The effect of silencing NKG2D through RNA interference on receptor functions in interleukin-2-activated human natural killer cells

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Natural killer (NK) cells are effectors of the innate immunity involved in tumor surveillance. NKG2D is a potent activating receptor eliciting cytokine and cytolytic NK responses upon recognition of tumor-associated ligands. We engineered primary interleukin (IL)-2-activated human NK cells to express constitutively low levels of NKG2D by lentiviral delivery of small interfering RNA. NKG2D-mediated effector functions were strongly impaired in NKG2D^{low} NK cells. Reduction of NKG2D surface expression to 15%, corresponding to receptor levels in resting NK cells, rendered cells fully insensitive to NKG2D triggering. These data underscore the importance of NKG2D receptor cell surface density and suggest a threshold of expression for optimal reactivity of human NK cells.

Key words: siRNA, activating receptor, interferon, cytotoxicity, innate immunity.

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The activating immunoreceptor NKG2D is expressed on all human natural killer (NK) cells, CD8$^+$ αβ and γδ T cell subsets. Triggering of NKG2D by its ligands, the UL16-binding proteins (ULBP) and the major histocompatibility complex class I chain-related (MIC) proteins, or by antibody-crosslinking leads to NK cell proliferation, cytokine production and cytotoxicity.[1] NKG2D levels on resting human peripheral blood NK cells can be strongly increased by exposure to interleukin (IL)-2 and IL-15 *in vitro*[2] and in response to IL-15 at inflammatory sites *in vivo*.[3] On the other hand, NKG2D can be systemically down-regulated by transforming growth factor-β1 and through endocytosis induced by soluble MIC proteins shed by solid cancers and leukemic blasts, possibly resulting in immunoevasion.[4-6] We have recently demonstrated that NKG2D expression is significantly reduced in NK cells from up to 50% of patients with acute myeloid leukemia (AML).[7] All these studies brought evidence of the significance of NKG2D levels in tumor recognition, but receptor levels limiting the NK cell response have not been defined. To study the specific contribution of NKG2D receptor density to cytokine production and cytotoxicity by human NK cells, we targeted NKG2D expression by small interfering (si) RNA. Here we provide evidence that a threshold for NKG2D cell surface expression must be exceeded to trigger NK cell effector functions and ensure immune surveillance. These data have implications for currently explored NK cell-based immunotherapies against leukemias and solid cancer.

Design and Methods

Lentiviral NKG2D-siRNA vector

The NKG2D siRNA (5'-GGACCAGGATTTACTTAAA-3') was selected based on GenScript's siRNA target finder (Genscript) and subcloned into modified pSUPER vector. pLV-siNKG2D was generated by inserting the NKG2D siRNA expression cassette into the ClaI site of lentiviral vector pWPXL upstream of the green fluorescence protein (GFP).[8] Lentiviruses were produced as described.[9]

Transduction and culture of primary human NK cells

NK cells (CD56$^+$CD3$^-$) were purified from peripheral blood of healthy donors.[7] After 2 days in Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA, USA), 5% human serum and 100 units/mL IL-2 (Novartis, Basel, Switzerland), 1×10^5 cells were transduced with lentiviral vectors pLV-siNKG2D and pWPXL at a multiplicity of infection (MOI)=20-30.[8] Five days later, transduced cells were restimulated with phytohemagglutinin (2 μg/mL; Murex Biotech Ltd., Dartford, England) and irradiated allogeneic mononuclear peripheral blood cell feeders.[7] pLV-siNKG2D-GFP$^+$ and control GFP$^-$ NK cell lines as well as control pWPXL-GFP$^+$ NK cell lines were purified by FACS sorting (FACSVantage; Becton Dickinson, San Diego, CA, USA). NK cell clones were generated by limiting dilution of GFP$^+$ NK cells. Cell lines and clones were maintained up to 3 months as independent cultures. Functional and phenotypic analyses were performed 10-24 days after stimulation.

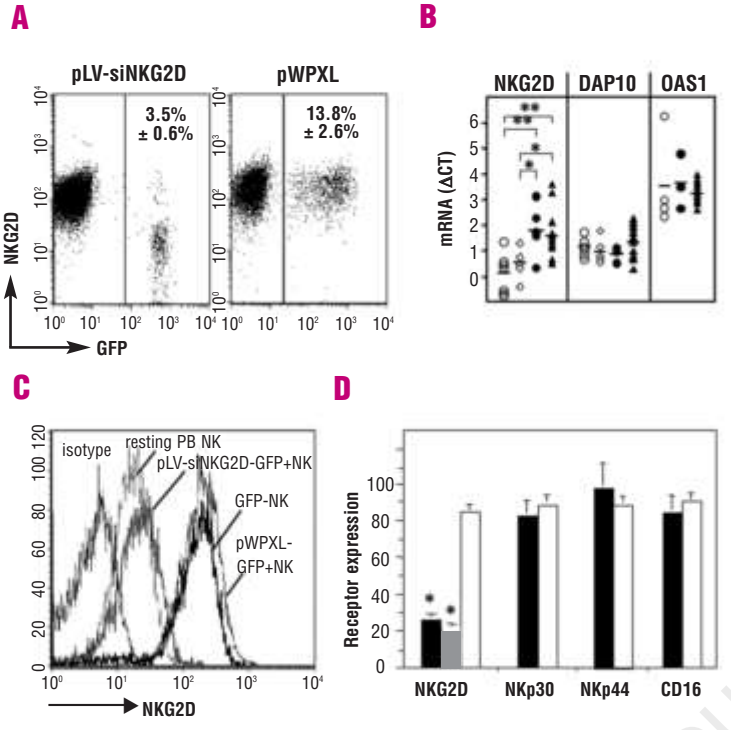

Figure 1. Gene silencing by lentiviral delivery of NKG2D siRNA. **A.** Efficiency of lentiviral transduction of IL-2-activated human NK cells. The percentages of GFP$^+$ cells after transduction with pLV-siNKG2D (n=4) and control pWPXL (n=6) vectors were determined by FACS. **B.** Quantitative RT-PCR analysis of NKG2D, DAP10 and OAS1 mRNA in FACS-sorter purified GFP$^-$ (○) and pWPXL-GFP$^+$ control NK cell lines (◇), and in LV-siNKG2D-GFP$^+$ NK cell lines (●) and clones (▲). ΔCT (cycle threshold) of single measurements and mean values (–) are shown (**p<0.01, *p<0.05; unpaired t-test). **C.** NKG2D expression profile of transduced NK cells and resting peripheral blood NK cells. **D.** Receptor expression in pLV-siNKG2D-GFP$^+$ NK cell lines (■) and clones (■) and pWPXL-GFP$^+$ NK cells (□). Receptor levels in GFP$^+$ cells were calculated as percentage of mean fluorescence intensity ratios compared to GFP$^-$ cells set as 100%. NKG2D levels were significantly reduced in pLV-siNKG2D- but not in pWPXL-transduced NK cells (*p<0.001). NKp30, NKp44 and CD16 surface levels were not changed. Error bars indicate the SEM of 19-24 measurements.

Flow cytometry (FACS)

Cells were stained with monoclonal antibodies against human NKG2D, CD3, CD16, CD56 (BD Pharmingen, San José, CA, USA), NKp30 and NKp44 (Beckman Coulter, Marseille, France) or isotype controls.[7] FACS data were analyzed with FACSCalibur and CellQuestPro software (Becton Dickinson).

RNA analysis

RNA was isolated with Trizol (Invitrogen). Reverse transcriptase polymerase chain reaction (RT-PCR) was carried out with primers for NKG2D (5'-CAGTGGGAAGATGGCTCC-3'; 5'-CAGTCTCC-CTTCTGCATTTCAAT-3'), DAP10 (5'-TCCCTGC-CTTTTACCCTGG-3'; 5'-CAGGAGCGGCAGAGA-GAGG-3') and 2'-5'-oligoadenylate synthase 1 (OAS1: 5'-TCCAAGGTGGTAAAGGGTGG-3'; 5'-AGGTCAGCGTCAGATCGGC-3'). The primer for the RPL19 reference gene and conditions for quantitative real time RT-PCR have been described elsewhere.[10]

Interferon-γ release and cytotoxicity assays

NK cells (1×10^5) were stimulated for 24 hours with plastic-coated ULBP1-Fc or Ig-Fc (10 μg/mL; R&D Systems, Minneapolis, MN, USA) or with 10 U/mL IL-12 and 100 ng/mL IL-18 (PeproTech, Rocky Hill, NJ, USA) as a control.[11] Interferon (IFN)-γ was measured in cell-free culture supernatants in duplicate by an enzyme linked immunosorbent assay (ELISA) using human IFN-γ-specific 43-11 and 45-15 monoclonal antibodies (provided by Ch. Heusser, Novartis). The cytotoxic activity of NK cells against human Daudi cells was determined using the calcein-acetyoxymethyl-based killing assay.[12] For re-directed killing assays, FcR$^+$ P815 target cells were preincubated with αNKG2D monoclonal antibody (3 μg/mL for 20 min at 37°C; clone M585, provided by D. Cosman, Amgen Inc., Seattle).[12]

Results and Discussion

Efficient silencing of NKG2D expression in human NK cells by specific siRNA

Stable silencing of NKG2D expression was achieved in human peripheral blood NK cells transduced with lentiviral vector pLV-siNKG2D containing the siRNA expression cassette and GFP reporter gene. Transduction efficiency of NK cells was 3.5%±0.6% with pLV-siNKG2D and 13.8%±2.6% with control vector pWPXL (Figure 1A). Three independent pLV-siNKG2D-GFP$^+$ NK cell lines and five clones were generated by FACS sorting. GFP$^-$ and pWPXL-GFP$^+$ NK cell lines were generated as controls. Transduction with pLV-siNKG2D significantly decreased NKG2D mRNA in GFP$^+$ as compared to in GFP$^-$ and pWPXL-GFP$^+$ NK cells (Figure 1B). The mRNA levels of DAP10, the adaptor for NKG2D receptor,[13] and OAS1, an IFN-stimulated gene, were not affected by lentiviral transduction. NKG2D cell surface levels in pLV-siNKG2D-GFP$^+$ NK cell lines and clones were strongly reduced to, respectively, 25%±3.5% and 19.6±1.0% of levels in GFP$^-$ cells, resembling NKG2D levels in freshly isolated resting peripheral blood NK cells (Figure 1C and 1D). Reduction

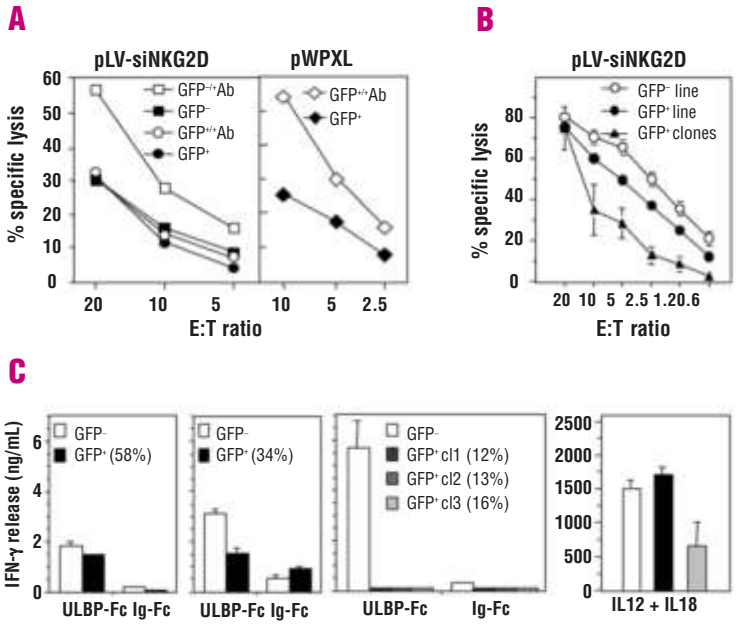

Figure 2. Suppressed NKG2D-mediated effector functions of siNKG2D-transduced NK cells. A. Re-directed killing of P815 cells by GFP⁻ control NK cells (□), by pLV-siNKG2D-GFP⁺ NK cells (○) expressing 15% of NKG2D levels, and by pWPXL-GFP⁺ control NK cells (◇) in the presence (open symbols) or absence (filled symbols) of αNKG2D monoclonal antibody. The mean values of specific lysis at the indicated effector: target (E:T) ratios measured in triplicate are shown. B. Cytolysis of Daudi cells by GFP⁻ NK cell line (○), pLV-siNKG2D-GFP⁺ NK cell line (●, as in Figure 2A) and clones (▲; cl1, 2 and 3, as in Figure 2C). The mean values of specific lysis ± SEM. C. IFN-γ release by GFP⁻ control (□) and by pLV-siNKG2D-GFP⁺ NK cell lines (■) and clones (cl1, 2 and 3; gray bars) upon stimulation with ULBP1-Fc protein, measured by ELISA. GFP⁺ NK cell lines with 58% and 34% and GFP⁺ NK cell clones with 12%-16% of NKG2D expression, compared to GFP- control, were used. Stimulation with Ig-Fc protein and with IL-12 and IL-18 served as negative and positive controls for the IFN-γ response of GFP⁻ NK cell lines (□), pLV-siNKG2D-GFP⁺ NK cell lines (■) and clones (gray bar). Error bars indicate standard deviations >0.05 ng/mL.

in surface NKG2D expression is unlikely to be due to limiting DAP10 molecules because DAP10 mRNA levels in transduced cells were unaffected. Silencing of NKG2D expression remained stable over an observation period of 3 months involving multiple stimulation cycles to expand transduced NK cells. In contrast to the NKG2D target, surface expression of other activating NK receptors, NKp30, NKp44 and CD16, was not altered. Control pWPXL-GFP⁺ NK cells expressed normal levels of receptors, including NKG2D, comparable to receptor levels in GFP⁻ cells, thus confirming the results of mRNA measurements (Figure 1D). These results demonstrate specific and persistent silencing of NKG2D in primary human NK cells.

Strongly diminished effector functions of siRNA-transduced NKG2Dlow human NK cells

The functional relevance of reduced levels of surface NKG2D was determined by measuring cellular cytotoxicity and IFN-γ production. The cytotoxic activity of transduced NKG2D^{low} NK cells was strongly diminished. In re-directed killing assays using P815 target cells coated with αNKG2D monoclonal antibody, NKG2D-specific triggering of cytolysis was induced by both control GFP- and pWPXL-GFP⁺ NK cells but not by pLV-siNKG2D-GFP⁺ NK cells expressing a residual 15% of NKG2D (Figure 2A). Accordingly, the capacity of pLV-siNKG2D-GFP⁺ NK cell lines and clones to kill Daudi cells was lower than that of GFP⁻ control cells over a wide range of effector: target ratios (Figure 2B). This reduction was partial, which likely reflects the involvement of other activating receptors, present at unchanged levels, on transduced NK cells.

When stimulated with recombinant ULBP1-Fc, pLV-siNKG2D-GFP⁺ NK cell lines expressing 58% and 34% of control NKG2D levels, produced 80% and 50% of IFN-γ compared to control cells (Figure 2C), i.e. the degree of NKG2D silencing in GFP⁺ NK cells was paralleled by a reduction in cytokine release. Notably, three GFP⁺ NK clones with NKG2D levels as low as 15% did not respond to ULPB1 triggering (IFN-γ release below the detection limit of 0.2 ng/mL). The failure of GFP⁺ NKG2D^{low} cells to produce IFN-γ may explain why freshly isolated resting peripheral blood NK cells from healthy donors with similarly low receptor levels (Figure 1C) are refractory to NKG2D stimulation.[14] High release of IFN-γ upon stimulation with IL-12 and IL-18 (>500 ng/mL; Figure 2C) indicated that IFN-γ production was not affected in NKG2D-silenced NK cells. We thus confirmed a recent report on reduced cytotoxicity of polyclonal NK cells after NKG2D-siRNA delivery[15] and extended the findings by showing that NKG2D^{low} cells, which are refractory to NKG2D-specific stimulation of cellular cytotoxicity, also fail to produce IFN-γ.

The importance of NKG2D for anti-tumor immunity is emphasized by the severely impaired function of murine NK cells *in vivo* upon NKG2D ligand-induced down-regulation of receptor levels.[16,17] In human cancer, NKG2D receptor expression below threshold level may be particularly detrimental for the recognition of those malignant cells, such as AML blasts, which express low levels of the ligands.[12] Our findings of abrogated cytokine and cytolytic responses by NK cells with limiting NKG2D levels have implications for the clinical efficacy of adoptively transferred NK cells to prevent relapses in leukemias and solid tumors.[18,19] Purified

healthy donor-derived NK cells used for infusions expressed NKG2D at levels resembling those on NKG2D^{low} transduced NK cells (*Stefan Diermayr and CK, unpublished observations, January 2006*). We therefore suggest that the use of cytokines, such as IL-2, to increase NKG2D expression above a critical threshold level may be necessary for optimal reactivity of NK cells in immunotherapy trials.

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