻), CD8 T-cells (CD8⁺CD19⁻), and NK T-cells (CD3⁺CD56⁺). *C*: Type 1 diabetic subjects possess fewer NK cells than normal control subjects. Complete blood-cell counts were used to determine the NK cell numbers present per liter of blood. ***$P < 0.0005$. Error bars represent the SD.
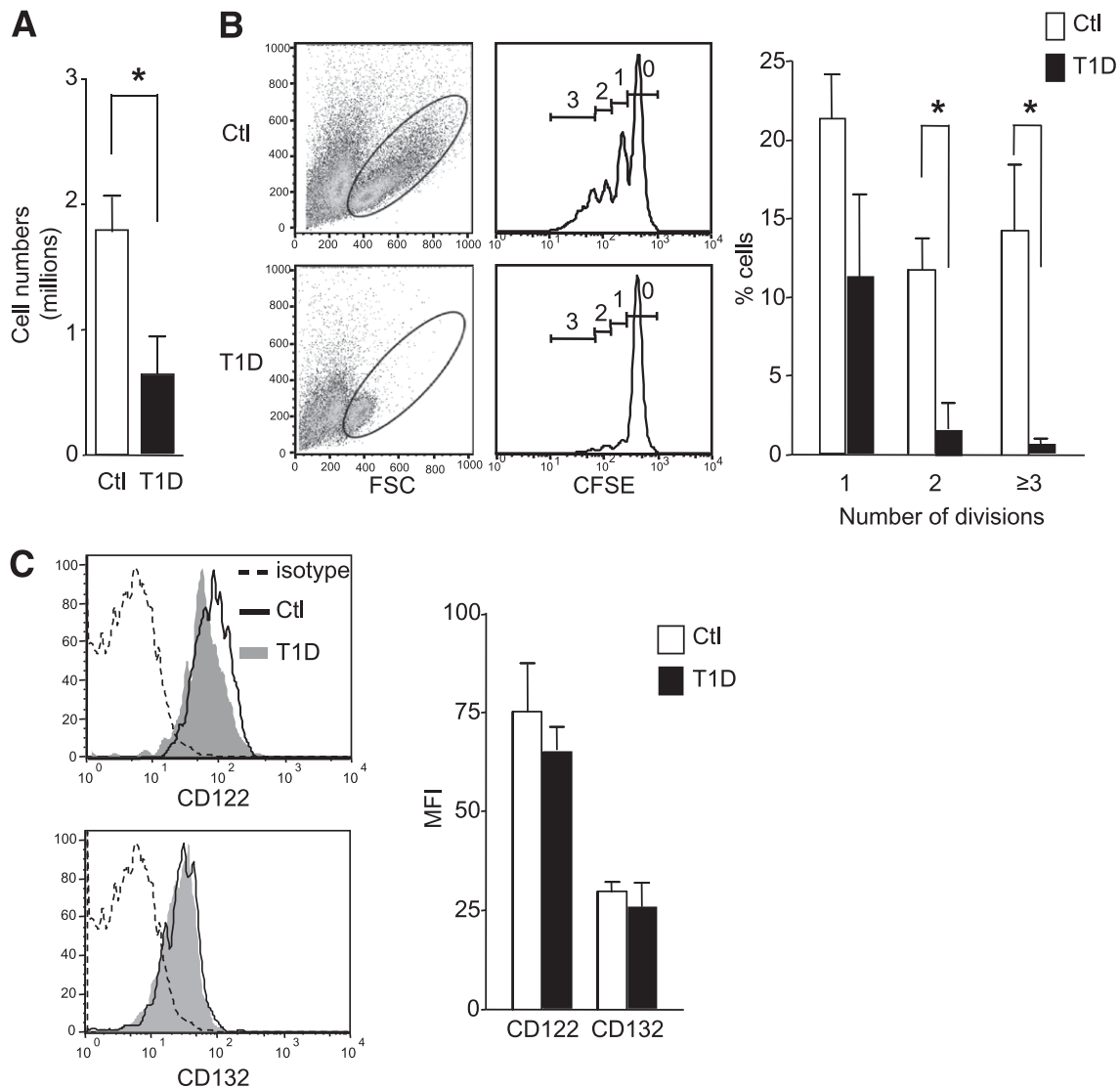
FIG. 2. Type 1 diabetic NK cells are poorly responsive to IL-2/IL-15 stimulation. *A*: One million NK cells from type 1 diabetic subjects (T1D; *n* = 8) and age-matched control subjects (Ctl; *n* = 4) were cultured for 1 week with rIL-2 and rIL-15 and were subsequently counted. *B*: Purified NK cells from the peripheral blood of type 1 diabetic subjects (■, *n* = 8) and age-matched control subjects (□, *n* = 4) were labeled with CFSE and cultured for 1 week with rIL-2 and rIL-15. Representative (histograms) and cumulative data (bar graphs) are shown for the cell-division history of cultured NK cell populations. *$P < 0.05$. *C*: Expression of IL-2Rβ/IL-15Rβ (CD122) and the common-γ chain receptor (CD132) on NK cells was determined directly ex vivo by flow cytometry. Cumulative data were plotted out as MFI values. Error bars represent the SD.

the signaling lymphocyte activation molecule family receptor, 2B4. Next, we assessed levels of NKG2D ligands on the surface of control and type 1 diabetic NK cells because previous experiments in diabetic NOD mice attributed their altered expression to NK cell dysfunction (27). Resting NK cells have been reported to express a MICA and MICB message (http://biogps.gnf.org) and MICA protein (36). Using a specific monoclonal Ab anti–MICA/B Ab (clone 6D4) for detection, we also detected MICA/B expression on control and type 1 diabetic NK cells directly ex vivo (Fig. 3*B* and *C*). However, upon activation in vitro, MICA/B levels on control NK cells were almost completely lost, whereas type 1 diabetic NK cells maintained strong MICA/B expression (control = 4.8 ± 0.3 MFI; type 1 diabetic = 30.3 ± 7.6 MFI; 6.3-fold change in MFI). Experiments performed with monoclonal anti-MICA Ab corroborated these conclusions (Ab clone 159227; data not shown). Despite retaining high MICA/B levels, activated type 1 diabetic NK cells expressed NKG2D levels that were comparable to

control NK cells (Fig. 3*B* and *C*). Together, these experiments reveal that type 1 diabetic NK cells exhibit dysregulated MICA/B but normal CD94 and 2B4 expression upon stimulation with IL-2/IL-15.
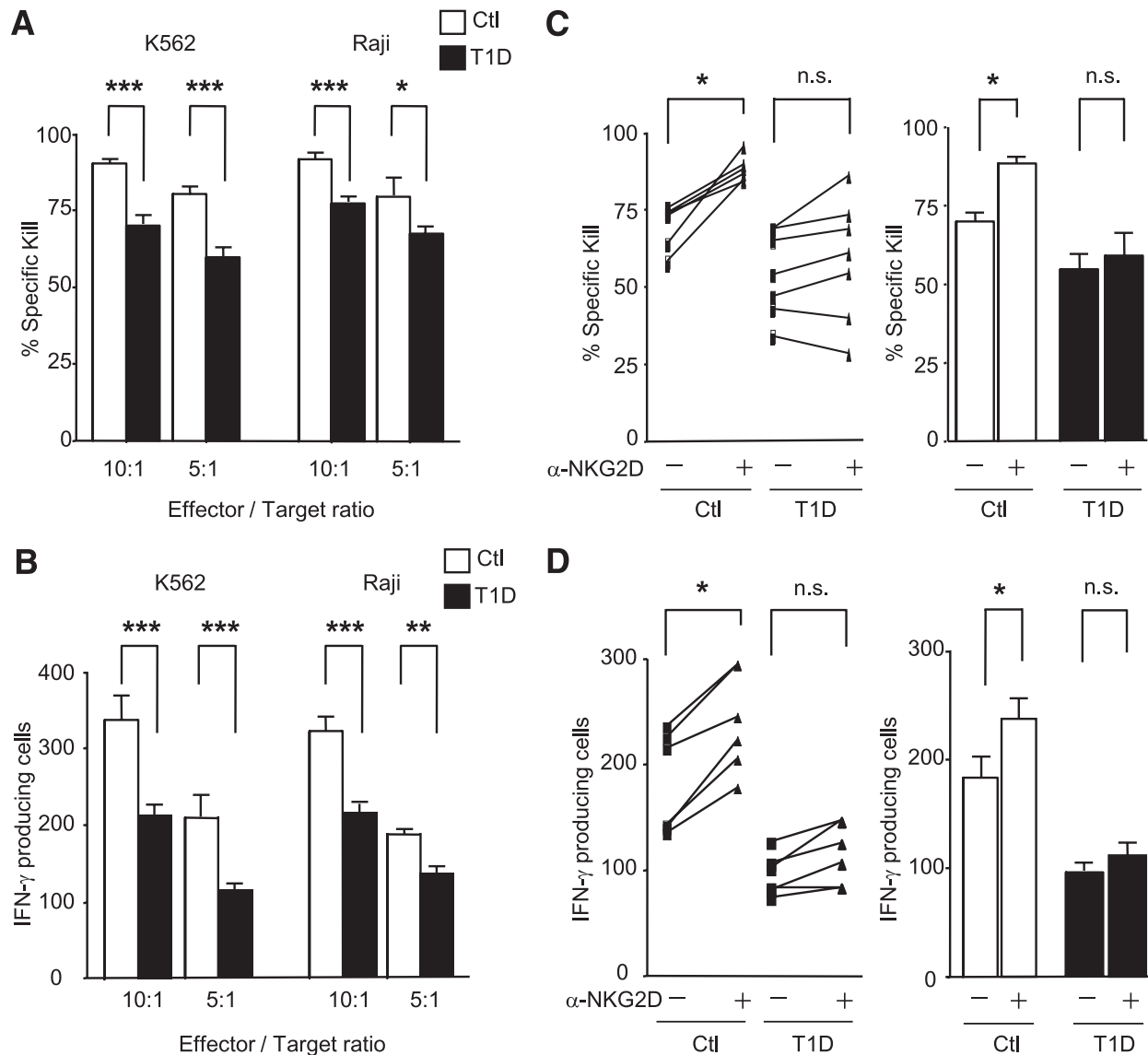
Type 1 diabetic LAK cells exhibit reduced cytotoxicity, IFN-γ secretion, and NKG2D function. To evaluate their effector function, purified type 1 diabetic and control NK cells were expanded with IL-2 to generate LAKs and were assessed for their ability to lyse either HLA-negative, NK cell–sensitive K562, or NK cell–resistant LAK-sensitive Raji targets using standard ^{51}Cr-release assays (Fig. 4*A*). Type 1 diabetic LAK cells were found to be at least two-fold less efficient killers of K562 cells on a per-cell basis than control LAKs (type 1 diabetic LAK = 70.4 ± 3.0% kill at 10:1 effector:target [E:T] ratio kill vs. control LAK = 80.4 ± 2.6 kill at 5:1 E:T ratio). In addition, a similar deficit in type 1 diabetic LAK cytotoxicity also was observed against Raji targets (type 1 diabetic LAK = 78.6 ± 1.8% kill at 10:1 E:T ratio kill vs. control LAK = 80.5 ± 5.7 kill at 5:1
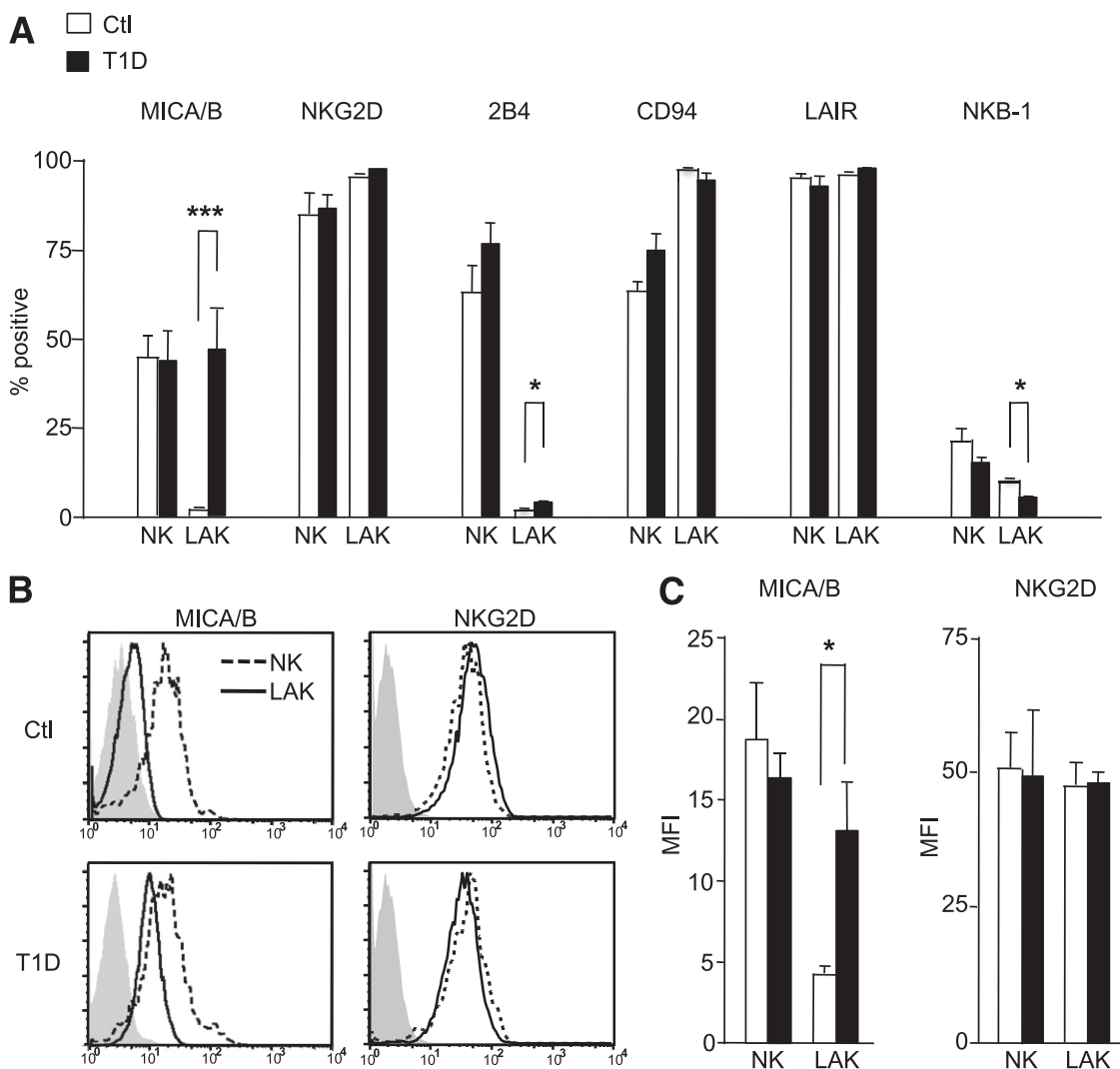
FIG. 3. Type 1 diabetic NK cells fail to downregulate the NKG2D ligands MICA/B upon activation. *A*: Surface marker analyses were performed on type 1 diabetic (T1D; *n* = 10) and age-matched control (Ctl; *n* = 10) NK cells either directly ex vivo (NK) or after 1 week of in vitro activation with IL-2/IL-15 (LAK). NK cells were pretreated with anti-CD16 Ab to block nonspecific FcR binding and were subsequently stained with Abs recognizing MICA/B, NKG2D, 2B4, CD94, LAIR, NKB-1, NKp46m or CD16, electronically gated on CD56$^+$CD3$^-$ cells and the percent positive for the indicated marker determined. *B*: Representative histograms illustrate staining with anti-NKG2D (IgG1) or anti-MICA/B (IgG2a) Abs, both directly conjugated with phycoerythrin (PE), on freshly isolated (NK) and 1-week-activated NK cells (LAK) from the peripheral blood of type 1 diabetic and age-matched control subjects. Shaded histograms represent staining with isotype-control Abs (IgG1 or IgG2a) bound to PE and include relevant antibodies from other channels to account for fluorescence spillover. *C*: Cumulative data comparing NKG2D and MICA/B expression, as net MFI values (MFI of specific Ab-stained cells minus MFI of isotype control Ab-stained cells), on type 1 diabetic (*n* = 10) and age-matched control (*n* = 10) NK cells directly ex vivo and 1 week after activation with IL-2/IL-15. *P < 0.05; ***P < 0.0001. Error bars represent the SD.

E:T ratio). Next, we assessed the ability of control and type 1 diabetic LAK cells to produce IFN-γ upon exposure to target cells (Fig. 4*B*). Control or type 1 diabetic LAK cells were incubated with either K562 or Raji cells for 24 h and IFN-γ cellular secretion enumerated by ELISpot assays. Similar to the cytotoxicity results, type 1 diabetic LAK cells demonstrated a twofold-decreased capacity to produce IFN-γ when stimulated with K562 targets (type 1 diabetic LAK = 220 ± 13 spots at 10:1 E:T ratio vs. control LAK = 210 ± 29 spots at 5:1 E:T ratio). Likewise, type 1 diabetic LAK cells also displayed marked reductions in IFN-γ secretion relative to control subjects when treated with either 10:1 (220 ± 12 vs. 320 ± 18) or 5:1 (140 ± 10 vs. 190 ± 4) ratios of Raji stimulators. Together, these findings demonstrate that LAK cells derived from type 1 diabetic subjects display reduced effector function compared with those derived from nondiabetic control subjects.

Previous work in NOD mice has suggested that the expression of NKG2D ligands on activated NK cells affects NKG2D signaling and results in decreased NKG2D-dependent cytotoxicity and cytokine production (27). Because activated type 1 diabetic NK cells possess unusually high levels of NKG2D ligands, we sought to examine whether these cells also exhibited defects in NKG2D function (Fig. 4*C*). To address this question, type 1 diabetic and control LAK cells were treated with either anti-NKG2D Abs or control murine Abs for 20 min, washed, and subsequently incubated with ^{51}Cr-labeled Daudi targets, a cell line known both to express NKG2D ligands and to be sensitive to NKG2D-mediated killing (37,38). Stimulation of control LAK cells with anti-NKG2D Abs resulted in markedly improved killing of targets versus control murine Abs (70.1 ± 2.8% vs. 88.4 ± 1.7%; 26.1% increase; P < 0.0005), whereas type 1 diabetic LAK cells were unaffected
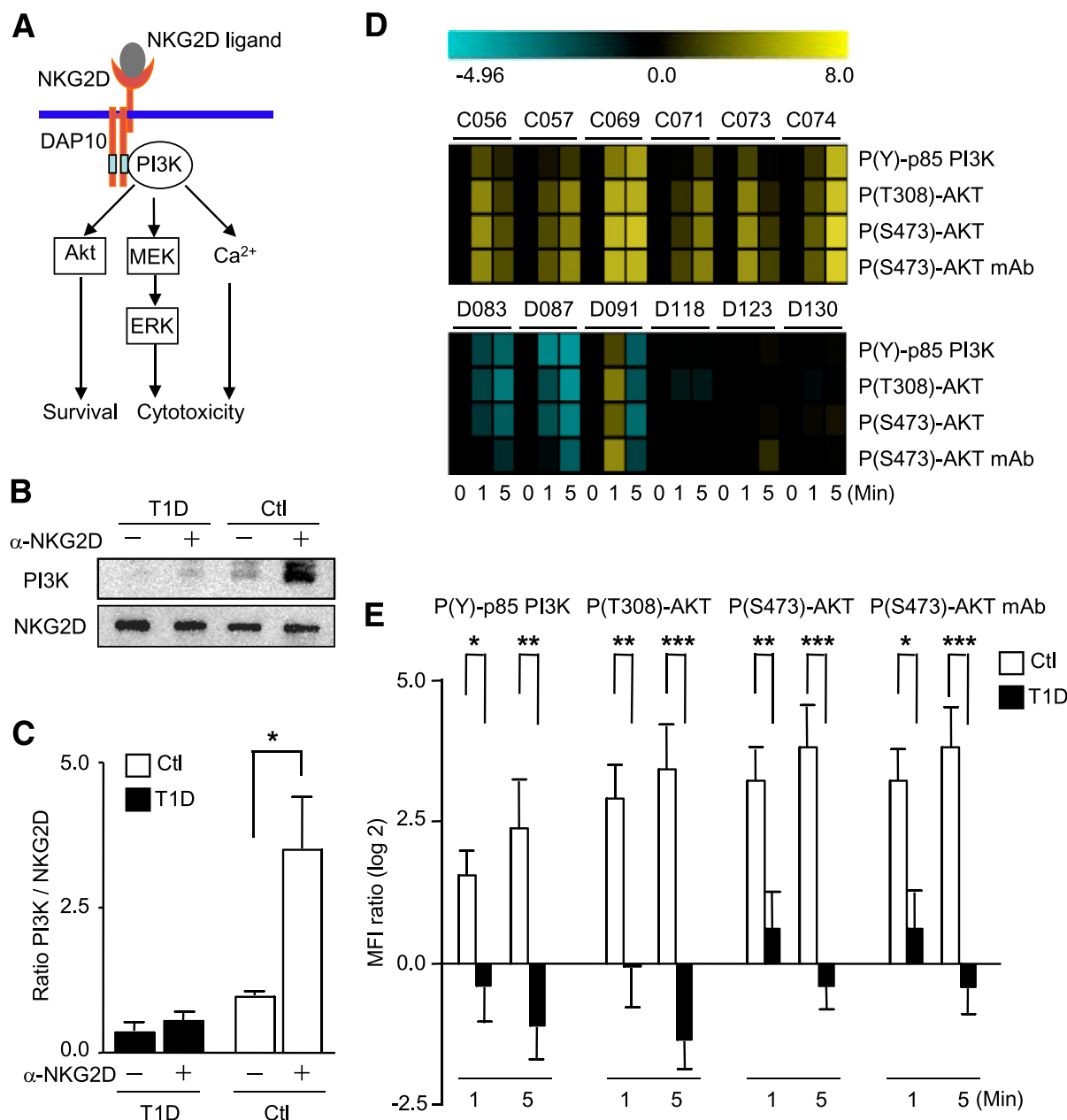
FIG. 4. Type 1 diabetic LAK cells exhibit reduced cytotoxicity, IFN-γ secretion, and NKG2D function. LAK cells were generated by treating purified NK cells with rIL-2/rIL-15 and were tested for effector function. *A*: The cytotoxicity of LAK cells from type 1 diabetic subjects (T1D; *n* = 8) and age-matched control subjects (Ctl; *n* = 14) was assessed using standard chromium-release assays with either K562 or Raji cell lines as targets and indicated numbers of E:T ratios. *B*: The capacity of type 1 diabetic (*n* = 8) and age-matched control (*n* = 14) LAK cells to produce IFN-γ following stimulation with K562 or Raji cells was measured with ELISpot assays, using the indicated E:T ratios. *C*: Type 1 diabetic (*n* = 6) and control LAK cells (*n* = 6) were cultured with ^{51}Cr-labeled Daudi target cells at a 5:1 E:T ratio, and cytolytic activity was assessed at the end of 4 h. Data are presented in both dot graph (■, control Ig; ▲, NKG2D) and bar graph (□, control; ■, type 1 diabetic) format. *D*: Type 1 diabetic (*n* = 6) and control (*n* = 6) LAK cells were treated with Daudi stimulators, and IFN-γ production was measured by an ELISpot assay. Data are presented in the same fashion as in *C* (dot graph and bar graph format). *P < 0.05; **P < 0.01; ***P < 0.0005. Error bars represent the SD.

by treatment (54.5 ± 5.1% vs. 59.0 ± 7.4%; 8.2% increase; P = 0.39). Using ELISpot assays, we also assessed the effect of anti-NKG2D Ab treatment on the ability of nondiabetic control and type 1 diabetic LAK cells to secrete IFN-γ after incubation with Daudi stimulators (Fig. 4*D*). As with the cytotoxicity results, anti-NKG2D Ab stimulation had a more profound and significant effect on IFN-γ production by control LAK cells (183 ± 19 vs. 241 ± 19 spots; 31.7% increase; P = 0.031). In comparison, type 1 diabetic LAK cells treated with anti-NKG2D Abs displayed an insignificant rise (96 ± 8 vs. 116 ± 12; 20.8% increase; P = 0.096). These results suggest that a defect in the NKG2D-dependent activation pathway of type 1 diabetic NK cells may be responsible for their diminished effector functions.

Type 1 diabetic LAK cells exhibit defective NKG2D signaling. NKG2D-mediated effector functions are triggered through its association with the transmembrane adaptor molecule DAP10 (26,39). Coupling of NKG2D to DAP10 leads to formation of a multimolecular signaling complex and the activation of multiple downstream signaling cascades, including the PI3K-AKT pathway (summarized in Fig. 5*A*), which is critically involved in effector function, cell growth, and cell survival (39,40). To investigate whether NKG2D signaling is altered in type 1 diabetic subjects, we first measured PI3K association with NKG2D-DAP10 complexes in control and type 1 diabetic LAK cells after treatment with either anti-NKG2D Abs or control murine Abs (Fig. 5*B* and *C*). After Ab stimulation, NKG2D-DAP10 complexes were pulled down by immunoprecipitation and

probed with either anti-NKG2D or anti-p85 subunit of PI3K Abs. Strikingly, NKG2D stimulation resulted in the efficient association of PI3K with NKG2D-DAP10 complexes in LAK cells from three nondiabetic control subjects but not from type 1 diabetic subjects. To measure the activation status of PI3K and the downstream-acting serine/threonine kinase AKT, we next used reverse-phase protein lysate microarrays to measure their phosphorylation with phospho-(P)–specific Abs, as previously described (30). NK cells

expanded from six type 1 diabetic subjects, and six nondiabetic control subjects were serum-starved for 4 h then stimulated with anti-NKG2D Abs over a time course of 5 min and their lysates probed with two P-AKT(S473)–, one P-AKT(T308)–, and one P-PI3K p85(Y458)-specific Abs (Fig. 5D). The use of two separate Ab clones, both recognizing P-AKT(S473), allowed us to assess the internal reproducibility of the assay. Robust phosphorylation of PI3K and AKT was detected in all six control samples

FIG. 5. Type 1 diabetic LAK cells exhibit defective NKG2D signaling. *A*: DAP10 phosphorylation at its "YINM" motif (blue box) results in the activation of the PI3K pathway. *B*: Type 1 diabetic (T1D; *n* = 3; C052, C053, and C068) and control (Ctl; *n* = 3; D068, D069, and D088) LAK cells were stimulated with either anti-NKG2D Ab or murine IgG for 15 min. NKG2D was pulled down with anti-NKG2D Abs and blotted with either anti-NKG2D or anti-PI3K Abs. *C*: Densitometric measurements are presented on NKG2D and PI3K band intensities and ratios of cumulative means ± SD. *P < 0.05. *D*: Purified NK cells from nondiabetic control subjects (C; *n* = 6) and type 1 diabetic subjects (D; *n* = 6) were stimulated with anti-NKG2D Abs for a time course. Reverse-phase protein lysate microarrays were used to detect phosphorylation of p85 PI3K and AKT. Results are expressed as log base 2 MFI ratio values and presented as heatmaps of phosphorylation changes over time, with yellow reflecting an increase, blue reflecting a decrease compared with baseline time zero, and black representing no change. *E*: Cumulative data from control and type 1 diabetic samples in *D*. *P < 0.05; **P < 0.01; ***P < 0.005. Error bars represent the SD.

over the sampled times. By contrast, five of six type 1 diabetic samples showed no evidence of stimulation-induced phosphorylation and three of six in this group exhibited stimulation-induced dephosphorylation. The cumulative mean phosphorylation by type 1 diabetic NK cells was significantly decreased relative to control samples at both 1 and 5 min after anti-NKG2D stimulation (Fig. 5E). Together, these findings suggest that impaired effector functions by type 1 diabetic LAK cells may be a consequence of aberrant signaling through the NKG2D receptor.

DISCUSSION

Our analysis of PBMCs from type 1 diabetic subjects revealed that NK cell frequency (CD3⁻CD56⁺) was decreased ~37% relative to age-matched nondiabetic control subjects (Fig. 1). Rodacki et al. (41) also have reported that NK cell frequencies were reduced in type 1 diabetic subjects, although in their study, the reduced frequencies were present in recent-onset (<1 month) but not in long-standing (>1 year; mean 10 years postdiagnosis) type 1 diabetic subjects. It is not clear why those data differ from our findings. Our observation that decreased NK cell frequencies in PBMCs from type 1 diabetic subjects were associated with impaired responsiveness to IL-2/IL-15 stimulation suggests that cell-intrinsic mechanisms may be responsible for their reduced frequencies (Fig. 2). Horng et al. (42) have proposed that murine NK cell homeostasis and NKG2D function are coregulated through the coupling of NKG2D and IL-15 receptors, suggesting that a common pathway may be responsible for defects in both cytokine responsiveness and NKG2D function exhibited by type 1 diabetic NK cells. Consistent with these findings, NKG2D-deficient mice possess perturbations to NK cell numbers, NK cell apoptosis, and NK cell proliferation, implying that NKG2D plays a critical role in the regulation of NK cell homeostasis (43).

The decreased responsiveness to IL-2/IL-15 led us to compare markers of NK cell activation and differentiation between type 1 diabetic and nondiabetic control NK cells directly ex vivo and after cytokine stimulation (Fig. 3). Of the NK cell markers assessed, the only difference seen between type 1 diabetic and control NK cells was in the failure of type 1 diabetic NK cell to downmodulate expression of the NKG2D ligands MICA/B. However, despite aberrant maintenance of MICA/B expression on activated type 1 diabetic NK cells, we did not see signs of NKG2D receptor downmodulation, a PI3K-dependent phenomenon seen in NOD NK cells (27). The finding that surface levels of NKG2D on type 1 diabetic NK cells continued to match closely those of nondiabetic control NK cells suggested that impaired NKG2D function by type 1 diabetic NK cells was a consequence of downstream (intracellular) signaling rather than insufficient receptor expression (Fig. 3). Consistent with this interpretation and with the diminished NKG2D-mediated effector function observed (Fig. 4C and D), type 1 diabetic NK cells were found to possess an intracellular signal transduction defect proximal to the NKG2D receptor affecting the PI3K-AKT pathway (Fig. 5). Additional examination of NKG2D signal transduction in type 1 diabetic NK cells, including the Grb2/Vav1 pathway, is being pursued.

NK cells share an increasing number of traits with the adaptive immune system, including the formation of self-tolerance and the generation of long-lived memory cells,

despite their exclusive use of germline-encoded antigen receptors (44,45). Continuous exposure of NK cells to ligands recognizing their activating receptors has been shown to result in NK cell tolerance and, therefore, argues that regulatory mechanisms exist to limit their autoimmune potential (46,47). Moreover, these experiments suggest that the expression of NKG2D ligands on activated type 1 diabetic NK cells could result in their chronic stimulation through the NKG2D receptor, inducing NK cell hyporesponsiveness. Notably, ectopic expression of the murine NKG2D ligand Rae-1ε in the epithelium of mice has been shown to result in NKG2D downregulation and defective NK cell cytotoxicity (48). Our findings of dysregulated NKG2D ligand expression on type 1 diabetic NK cells are reminiscent of a previous report (27) describing the expression of NKG2D ligands on activated NK cells from diabetes-prone NOD, but not diabetes-resistant C57BL/6, mice. In NOD mice, it has been postulated that the expression of NKG2D ligands by activated NK cells results in chronic NKG2D stimulation, NKG2D downmodulation through PI3K-dependent ligand-induced internalization, and, eventually, desensitization (27). As a consequence of the aforementioned study, as well as our own, we hypothesize that chronic exposure to NKG2D ligands, either on the same cell (cis) or on an adjacent NK cell (trans), may result in prolonged signaling and eventually lead to NK cell dysfunction.

Given their propensity to produce IFN-γ and kill other cells, NK cells may influence the development of autoimmune diseases through direct tissue destruction or indirectly via the regulation of adaptive immune responses or the modification of antigen-presenting cells (49). Examples of NK cells playing a causative role in disease exist (5); for instance, NK cells have also been suggested to mediate a protective function in subjects with multiple sclerosis and their depletion in rodent models of experimental autoimmune encephalomyelitis exacerbates autoimmunity (50,51). In addition, low NK cell activity has been observed in other autoimmune settings, including systemic lupus erythematosus (SLE) subjects and the lpr murine model of SLE. Adoptive transfer of NK1.1⁺ cells into lpr mice has been found to slow down the lupus-like disease process (52–54). With respect to type 1 diabetes, we have previously shown that enhancement of NK cell function through CFA treatment, resulting in improved NKG2D receptor levels and decreased NKG2D ligand expression, reduces autoreactive CTL numbers and protects NOD mice from disease (28,29). The data above indicate that NK cells in type 1 diabetic subjects are defective in number, signaling, and function and suggest that augmentation of NK cell function may prove valuable as an immune-modifying therapy for type 1 diabetes or other autoimmune diseases.

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