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IL-21 Down-Regulates NKG2D/DAP10 Expression on Human NK and CD8$^+$ T Cells[1]

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IL-21 is a recently described cytokine, produced by activated Th cells, that shares significant homology with members of the IL-2 family of cytokines. IL-21 mediates its biological effects via the IL-21R in conjunction with the common receptor γ-chain that is also shared by members of the IL-2 family. We report that culture of human primary NK and CD8$^+$ T cells with IL-21 in combination with IL-2 results in significant reduction of the cell surface expression of NKG2D, compared with that in cells treated with IL-2 alone. The reduced expression of NKG2D after IL-21 culture had functional consequences for NK cell function, as assessed by NKG2D-mediated redirected lysis assays and degranulation assays, compared with NK cells treated with IL-2 alone. IL-21-mediated NKG2D down-regulation in human NK cells correlated with a marked reduction of DNAX-activating protein of 10 kDa (DAP10) transcription in cells treated with IL-2 in combination with IL-21 compared with cells stimulated with only IL-2. This was attributed to a dramatic reduction in DAP10 promoter activity, as assessed by a DAP10 luciferase reporter construct. In contrast to NKG2D expression, IL-21 was able to induce the expression of the NK activation receptors NKp30 and 2B4 as well as the costimulatory receptor CD28 on CD8$^+$ T cells. These data indicate that IL-21 is able to channel NK and CD8$^+$ T cell function by altering the expression pattern of activation/costimulatory receptors. *The Journal of Immunology,* **2006, 176: 1490–1497.**

Natural killer cells are components of the innate immune system and function in part to eradicate virally infected or tumorigenic cells without previous sensitization. To achieve accurate target cell lysis, NK cells express a wide array of activation receptors (1, 2). NKG2D is an activation receptor expressed on all NK cells, γδ T cells, and the majority of CD8$^+$ T cells (3). The ligands for human NKG2D are structurally diverse and include the MHC class I chain-related proteins A/B and the UL16-binding proteins (4–6). The ligands for NKG2D are usually absent on normal cells, but are often up-regulated in many types of cancers (7) and, upon ligand recognition, can lead to NKG2D-mediated tumor clearance (8). NKG2D has no identifiable signaling motifs, but, instead, attains signaling capability through association with DNAX-activating protein of 10 kDa (DAP10),[3] which contains an YxxM motif that can activate the PI3K signaling pathway upon NKG2D ligation (9).

It has been reported that IL-2, IL-7, and IL-15 can induce and maintain NKG2D/DAP10 expression in T cells (10, 11). IL-21 is a recently described member of the IL-2 family and shares significant homology with this group of cytokines (12). It is expressed by activated Th cells (13) and binds to a heterodimeric receptor composed of a specific IL-21R chain and a γ-chain that is also shared by IL-2, IL-4, IL-7, IL-9, and IL-15 cytokine receptors (14). Significant interest has recently been focused on the potential of IL-21 to activate NK cells and CTL, thereby promoting antitumor activity (15–17). It has previously been reported that culture of human NK cells with IL-21 can induce NK cell maturation (18) and up-regulate various NK cell receptors, including the NKp46, NKp30, and CD16 triggering receptors (19). However, the potential of IL-21 to regulate NKG2D/DAP10 expression is currently unknown.

We report the novel observation that stimulation of primary human NK and CD8$^+$ T cells with IL-21 in combination with IL-2 results in the down-regulation of cell surface NKG2D/DAP10 receptor expression compared with that in cells treated with IL-2 alone. Suppression of NKG2D/DAP10 expression in human primary NK cells occurred at the level of transcription, because IL-21 was able to reduce DAP10 transcript expression, as shown by real-time RT-PCR, and also the reporter activity of a DAP10 luciferase construct. IL-21-mediated reduction of NKG2D/DAP10 expression in human primary NK cells led to decreased cytolytic function. These results suggest that the increased NK cytolytic activity observed after culture with IL-21 is likely to be independent of NKG2D/DAP10. Thus, IL-21 can act to differentially regulate NK cell activation receptor expression, perhaps to skew the specificity of NK cell target recognition.

Materials and Methods

Abs and flow cytometry

PE-conjugated anti-NKG2D, anti-IL-21R (R&D Systems), anti-NKG2A, anti-2B4 (CD244), and anti-CD56 (Beckman Coulter) were used for phenotypic analyses. Anti-NKp30 (R&D Systems) and PE-conjugated goat anti-mouse IgG (Beckman Coulter) were used in the indirect immunofluorescence assay. Purified anti-NKG2D (clone 149810, mouse IgG1; R&D Systems) was used for cell stimulations. The anti-phospho-STAT sampler kit (Cell Signaling Technology), anti-phospho STAT4 (Invitrogen Life Technologies), and anti-β-actin (Sigma-Aldrich) were used for cytokine signaling studies. Flow cytometric analyses were performed on a FACSort

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[3] Abbreviations used in this paper: DAP10, DNAX-activating protein of 10 kDa; MFI, mean fluorescence intensity; NCR, natural cytotoxic receptor.

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cytofluorometer (BD Immunocytochemistry Systems). PE-conjugated isotype-matched control mAb (e-Bioscience) was used to monitor background staining levels. FITC-conjugated anti-CD107a and anti-CD107b (BD Biosciences) were used for NK cell degranulation assays.

Cell isolation and culture conditions

Polyclonal NK and $CD8^+$ T cells were isolated by negative selection from peripheral blood using an NK or $CD8^+$ T cell isolation kit (Miltenyi Biotec). The human NK and $CD8^+$ T cells used in this study were routinely found to be >97% pure. Freshly purified NK and $CD8^+$ T cells were cultured in IMDM (BioWhittaker) supplemented with 100 U/ml rIL-2 (Biological Resources Branch, National Cancer Institute, Frederick Cancer Research and Development Center), 10% human AB serum (BioWhittaker), and 2 mM GlutaMax (BioSource International). The human NK leukemia cell line, NKL, was grown in RPMI 1640 (BioSource International) supplemented with 2 mM GlutaMax, 1 mM sodium pyruvate (Invitrogen Life Technologies), 200 U/ml rIL-2, 5 μg/ml plasmocin (InvivoGen), and 10% FBS (BioWhittaker). P815 cells were cultured in RPMI 1640 supplemented with 2 mM GlutaMax, 1 mM sodium pyruvate, 10% FBS, and 5 μg/ml plasmocin. All cells were cultured at 37°C under an atmosphere of 5% CO_2.

Where appropriate, cells were treated with IL-21 (Biosource International) at different concentrations (1–100 ng/ml) for 5–7 days before analysis. Cells were restimulated with 100 U/ml IL-2 and IL-21 (1–100 ng/ml) on day 3 of the experiment.

Plasmid construction

All DNA fragments were derived by PCR using platinum *Taq* polymerase (Invitrogen Life Technologies) with human genomic DNA (Promega) as template. A DAP10 luciferase construct was subcloned into the *Nhe*I restriction site of the pGL3-basic reporter plasmid (Promega). The primers used to generate the DAP10 luciferase constructs were: DAP10 forward, 5′-CTAGCTAGCTTCTTGGCCCTACCTCC-3′; and DAP10 reverse, 5′-CTAGCTAGCGCATGTTGATGTAGACTTTGCC-3′. All plasmid DNA used for transfections were purified with a Qiagen Plasmid Mega kit, according to the manufacturer's protocol. Restriction enzymes were purchased from New England Biolabs. Custom-synthesized oligonucleotides were supplied by Sigma-Genosys. Plasmid inserts were verified by sequence analysis.

Transfection and luciferase assay

NKL cells (2.5×10^6) were transiently transfected with 2.5 μg of the reporter plasmid and 0.25 μg of the pRL-CMV *Renilla* control vector (Promega), which acts as an internal control for the normalization of transfection efficiency. Electroporation was performed using the Amaxa nucleofection system as previously described (20). Cells were harvested 48 h after transfection and lysed. The specific luciferase activity of cell lysates was measured on a Monolight 3010 luminometer (Analytical Luminescence Laboratory). The dual-luciferase, double-reporter assay system and substrates were purchased from Promega.

RNA isolation and RT-PCR

Total RNA was isolated from cultured human primary NK cells using the RNAqueous-4PCR kit (Ambion), including DNase treatment, according to the manufacturer's instructions. Synthesis of cDNA was performed by the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies). Samples were analyzed by real-time PCR (MJ Research; DNA Engine Opticon 2) using the QuantiTect SYBR Green PCR kit (Qiagen). A melting curve was performed at the end of each run to verify that there was a single amplification product and a lack of primer dimers. Standard curves obtained using a serial 10-fold dilution of human primary NK cell cDNA were generated to determine the level of each amplified transcript, and all samples were normalized to the amount of GAPDH transcript present in each sample. Primers for GAPDH were the RT-PCR GAPDH Primer Set (SuperArray Bioscience). Primers for DAP10 were; forward, 5′-TCCATCTGGGTCACATCCTCTTCC-3′; and reverse, 5′-AAGAGCCTGAAGTGCCAGGGTAAAAG-3′. Real-time PCRs were performed in triplicate.

Western blot analysis of IL-21-mediated STAT phosphorylation

Primary NK cells (1×10^7) were stimulated overnight with IL-2 (100 U/ml), IL-21 (25 ng/ml), or both cytokines in combination or were left in NK cell medium alone as an unstimulated control. Nuclear extracts were isolated using a Nuclear Extract Kit (Active Motif), and samples were analyzed by SDS-PAGE, followed by immunoblotting as previously described (20). Band intensities were quantified using UN-SCAN-IT gel software (Silk Scientific).

Cytotoxicity assays

NK cells were cultured with IL-2 or IL-2/IL-21 in combination for 5–7 days as described above. The cytotoxic assays were performed as previously described (21). Briefly, the FcγR-positive P815 mouse mastocytoma cell line labeled with 10 μM CFSE (Molecular Probes) was used as a target cell. CFSE-stained P815 cells were washed three times with IMDM medium containing 10% FBS and 1% GlutaMax, followed by incubation at 37°C for 2 h. After that, P815 cells were washed twice to remove the excess dye and adjusted to a concentration of 10^5 cells/ml. Cytokine-treated NK and target cells were mixed in 200 μl of complete medium at an E:T cell ratio of 10:1 in the absence or the presence of different concentrations of anti-NKG2D or isotype control mAb. Cells were incubated at 37°C for 3–4 h, then 200 μl of a solution of 0.5 μg/ml propidium iodide (BD Biosciences) was added, and dead target cells were quantified by flow cytometric analysis.

NK cell degranulation assays

NK cells were cultured with IL-2 alone or in combination with IL-21 for 5–7 days as described above. Degranulation assays were performed as previously described (22). Briefly, cytokine-treated NK cells were washed in PBS and resuspended at 1×10^6 cells/ml in NK cell medium, then aliquoted onto 24-well plates coated with different concentrations (0.1–5 μg) of anti-NKG2D or anti-isotype control mAb and incubated for 2 h at 37°C in the presence of CD107a/b-FITC mAb. The secretion inhibitor, monensin (BD Biosciences), was added to the culture after 1 h. NK cell degranulation was assessed by cell surface staining for the lysosomal markers CD107a/b by flow cytometry.

Results

Expression of IL-21R on NK cells and effect of IL-21 on NK cell proliferation

IL-21R has been shown to be expressed on T, B, and NK cells (23). We confirmed the presence of IL-21R on freshly isolated primary human NK cells (Fig. 1*A*). IL-21R was expressed at low. but detectable. levels. with about a 3-fold greater staining intensity

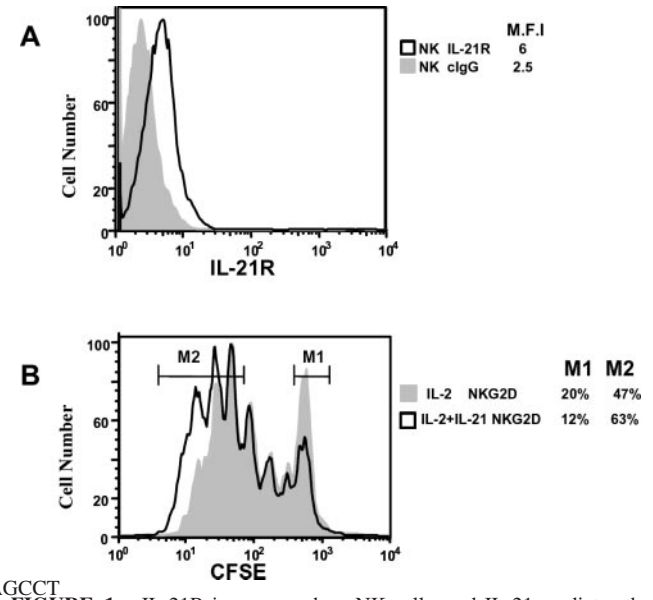

FIGURE 1. IL-21R is expressed on NK cells, and IL-21 mediates the expansion of IL-2-stimulated NK cells. *A*, Freshly isolated human NK cells were stained with anti-IL-21R mAb to determine cell surface expression of IL-21R by FACS analysis. Mean fluorescent intensities (M.F.I) are indicated. *B*, CFSE-labeled primary NK cells were cultured in the presence of IL-2 (100 U/ml) with and without IL-21 (25 ng/ml) for 5 days and analyzed by FACS to determine the rate of NK division. Quantification of $CFSE^{high}$ (nondividing cells) and $CFSE^{low}$ NK cells is provided. Data shown are representative of three independent experiments.

over the isotype control Ab. IL-21 is known to act synergistically with IL-15 in NK cell (24) and CD8$^+$ T cell (25) expansion, but it does not enhance the expansion of IL-2-stimulated CD8$^+$ T cells (25). In this regard, we assessed the role of IL-21 in mediating the expansion of IL-2 (100 U/ml)-stimulated primary human NK cells. Culturing CFSE-labeled primary human NK cells with IL-21 in combination with IL-2 for 5 days resulted in a modest increase in cell division compared with that in IL-2-treated cells (Fig. 1*B*). The observed increase in IL-2- plus IL-21-mediated division, although moderate, was supported by an increase in total cell numbers of NK cells (typically, a 1.5- to 2-fold increase in cell numbers in IL-2/IL-21-cultured cells, compared with IL-2-cultured cells). Culture of primary NK cells with IL-21 alone for 5–7 days did not induce any observable cell proliferation (data not shown). We also found a larger and more blast-like appearance, as observed by increased forward and side scatter of IL-2- plus IL-21-cultured NK cells compared with cells cultured with IL-2 alone (data not shown). Thus, all future experiments with IL-21 in this study, unless otherwise stated, were performed in the presence of IL-2.

Culture of primary NK and CD8$^+$ T cells with IL-21 down-regulates cell surface NKG2D receptor expression

The cytokines IL-2, IL-7, and IL-15, all of which share the γ-common chain, have previously been shown to induce and maintain NKG2D expression in CD8$^+$ T cells (10, 11). We therefore investigated the effect of IL-21 on the ability to regulate cell surface expression of NKG2D. We observed consistent and significant decreases in cell surface NKG2D expression on human primary NK cells after culture with IL-21 (25 ng/ml) in combination with IL-2 compared with cells treated with IL-2 alone (Fig. 2*A*). IL-21-mediated down-regulation of NKG2D was observed for multiple human donors (Fig. 2*B*) and also in the NKL leukemic cell line (data not shown). In addition, we noted that a range of IL-21 concentrations from 1–100 ng/ml could decrease cell surface expression of NKG2D (data not shown). IL-21-mediated down-regulation of NKG2D was only noticeable after 4–7 days of IL-2 plus IL-21 treatment (Fig. 2*C*), which suggests that the IL-21-mediated NKG2D receptor down-regulation becomes apparent only after cell surface NKG2D, which is present before IL-21 stimulation, is degraded.

We repeated similar experiments with primary human CD8$^+$ T cells. Culture of human CD8$^+$ T cells for 5–7 days with IL-2 and IL-21 also resulted in a reduction (~50% reduction) in cell surface NKG2D (Fig. 3) compared with cells stimulated with IL-2 alone. In contrast, however, we observed a consistent increase (25%) in CD28$^+$CD8$^+$ T cells after IL-2 plus IL-21 cytokine stimulation compared with IL-2 alone (Fig. 3*B*). The increase in CD28 expression after IL-21 stimulation of IL-15-treated human naive CD8$^+$ T cells has been recently reported (26). Our data indicate that IL-2 plus IL-21 combined cytokine stimulation of CD8$^+$ T cells can result in differential regulation of the costimulatory receptors CD28 and NKG2D.

IL-21 stimulation of NK cells results in phosphorylation of multiple STAT proteins

IL-21 binds a heterodimeric receptor composed of the IL-21R and the common γ-chain receptor, an indispensable requirement for IL-21 signaling, which, in turn, activates JAK3 kinase, leading to phosphorylation of STAT proteins (27). To define a mechanism for IL-21-mediated NKG2D down-regulation, we investigated the STAT phosphorylation profile in primary human NK cells after 12-h stimulation with IL-2 (100 U/ml) alone or in combination with IL-21 (25 ng/ml). Using phospho-specific Abs to various

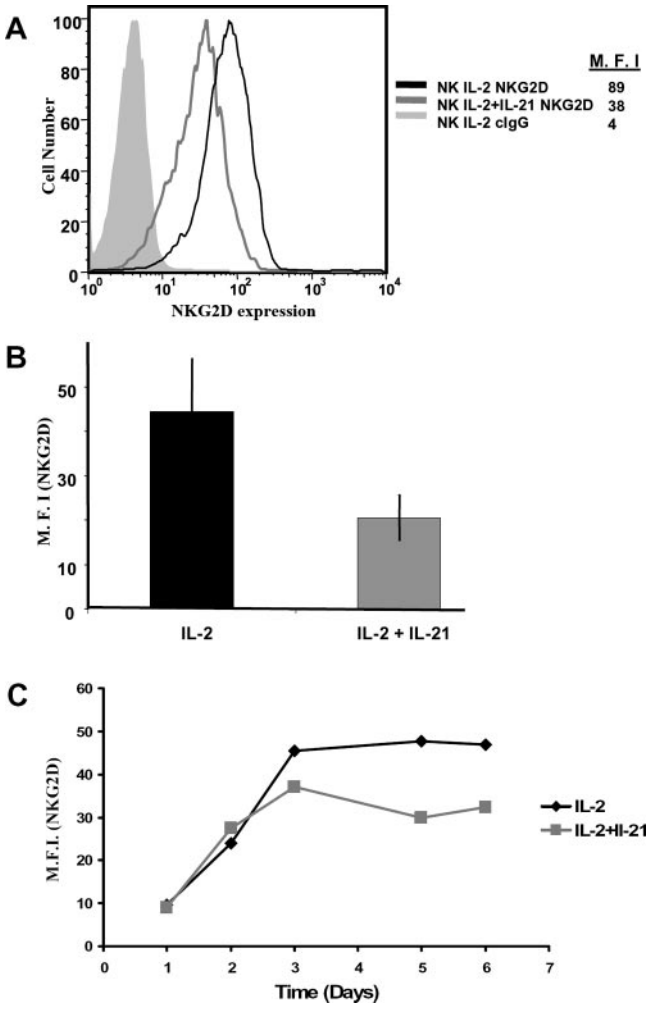

FIGURE 2. Human primary NK cells cultured with IL-2 plus IL-21 down-regulate NKG2D expression. *A*, Primary NK cells (1×10^6) were cultured with IL-2 (100 U/ml) with and without IL-21 (25 ng/ml) for 5 days, stained with anti-NKG2D mAb, and analyzed by FACS. The numbers in the histograms represent the M.F.I. *B*, Average IL-21-mediated down-regulation of NKG2D on NK cells from eight donors. *C*, Time course of NKG2D down-regulation in human NK cells after stimulation with IL-21. Data are representative of two independent experiments.

STAT proteins, we observed that IL-21 stimulation led to increased phosphorylation of STAT 1 (residue Tyr^{701}) in human NK cells compared with IL-2 stimulation alone (Fig. 4). Combined IL-2 and IL-21 stimulation had no observable additive effect on STAT1 phosphorylation. Stimulation with IL-21 alone induced specific phosphorylation of STAT3 (residues Tyr^{705} and Ser^{727}), whereas IL-2 stimulation alone showed little effect alone or in combination with IL-21 (Fig. 4). The phosphorylation of STAT3 and STAT1 induced by IL-21 supports previously published observations (28). IL-2 or IL-21 cytokine stimulation alone of NK cells led to phosphorylation of STAT4 (Tyr^{693}), with a novel synergistic effect observed when both cytokines were used in combination (Fig. 4). IL-21 alone revealed low detectable levels of STAT5A/B phosphorylation (residue Tyr^{694}) that were enhanced by costimulation with IL-2 (Fig. 4). In addition, we could not detect phosphorylation of STAT2 (Tyr^{690}), STAT6 (Tyr^{641}), or STAT5A/B (Ser^{726}) with either IL-2 or IL-21 stimulation, alone or in combination (data not shown). Taken together, the data suggest that IL-21 stimulation alone can regulate phosphorylation of multiple STAT proteins, with STAT3 being specifically phosphorylated by IL-21 stimulation, compared with IL-2. We also report

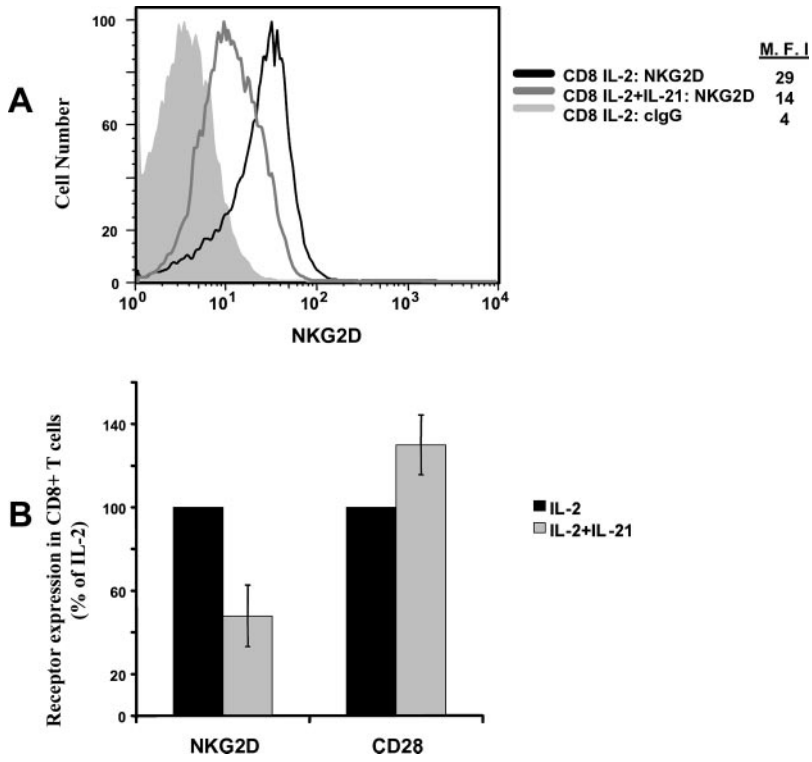

FIGURE 3. IL-21 culture of human primary $CD8^+$ T cells down-regulates NKG2D expression. *A*, $CD8^+$ T cells (1×10^6) were cultured with IL-2 (100 U/ml) with and without IL-21 (25 ng/ml) for 5 days, stained with anti-NKG2D mAb, and analyzed by FACS. Mean fluorescent intensities (M.F.I.) are indicated. *B*, $CD8^+$ T cells were cultured with IL-2 alone or in combination with IL-21 and analyzed for NKG2D and CD28 expression by FACS. Data are expressed as the percent difference from IL-2-stimulated cells (taken as 100%) and are mean averages from five donors.

novel data showing that IL-2 and IL-21 combined stimulation can induce synergistic/additive phosphorylation of STAT proteins in human primary NK cells.

IL-21 negatively regulates the expression of DAP10 transcripts and a DAP10 luciferase reporter construct

NKG2D mediates signal transmission through the association of adapter molecules, namely, DAP10 and DAP12 in mice, and only DAP10 in humans (8). Analysis of the *DAP10* gene revealed multiple potential STAT-binding motifs. Given the importance of DAP10 in NKG2D expression (29), we investigated, by real time RT-PCR, the regulation of DAP10 transcripts in primary NK cells after stimulation with IL-2 alone or in combination with IL-21. We observed a significant decrease in DAP10 transcripts after both 1 and 5 days of culture with IL-2 plus IL-21 compared with cells cultured with IL-2 only (Fig. 5). After 5 days of culture with IL-2 and IL-21, we noted, on the average, a 65% reduction in DAP10 transcripts over that in IL-2-stimulated cells. These data suggest that IL-21 may regulate cell surface expression of NKG2D in part by negatively regulating *DAP10* gene expression.

To confirm that IL-21 negatively regulates DAP10 gene expression, we cloned a DAP10 gene fragment into a pGL3basic luciferase reporter construct. Specifically, we cloned from human genomic DNA a fragment beginning 0.6 kb upstream of the translational start site of the *DAP10* gene that contains exons I, II, and III as well as the first two introns of the *DAP10* gene. As shown in Fig. 6, transfection of NKL cells with the DAP10 luciferase construct led to a 75-fold increase in the relative luciferase activity compared with pGL3basic in the presence of IL-2-containing medium. Addition of IL-21 to the IL-2-containing medium resulted in a drastic decrease (70% reduction) in luciferase activity, compared with transfected NKL cells treated with only IL-2 alone (Fig. 6). Taken together, these data provide compelling evidence that IL-21 negatively regulates DAP10 expression at the level of transcription, which, in turn, affects the cell surface expression of NKG2D.

IL-21 inhibits NKG2D-mediated cytotoxicity in primary human NK cells

NKG2D ligation can induce activation of NK cells and cytotoxicity toward NKG2D-sensitive targets (30). Because IL-21 appears to mediate a reduction in NKG2D cell surface expression, we assayed the cytotoxic activity between IL-2-treated cells or IL-2- and IL-21-treated cells for lysis of P815 cells ($Fc\gamma^+$) in the presence of anti-NKG2D mAb. We observed a moderate, but consistent,inhibition of NKG2D-mediated cytotoxicity toward P815 target cells of IL-21- plus IL-2-cultured NK cells compared with IL-2-cultured NK cells (Fig. 7*A*).

Another method was used to indirectly assess NK cell activation. NKG2D stimulation via plate bound anti-NKG2D Ab can activate NK cells and induce NK cell degranulation, leading to exposure of the lysosome-associated adapter molecules 1 and 2, also known as CD107a/b, to the plasma membrane. This marker can be detected by staining with anti-CD107a/b-FITC-conjugated mAb by FACS analysis. As shown in Fig. 7*B*, IL-2- and IL-21-treated NK cells upon stimulation with anti-NKG2D Ab resulted in a reduction in the expression of the degranulation markers CD107a/b, compared with IL-2-cultured cells (Fig. 7*B*). Thus, as assessed by two independent assays, combined IL-2/IL-21 stimulation of primary human NK cells can reduce NKG2D-mediated activation.

IL-21 up-regulates expression of NK cell activation receptors 2B4 and NKp30 in human primary NK cells

The fact that IL-21 promotes both murine and human NK cell IFN-γ production and increased cytotoxicity toward tumors is well documented (16). NK cell precursors derived from umbilical cord donors treated with IL-21 are known to mature toward a phenotype that includes increased expression of the natural cytotoxic receptor

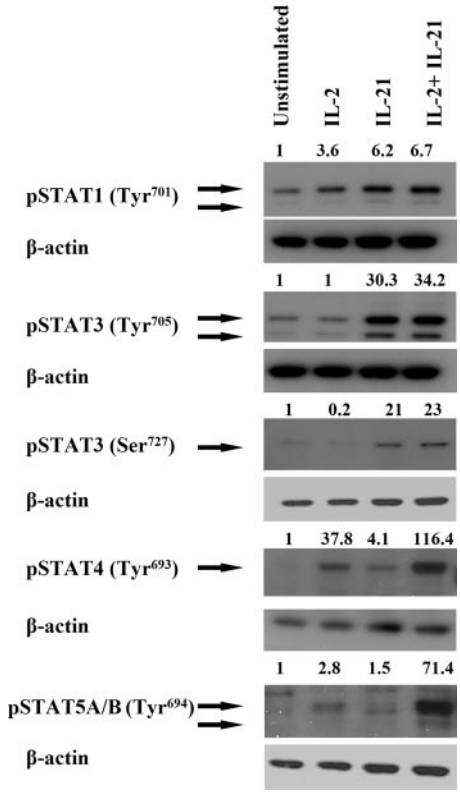

FIGURE 4. IL-21-mediated phosphorylation of STAT proteins in NK cells. Human primary NK cells (1×10^7) were stimulated overnight with IL-2 (100 U/ml) or IL-21 (25 ng/ml), alone or in combination. Nuclear extracts were prepared from harvested cells, and samples analyzed by SDS-PAGE and immunoblotted with various anti-phospho-STATs mAb. The presence of two bands in certain gels probably represents long and short forms of the STAT proteins. Equal loading was verified with anti-β-actin mAb. Numbers at the top of the lanes represent quantification of band intensity relative to β-actin. Data are representative of three independent experiments.

NKp46 (19). We confirmed the published observations of Sivori et al. (19), in that we observed increased expression of NKp46 (data not shown), but, in addition, we observed induction of another NCR, NKp30 (Fig. 8). We also observed increased expression of 2B4 (CD244), a receptor known to be expressed on all NK cells and a subset of $CD8^+$ T cells, which upon ligand engagement can either activate/costimulate human NK cells (31) or, in certain cases, provide an inhibitory signal to NK cells (32). IL-2 and IL-21 culture of NK cells over a 5-day period resulted in a 50% increase in cell surface expression of 2B4 compared with cells treated with IL-2 alone (Fig. 8). Taken together, these data suggest that IL-21, a product of activated Th cells, channels NK cell target recognition away from those cells that are NKG2D sensitive.

Discussion

In this paper we present novel evidence that culture of human primary NK and $CD8^+$ T cells with the cytokine IL-2 in combination with IL-21 significantly down-regulates cell surface expression of NKG2D, compared with that in cells treated with IL-2 alone. Indeed, this is the first such report that IL-21 can inhibit the surface expression of an NK cell activation receptor. This was initially a surprising observation, because other members of the IL-2 cytokine family, namely, IL-2, IL-7, and IL-15, have been previously shown to induce and maintain NKG2D expression on human $CD8^+$ T cells (10, 11).

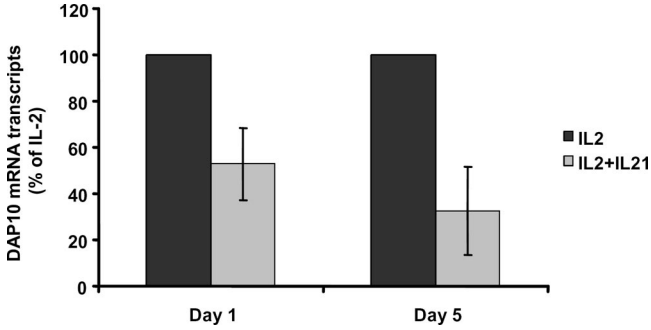

FIGURE 5. IL-21 down-regulates DAP10 transcripts in NK cells. NK cells (1×10^6) were cultured with IL-2 with or without IL-21. On days 1 and 5, cells were harvested, and total RNA was isolated. Samples were analyzed for DAP10 transcript expression by quantitative PCR. Data shown are the mean percent reduction in DAP10 expression taken from five independent experiments.

NKG2D is a well-characterized primary activation receptor expressed by NK cells and is also capable of providing costimulatory signals for $CD8^+$ T cells (10, 33). After ligand engagement, NKG2D-mediated activation of NK and $CD8^+$ T cells is an important signal required for cytotoxicity toward tumors (34). Because of these observations, the determination of the factors that regulate NKG2D/DAP10 expression is an area of significant interest. In this regard, recent studies have revealed that tumors can evade immunity by shedding soluble NKG2D ligands that bind NKG2D expressed by NK and $CD8^+$ T cells, thereby mediating receptor internalization and thus reducing the activation potential of NK/$CD8^+$ T cells (35–37). In addition, elevated levels of TGF-β in cancer patients inhibit the expression of NKG2D, impairing NK cytotoxicity (38). Such studies highlight the effect of down-regulation of NKG2D on tumor immunity. Moreover, aberrant NKG2D signaling and inappropriate activation of NK/$CD8^+$ T cells by NKG2D have been implicated for various human diseases, including celiac disease (39), rheumatoid arthritis (40), and autoimmune diabetes in NOD mice (41). Thus, the modulation of NKG2D receptor expression or blockade of NKG2D signaling may have therapeutic implications for a variety of disease states.

IL-21 is currently being investigated as a potential therapeutic agent for the treatment of cancer (42, 43). A recent report has stated that IL-21 is able to enhance tumor rejection through an NKG2D-dependent mechanism (17). Observations by Takaki et al. (17) show that mice treated with IL-21 reject tumor cells more efficiently, and that rejection is NKG2D dependent, because blocking Abs to NKG2D lead to reduced tumor cytotoxicity. Furthermore, in contrast to our findings using primary human NK cells, where IL-21 down-regulates NKG2D expression, there are two reports that IL-21 stimulation of murine NK cells does not alter NKG2D expression (15, 17). An explanation for this could be due to the possibility that DAP12 can compensate for the down-regulation of DAP10 in murine NK cells by serving as the adapter protein for NKG2D, thus promoting the cell surface expression of the NKG2D/DAP12 heterodimer (44). Because DAP12 cannot bind NKG2D in human NK cells (45), IL-21 is able to freely inhibit NKG2D expression. These data suggest that there may be species-specific variation, at least between humans and mice, in the effect of IL-21 stimulation on NKG2D expression. Nevertheless, our data suggest that increased cytotoxicity of human NK cells after IL-21 stimulation is likely to be NKG2D independent, and contrary to the suggestion by Takaki et al. (17), IL-21 immunotherapy may not be the best approach for generating NKG2D-enhanced tumor rejection.

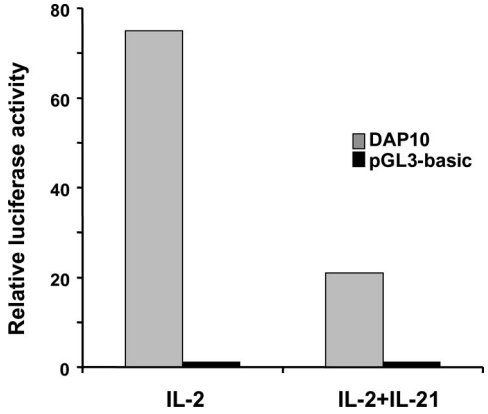

FIGURE 6. IL-21 inhibits the promoter activity of a DAP10 luciferase reporter construct. NKL cells were transfected with DAP10 luciferase or empty vector control and stimulated with IL-2 alone or IL-21 (25 ng/ml). The luciferase activities were measured 40 h after transfection and were normalized to pGL3basic. The luciferase activities shown are representative of four independent experiments performed.

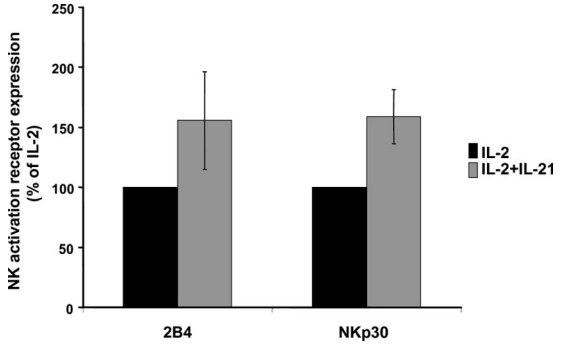

FIGURE 8. IL-21 up-regulates NK cell activation receptors 2B4 and NKp30 in human primary NK cells. Freshly isolated human NK cells were cultured with IL-2 (100 U/ml) with or without IL-21 (25 ng/ml) for 5–7 days. Cell surface expression of NK recognition receptors was determined by staining with anti-NKp30 and anti-2B4 mAb, followed by FACS analysis. The graphs represent the relative receptor expression of two (NKp30) and three (2B4) independent experiments.

As mentioned above, NKG2D associates with DAP10 in human NK and $CD8^+$ T cells, whereas in murine NK cells (but not in murine $CD8^+$ T cells, where DAP12 is not expressed), NKG2D can bind with an additional adapter, DAP12 (44). The expression of NKG2D in murine $CD8^+$ T cells is wholly dependent on DAP10, because $CD8^+$ T cells from DAP10-deficient mice do not express NKG2D, although a functional NKG2D receptor can still be expressed in NK cells of DAP10-deficient mice due to association with DAP12. Using DAP10 luciferase reporter constructs and real-time RT-PCR, we show that IL-21 down-regulates NKG2D at least in part by transcriptional down-regulation of the adapter protein DAP10. After 5 days of culture with IL-2 in combination with IL-21, we observed a 65% reduction in DAP10 transcripts, compared with that in IL-2-treated cells. Our preliminary data, using quantitative real-time PCR, indicate that IL-21 can also down-regulate NKG2D transcript expression (data not shown); however, this analysis has proven to be technically difficult. Thus, we show a potential mechanism for IL-21-mediated NKG2D down-regulation.

The signals that participate in IL-21-mediated inhibition of NKG2D expression probably involve the JAK/STAT signaling pathway. In T cells, IL-21 is known to activate JAK1 and JAK3, which phosphorylate STAT1, STAT3, STAT4, and STAT5 (23, 28). In our study we observed a similar STAT phosphorylation profile with human primary NK cells stimulated overnight with IL-21. However, stimulation of human NK cells with IL-21 in combination with IL-2 resulted in a synergistic phosphorylation of STAT4 and STAT5A/B, but, interestingly, not of STAT1 or STAT3. We predict that IL-21-induced STAT binding to the regulatory regions of the DAP10 promoter may mediate inhibition of DAP10, contributing to the down-regulation of NKG2D. Sequence homology plots of DAP10 reveal many potential STAT-binding consensus motifs (data not shown) within the DAP10 gene. The possibility of cytokine-induced STAT down-regulation of the DAP10 gene is currently under investigation. Although STAT proteins are often known for up-regulating gene expression, a recent

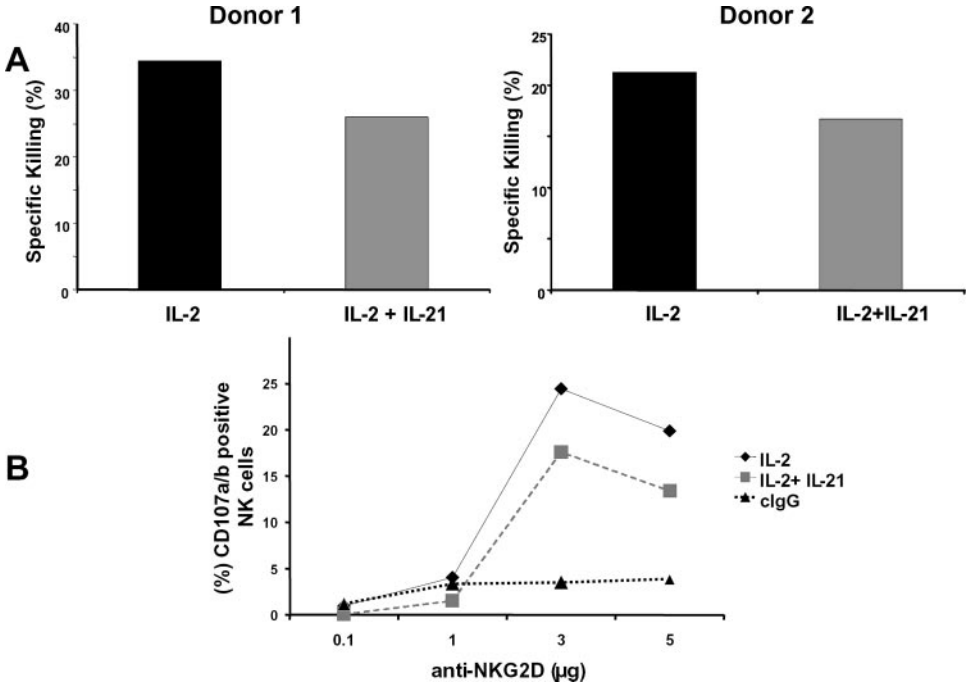

FIGURE 7. IL-21 inhibits NKG2D-mediated cytotoxicity. *A*, Human primary NK cells were cultured with IL-2 with or without IL-21 (25 ng/ml) for 5 days and were analyzed for cytotoxicity toward P815 target cells in an NKG2D-mediated redirected lysis assay as described in *Materials and Methods*. Data for two independent experiments are shown. *B*, IL-2- and IL-2 plus IL-21-cultured NK cells were assessed in a degranulation assay. Cytokine-treated cells were stimulated with varying concentrations of anti-NKG2D mAb as indicated. Three hours after stimulation, cells were harvested, and cell surface expression of the lysosomal markers, CD107a/b, were determined by FACS. Data are representative of three independent experiments, each showing the same trend.

study has shown that IL-10-induced activation of STAT3, which is phosphorylated by IL-21, but not by IL-2, can mediate selective inhibition of the transcription of genes involved in inflammation (46).

To date, IL-21 is generally considered to be an activating cytokine leading to the proliferation, maturation, and activation of effector lymphocytes (24, 47). We also found that IL-21 is able to increase IL-2 expansion of human NK cells and induce effector functions, yet at the same time, IL-21 down-regulates a key activation receptor expressed by these cells. A previous report of relevance to this study has shown that IL-21 treatment of murine NK cells can also down-regulate the expression of the activation receptor NK1.1, yet still enhance in vivo tumor immunity (24). The down-regulation of NKG2D is in opposition to the up-regulation of other NK activation receptors examined, i.e., NKp30, NKp46, and 2B4. The functional purpose for the differential regulation of NK activation receptors by IL-21 is not obvious in light of the apparent overall activation of NK cells. In the case of $CD8^+$ T cells, a recent study has shown that IL-21 is able to sustain CD28 expression on naive human IL-15-activated $CD8^+$ T cells (26). We also confirm these findings with a consistent 25% average induction of CD28 in human $CD8^+$ T cells after 5 days of culture with IL-21. Thus, it appears that activated Th cells, which are the primary producers of IL-21, have the potential to differentially regulate the $CD8^+$ T cell costimulatory receptors CD28 and NKG2D, perhaps leading to functionally different outcomes after $CD8^+$ T cell activation. In summary, we show novel data that culture of human primary NK and $CD8^+$ T cells with IL-2 and IL-21 results in down-regulation of the NKG2D receptor, which can be explained at least in part by the down-regulation of DAP10 gene expression. Significantly, these data suggest that using IL-21 as a potential immunotherapy for cancer may not be as effective for NKG2D-ligand expressing tumors as previously thought. However, IL-21 mediated down-regulation of NKG2D on human NK and $CD8^+$ T cells may have beneficial therapeutic effects on a variety of autoimmune diseases in which NKG2D signaling is known to participate.

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Disclosures

The authors have no financial conflict of interest.

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