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Tumor-Induced Apoptosis of Human IL-2-Activated NK Cells: Role of Natural Cytotoxicity Receptors

Alessandro Poggi, Anna-Maria Massaro, Simone Negrini, Paola Contini, and Maria Raffaella Zocchi

We provide evidence that tumor cells can induce apoptosis of NK cells by engaging the natural cytotoxicity receptors (NCR) Nkp30, Nkp44, and Nkp46. Indeed, the binding between NCR on NK cells and their putative ligands on tumor target cells led to NK cell apoptosis, and this event was abolished by blocking NCR/NCR-ligand interaction by anti-NCR-specific mAbs. The engagement of NCR induced up-regulation of Fas ligand (FasL) mRNA, FasL protein synthesis, and release. In turn, FasL interacting with Fas on NK cell surface causes NK cell suicide, as apoptosis of NK cells was inhibited by blocking FasL/Fas interaction with specific mAbs. Interestingly, NK cell apoptosis, but not killing of tumor target cells, is inhibited by cyclosporin A, suggesting that apoptosis and cytolyis are regulated by different biochemical pathways. These findings indicate that NCR are not only triggering molecules essential for antitumor activity, but also surface receptors involved in NK cell suicide. The Journal of Immunology, 2005, 174: 2653–2660.

Among antitumor lymphocytes, NK cells would represent the most powerful cytolytic effector cells with which we are provided. Indeed, NK cells are the main peripheral lymphocyte subset able to lyse tumor cells without any additional stimulus, and they acquire a highly efficient antitumor cytolytic activity when exposed to IL-2 (1, 6–8). Furthermore, the recent discovery of natural cytotoxicity receptors (NCR), represented by Nkp30, Nkp44, and Nkp46, has clearly indicated that NK cells in humans are specifically provided by a unique series of triggering surface receptors responsible for the elimination of different tumor target cells (14).

We have demonstrated recently that NK cell triggering via HLA-I receptors, including CD8 Ag and the activating isoforms of inhibitory receptor superfamily (IRS) members, induces FasL release, which in turn leads to NK cell death (15, 16). This finding would suggest that, not only FasL from tumor cells (13), but also FasL produced and released by NK cells can induce NK cell death. Thus, we tested the hypothesis that any time an NK cell kills a tumor target, it also triggers its own death through the activation of apoptosis.

We found that NK cell suicide is triggered when NK cells interact with tumor target cells, and that Nkp30, Nkp44, and Nkp46 are responsible for this effect. The molecular mechanism triggered by the engagement of NCR involves the production and release of FasL and the consequent interaction of FasL with Fas on the NK cell surface, leading to the activation of caspase 3.

Materials and Methods

mAbs and reagents

The anti-CD16 (NK1, IgG1, VD4, IgG1; KD1, IgG2a) mAb (10) and the anti-CD56 (TA181H12, IgG2a) mAb were obtained, as described (6, 14, 17, 18). The anti-CD3 mAb (Leu4, IgG4), the anti-CD4 mAb (Leu3a, IgG1), the anti-CD8 mAb (Leu2a, IgG1), and the anti-CD56 mAb (Leu19, IgG1) were from BD Biosciences, and the anti-FasL (NOK-1, IgG1) was from BD Pharmingen. The anti-CD54 mAb (ICAM-1, 14D12D2, IgG1) was a gift of S. Carrel (Ludwig Institute for Cancer Research, Lausanne Branch, Epalinges, Switzerland). The blocking anti-Fas mAb ZB4 (IgG1) and the apoptosis-inducing anti-Fas mAb CH11 (IgM) were from Medical and Biological Laboratories; the anti-NKp30 mAb (clone Z25, IgG1), the anti-NKp44 mAb (clone Z231, IgG1), the anti-NKp46 mAb (clone BAB281, IgG1), either in purified azide-free form or PE conjugated, and the anti-human Fas mAb UB2 were from Beckman-Coulter; the anti-FasL mAb Alf-2.1a was from Ancell; and the anti-Fas mAb (M38, IgG1) was
from American Type Culture Collection. Annexin V FITC was from Boehringer Mannheim, and propidium iodide (PI) was from Sigma-Aldrich. The affinity-purified goat anti-mouse (GAM) anti-iso-type-specific antiserum was from Southern Biotechnology Associates. Purified GAM anti-Ig (H + L) was purchased from Valence Pharmaceuticals; immunomagnetic beads were from Dynal Biotech; and rIL-2 was from Chiron (Proleukin). Cells were cultured in RPMI 1640 medium (Biochrom) supplemented with 10% of FCS (Sigma-Aldrich) and with glutamine and penicillin-streptomycin (Biochrom). The L-type calcium channel blocker verapamil was from Sigma-Aldrich and used in functional assays at 10, 1, and 0.1 μM concentration, as described (19).

**Indirect immunofluorescence**

Single fluorescence staining was performed, as described elsewhere (16, 17). Briefly, aliquots of 10^7 cells were stained with the corresponding mAb, followed by PE-conjugated anti-iso-type-specific GAM serum (Southern Biotechnology Associates), or with an unrelated mAb (BD Biosciences), followed by the fluorescent second reagent. In some experiments, anti-NKp30, anti-NKp44, and anti-NKp46 mAbs directly conjugated with PE were used. Samples were analyzed on a flow cytometer (FACSsort; BD Biosciences) equipped with an argon ion laser exciting PE at 488 nm. Data were analyzed using CellQuest computer program. Calibration was assessed with CaliBRITE particles (BD Biosciences). Results are expressed as log red mean fluorescence intensity in arbitrary units (a.u.) (x-axis) vs number of cells (y-axis).

**Isolation and culture of polyclonal and clonal NK cell populations**

NK cells were isolated with the RosetteSep NK isolation kit (StemCell Technologies) from heparinized blood of healthy volunteers, according to the kit protocol. The resulting cell population was 50–70% CD16^+ (range of eight different experiments), but 99% CD3^- . Highly purified CD3^- cells were stimulated with 10 μg/ml PHA and cultured in 96-well U-bottom microplates (Greiner Labortech) with RPMI 1640 medium supplemented with 10% FCS in the presence of 100 U/ml rIL-2 in a final volume of 200 μl/well in the presence of 10^3/well irradiated allogeneic PBMC and 10^4/well 721.221 lymphoblastoid cell line (16). Under these culture conditions, by day 15 all cells expressed CD16 and CD56 Ags. CD3^-CD16^- clones were obtained by culturing highly purified CD3^- NK cells under limiting dilution conditions, as previously reported (16). In particular, decreasing number of NK cells (100, 50, 25, 12, and 6) was seeded in 200 μl/well in 96-well plates. After 10–12 days, wells positive for cell proliferation were selected, and cells were expanded for additional 30–45 days. Cloning efficiency was 5–10% (20). Cell cultures obtained at 6, 12, and 25 cells/well were then analyzed for the expression of the different NCR: NKp30, NKp44, and NKp46 with specific PE-conjugated mAb by direct immunofluorescence. Only cell cultures that resulted homogeneously positive for these receptors (>95% of positive cells) were further used in functional assays.

**Cytolytic assays**

Cytolytic activity of CD3^-CD16^- NK cell clones or bulk populations was analyzed in a 4-h 51Cr release assay against a panel of tumor target cells: the lymphoblastoid cell line 721.221, the hepatocarcinoma SMMC, the melanoma F01, the cervical carcinoma Hela, the neuroblastoma IMR5, and the lung adenocarcinoma P71. Target cells were labeled with ^51Cr and used at an E:T ratio of 10:1 to 0.5:1, in a final volume of 200 μl of culture medium in V-bottom microcwell in a 4-h ^51Cr release assay. One hundred microliters of supernatant (SN) were counted in a gamma counter, and percentage of ^51Cr-specific release was calculated, as described previously (16). In the redirected killing assay, the Fe-cyR^- murine mastocytoma cell line P815 was used as target in the presence of saturating amounts (3 μg/ml) of the indicated mAb (16, 17) or in medium alone (nil) at the E:T ratio of 1:1.

**FIGURE 1.** Interaction between NK cells and tumor target cells induces NK cell apoptosis. A. Microscopic analysis of NK cells (bulk population NK1) (upper left quadrant, ×200 magnification) or NK cells cocultured with F01 for 2 h (upper right quadrant, ×200) or 48 h (lower left, ×200; lower right, ×400). B. Cultures of NK cells (bulk population NK1) (upper quadrants) or NK cells with F01 cells for 24 h (middle and lower quadrants) were stained with FITC annexin V (right quadrants). Samples were analyzed by flow cytometry after gating on NK cells (upper and middle quadrants) or on F01 cells (lower quadrants). NK cells and F01 cells were identified on the basis of their FSC and SSC. Percentage of annexin V^- cells is shown. C. Annexin V staining and FACS analysis of NK cells (bulk population NK1) (upper left histogram) or NK cells cocultured with F01 for 24 h at different E:T ratio (.5, 1, 2, 5, 10:1), as indicated. Gate was performed on NK cells, as in B. Percentage of annexin V^- NK cells is shown. D. Apoptosis of NK cells alone (bulk population NK1) (lower left histogram) or cocultured for 72 h with F01 target cells (lower right histogram), at the E:T ratio of 1:1, was analyzed by gating on CD16^- NK cells (R1 middle quadrants) after staining with PI of Formalin-fixed cells in the presence of Nonidet P-40 detergent to permeabilize cells (lower quadrants). Percentage of apoptotic NK cells is indicated in lower panel. Apoptotic cells: cells with a PI content >2n. FSC and SSC of cultures of NK or NK + F01 cells are shown (upper dot plots). Similar results to those depicted in A–D were obtained with three additional bulk populations (NK2, NK3, and NK4).
Determination of soluble FasL (sFasL) in culture SN

sFasL present in culture SN after incubation with anti-NCR mAbs (anti-NKp30, anti-NKp44, anti-NKp46) either in soluble form or with GAM-coated beads or on GAM-coated plates was evaluated by ELISA, as previously described (15, 16). Briefly, 100 μl of culture SN derived from NK cell clones after different incubation times (16), with medium alone, apoptosis-inducing anti-Fas mAb (CH-11, 3 μg/ml), or 4-per-cell GAM-coated magnetic beads on NK cells preincubated with anti-NKp30 mAb, anti-NKp44 mAb, anti-NKp46 mAb, anti-CD16 mAb, or anti-CD54 mAb (14D12D2) were collected, added to a microtiter plate coated with anti-FasL mAb NOK-2, and incubated for 45 min at room temperature (RT). After five washes with PBS/0.2% Tween 20, biotinylated anti-FasL mAb NOK-1 was added for additional 45 min at RT. Plates were then washed, and streptavidin-HRP (Pierce) was added for 1 h at RT. The reaction was developed for 15 min at RT in the dark with p-phenylenediamine (40 μg/ml) in phosphate citrate buffer (pH 5.0) supplemented with 0.04% (v/v) H2O2. OD was read with a spectrophotometer at 490 nm against reagent blank (PBS/BSA 5%). Standard curve was obtained using progressive dilution of rFasL from Alexis. Results were expressed as mean ± SD of triplicate wells. SN harvested from NK cells incubated under the above mentioned conditions were analyzed for their ability to induce apoptosis of the human lymphoid cell line Jurkat (CD3/TCR− Fas−) to determine whether FasL present was functional (16).

Induction and detection of apoptosis

Bulk NK cell populations or NK cell clones (10^6/ml) were cultured in RPMI 1640 culture medium supplemented with 10% FCS in the presence of different concentrations of rFasL from Alexis. Results were expressed as mean ± SD of triplicate wells. SN harvested from NK cells incubated under the above mentioned conditions were analyzed for their ability to induce apoptosis of the human lymphoid cell line Jurkat (CD3/TCR− Fas−) to determine whether FasL present was functional (16).

Isolation of RNA, reverse transcription, and PCR amplification

Total RNA was isolated from NK cell pellets stained with anti-NKp30, anti-NKp44, anti-NKp46, or anti-ICAM-1 mAb, followed by incubation with GAM-coated beads (four beads per cell) by using the RNAzol B (Biotecx Laboratories) method according to the manufacturer’s protocol. cDNA (corresponding to 2 μg of RNA) was synthesized from oligo(dT)- primed RNA in 20 μl of reverse-transcriptase buffer and 200 U of Moloney murine leukemia virus reverse transcriptase (PerkinElmer/Cetus) incubated at 42°C for 45 min and at 52°C for 45 min. The PCR mixture contained 2 μl of cDNA, 2.5 mM MgCl2, 2 mM dNTP, 50 μM 5’ and 3’ oligonucleotide primers, and 2.5 μl of Amplitaq Gold Polymerase (PerkinElmer). The PCR mixture was amplified by 35 cycles of denaturation at 99°C for 5 min and at 42°C for 1 h, annealing at 60°C for 30 s, and extension at 72°C for 30 s in 0.2 ml of thin-walled tubes in a total volume of 40 μl. Primer sequences used were: β-actin, 5’-AAGACACCAGATGGTGCGC-3’; FasL, 5’-ACCTGCCTGGTGGTCAAG-3’; B-actin, 3’-CATACCTGCTGGTCTGGCTAC-5’ (228-bp fragment); FasL, 5’-CAAGTCACTACTAGCTGTCAGCA-3’; FasL, 3’-CATGTGCTAGTGGCGAGGAGAC-3’; and 3’-CGTGGCATAGCTCGAGAGAG-3’ (350-bp fragment) (16). PCR products were size fractionated by agarose electrophoresis and normalized according to the concentration in PBS (16). NK cells were identified on the basis of their specific reactivity with anti-CD16 mAb, followed by anti-isotype-specific GAM-FITC conjugated. Some experiments were performed in the presence of 0.1, 1, and 10 μM verapamil diluted in DMSO or with the same amount of DMSO solvent as control, or with 2 mM EGTA (calcium chelator) to determine the role in target cell-induced NK cell apoptosis of extracellular calcium. FACSort calibration was assessed with CalibRITE particles (BD Biosciences) using CellQuest computer program. None of the target cell lines used in these experiments reacted with anti-CD16 mAb, indicating that gated CD16+ cells were NK cells. CD16+ NK cells with a PI content <2n compared with control samples (NK cells alone) were considered as apoptotic cells. At least 106 events for each sample were analyzed. Anti-Fas mAb (M38) and/or anti-FasL mAb (Alf-2.1a) were added (3 μg/ml) for each NK cell population and NK-target cell cocultures. In some experiments, cells were incubated with anti-NKp30 or anti-NKp44, anti-NKp46 or anti-CD16, or anti-Fas or anti-ICAM-1 mAb for 30 min at 4°C, washed, and incubated for different times (24, 48, and 72 h) in medium alone or with 4-per-cell of GAM-coated magnetic beads or on plates coated with 10 μg/ml GAM. Again, anti-Fas mAb and/or anti-FasL mAb were added (3 μg/ml) at the onset of cell culture. In all of these experiments, apoptosis was analyzed by PI staining (see above) and by DNA laddering (16).

FIGURE 2. Interaction between NK cells and tumor target cells induces NK cell apoptosis. A. Kinetics of NK cell (bulk population NK3) apoptosis upon incubation with tumor targets (Foi, squares; Hela, triangles) was evaluated as in Fig. 1D by gating on CD16+ NK cells permeabilized and stained with PI after incubation with a given target for the indicated period of time (1, 6, 24, 36, and 72 h). Apoptosis of NK cells alone is shown for comparison (diamonds). B. NK cell apoptosis (bulk population NK3) evaluated as in A after 72 h of incubation with Hela target cells at the indicated E:T ratio. □. Specific lysis of Hela cells exerted by NK cells at the same E:T ratio. Similar results as those depicted in A and B were obtained with two additional NK cell populations (NK1, NK2). C. Apoptosis of two bulk NK cell populations (NK1 or NK2) or the NK cell clone S26 (from donor 1), evaluated as in A, incubated for 72 h with the indicated target cell lines (IMR5, Foi, P71, SMMC, Hela, and 721.221) at the E:T ratio of 1:1. None, Apoptosis of NK cells in the absence of any target. D. Cytolytic activity of the two bulk NK cell populations NK1 or NK2 or the S26 NK cell clone against the same target cells of C at the E:T ratio of 1:1. Results in A–C are shown as percentage of apoptotic NK cells, that is, NK cells with a PI content <2n; in B and D, cytosis was expressed as percentage of 51Cr-specific release.
amount of β-actin detected in the same mRNA sample. Densitometric analysis of each band was performed by Gel Pro Analyzer 3.1 (Media Cybernetics), and results are expressed as number of pixel in a.u.

**Statistical analysis**

Data from NK cell populations (n = 8) (expressing >95% NKp30, NKp44, and NKp46 receptors) and from NK cell clones (n = 45) (>95% NKp30/NKp44/NKp46+) from six different donors have been analyzed by Student’s t test.

**Results**

**Interaction between tumor cells and NK cells induces NK cell apoptosis**

To investigate whether NK cell interaction with tumor cells could induce NK cell death, polyclonal or clonal NK cell populations were cocultured with melanoma F01 target cells for different periods of time (1, 6, 24, 48, and 72 h). We found that NK cells bound to F01 (Fig. 1A, upper right panel) within 15–30 min and killed them (90% specific lysis) at 120 min (data not shown). It is of note that in the same well the large majority of cells after 48 h appeared as round shaped with some nuclear blebs (Fig. 1A, lower right panel), and no NK cell blasts were detectable (Fig. 1A, compare lower panels with the upper left panel). This finding suggests that upon killing of F01 melanoma cells, NK cells die by apoptosis.

Furthermore, we stained NK-F01 cocultures with the early apoptotic cell marker annexin V, and these samples were analyzed on a flow cytometer gating on either NK or F01 cells on the basis of their different FSC and SSC (Fig. 1B). We found that a large portion of NK cells was annexin V+ after 24 h of incubation with F01 (p < 0.001, data from eight polyclonal NK cell populations). Interestingly, we noticed that the percentage of apoptotic NK cells decreased by increasing the E:T ratio (Fig. 1C). That NK cells died by apoptosis during the coculture with F01 was further demonstrated by PI staining on permeabilized CD16+ NK cells (Fig. 1D, compare lower right panel with the lower left panel, which represents control NK cells). Importantly, the melanoma F01 did not express CD16 Ag, indicating that gated CD16+ cells were actually NK cells (data not shown).

Kinetics experiments showed that NK cells undergo cell death starting from 24 h, reaching a maximum at 48 h of incubation with F01 or Hela tumor target cells (Fig. 2A). Again, the percentage of apoptotic NK cells increased when the ratio between NK and Hela cells decreased (Fig. 2B). Indeed, almost all NK cells (80%) died upon incubation with the Hela cell line at an E:T ratio of 1:1, while at higher E:T ratios (18%) of NK cells underwent apoptosis when incubated with the same target at 10:1 E:T ratio. It is conceivable that at 1:1 E:T ratio, at least one NK cell binds to a target cell to deliver the lethal hit, while at higher E:T ratios only a fraction of NK cells actually interacts with target cells, and this fraction represents a minority of the total amount of effector cells. Interestingly, the same NK cell clone (clone S26) or NK polyclonal population (NK1 and NK2), which underwent apoptosis when incubated with an NK-sensitive target (F01, P71, SMMC, Hela, and 721.221), did not die when incubated with highly resistant target cells, such as the neuroblastoma cell line IMR5 (Fig. 2, C and D).

**Target-induced NK cell apoptosis is calcium dependent and is mediated by FasL/Fas**

Given the correlation between the degree of apoptosis of NK cells and their lytic activity, we further analyzed whether blocking of NK cell-mediated cytolysis could affect NK cell death as well. It has been shown that calcium ions play a key role both in NK-target cell binding and in the delivery of lethal hit (19). Thus, we first analyzed whether the calcium chelator EGTA or the calcium channel blocker verapamil affects target-induced effector cell apoptosis or cytolysis. It is noteworthy that apoptosis of NK cells incubated with F01 or Hela cells was strongly reduced in the presence of either EGTA or verapamil (Fig. 3A) (p < 0.001 data from six polyclonal NK cell populations). Moreover, the lysis of these two cell lines was inhibited by blocking calcium entry from the extracellular medium (Fig. 3B). These findings suggest that intracellular calcium increase is needed both for tumor cell killing and for tumor-induced NK cell apoptosis.

Then, we investigated the molecular mechanism by which tumor targets induced the apoptosis of NK cells. It has been shown that NK cells undergo apoptosis through the engagement of Fas molecule by FasL (16, 17). Thus, we analyzed whether FasL/Fas interaction was involved in the NK cell death following the binding with tumor targets. We found that blocking anti-Fas mAb or anti-FasL mAb reduced by 35–55% the apoptosis of NK cells induced by the incubation with Hela or F01 tumor cells (Fig. 3, C and D). Importantly, this inhibitory effect reached 60–70% inhibition when anti-Fas and anti-FasL mAbs were added together to NK tumor cell cocultures (Fig. 3) (p < 0.001, data from six polyclonal NK cell populations). This finding suggests that NK cell apoptosis is triggered by the Fas-mediated cell death pathway.

**Role of NCR in tumor target-induced NK cell apoptosis**

It has been reported that the NCR NKp30, NKp44, and NKp46 are responsible for the lysis of tumor cells (14). Indeed, covering of different NCR on NK cells with specific mAbs can strongly reduce NK cell-mediated lysis of a given target cell, because the interaction between NCR and their putative ligands on the targets is
impaired (14). It also has been reported that the lysis of tumor cells can be abolished by the simultaneous covering of all three NCR (14). Thus, we analyzed whether covering of NCR could reduce the degree of apoptotic effect observed upon interaction of effector NK cells and target cells. To this aim, we selected a series of NK cell clones expressing functional NCR and we tested the ability of anti-NCR mAb specific for NKp30, NKp44, or NKp46 alone or in combination, to block target-induced NK cell apoptosis. We found that preincubation of NK cells with either anti-NKp30 or anti-NKp46 mAbs strongly reduced (>50% for each anti-NKp mAb) (Fig. 4A) NK cell apoptosis elicited by incubation with Fo1 melanoma cells, while the covering of NKp44 receptor had a lower effect (Fig. 4A) (p < 0.001, data from 25 NK cell clones from four donors). Importantly, inhibition of apoptosis of NK cells after covering all three NCR was almost complete (Fig. 4A). Along with the decrement in NK cell death found using anti-NKp30 and NKp46 mAbs, we observed that the lysis of melanoma Fo1 target cells was strongly inhibited by covering these receptors (data not shown), in keeping with previous report (14).

We analyzed the effect of covering NCR on NK cell apoptosis induced by other tumor targets, including the cervical carcinoma cell line Hela, and the lymphoblastoid cell line 721.221. Again, we observed that the incubation with anti-NKp30 and NKp46 mAbs inhibited the apoptosis of NK cells (Fig. 4A) (p < 0.001, data from 25 NK cell clones from 4 donors) and, at the same time, the lysis of a given target cell (data not shown). Taken together, these findings suggest that, upon interaction with their ligands expressed on tumor cells, the NKp30 and NKp46, and to a lesser extent the NKp44 receptors present on NK cells delivered an apoptotic signal that ultimately leads to NK cell death.

**The engagement of NCR with specific mAbs directly triggers NK cell apoptosis**

It is conceivable that during interaction between NK lymphocytes and tumor target cells additional surface molecules, besides NCR, may play a role in activating NK cell apoptosis (1, 15). This does not allow us to consider NCR as molecules that directly trigger apoptosis. To clarify this point, we selected a series of NK cell clones and we applied different experimental settings to determine whether cross-linking of NCR was necessary to deliver an efficient apoptotic signal (Fig. 4B). Indeed, we found that in soluble form anti-NKp30, anti-NKp44, or anti-NKp46 mAbs did not induce apoptosis (data not shown). However, when NKp30, NKp44, or NKp46 receptors were cross-linked using GAM-coated beads, an apoptotic effect was detectable starting from 60 min of incubation (by annexin V staining; Fig. 4B), reaching a maximum between 24 and 48 h (analyzed by staining with PI; Fig. 4C) (p < 0.001; n = 9, data from two polyclonal NK cell populations and seven clones from three donors). Similar results were obtained when NCR cross-linking was achieved with GAM-coated plates (data not shown). Interestingly, the degree of apoptosis was directly proportional to the degree of lysis of P815 target cells observed in a redirected killing assay using anti-NKp30, anti-NKp44, or anti-NKp46 mAbs (Fig. 4D), further

![FIGURE 4. NCR NKp30, NKp44, and NKp46 are involved in target-induced NK cell death. A, NK cells (NK cell clone S26 from donor 1) were incubated for 72 h with the indicated tumor target cells (Hela, Fo1, and 721.221) in the absence (nil) or in the presence of saturating amounts (3 μg/ml) of anti-CD56, anti-NKp30, anti-NKp44, or anti-NKp46, or a mixture of anti-NKp30, anti-NKp44, and anti-NKp46 mAbs (mix anti-NCR, 3 μg/ml each mAb) at the E:T ratio of 1:1. Anti-CD56 mAb was used as isotype-matched control. Percentage of apoptotic NK cells was assessed by PI staining as in A. B, NK cells (bulk population NK4) were stained with saturating amounts of the indicated mAbs (Fig. 4A) (p < 0.001, data from 25 NK cell clones from 4 donors) and, at the same time, the lysis of a given target cell (data not shown). Taken together, these findings suggest that, upon interaction with their ligands expressed on tumor cells, the NKp30 and NKp46, and to a lesser extent the NKp44 receptors present on NK cells delivered an apoptotic signal that ultimately leads to NK cell death.](http://www.jimmunol.org/DownloadedFrom/)
products were size fractionated by agarose electrophoresis and normalized according to the amount of ICAM-1 with an isotype-matched mAb is shown for comparison. Total mRNA was isolated and subjected to PCR with primers for FasL or cells of FasL. Superimposable results were obtained using two additional NK cell populations (NK2, NK3).

Engagement of NCR induces mRNA FasL up-regulation, release of FasL protein, and activation of caspase 3

It is well known that lymphocytes can die by apoptosis upon ligation at the cell surface of Fas molecule by FasL, and activation of caspase 3 (21). Thus, to determine the molecular mechanism underlying the triggering of NK cell apoptosis induced by NCR engagement, we first analyzed whether ligation of these receptors could elicit FasL mRNA synthesis and the consequent production and release of FasL in the extracellular milieu. As shown in Fig. 5A, we found that the cross-linking of NCR (NKp30, NKp44, or NKp46) could up-regulate, within 3 h, FasL mRNA in NK cells. sFasL was found in NK cell culture SN starting from 8 h (data not shown), reaching a maximum after 18 h from ligation of NCR (Fig. 5B). FasL release was obtained only when NCR were cross-linked by immobilized mAbs on GAM-coated beads or GAM-coated plates (although to a lesser extent), while no sFasL was detectable when anti-NCR Abs were used in soluble form (Fig. 5B).

By flow cytometry, we assessed the engagement of NCR on NK cells. Percentages of apoptotic cells (25) were analyzed after incubation for 72 h with GAM-coated beads (four beads per cell) alone (none, FasL present in culture SN of NK cells was evaluated by ELISA. The bulk NK cell population NK1 was stained with the indicated mAbs (3 μg/ml) to the indicated surface receptors, followed by GAM-coated beads (four beads for each NK cell) for 3 h. The engagement of ICAM-1 with an isotype-matched mAb is shown for comparison. Total mRNA was isolated and subjected to PCR with primers for FasL or β-actin. PCR products were size fractionated by agarose electrophoresis and normalized according to the amount of β-actin detected in the same mRNA sample. A, Densitometric analysis of each band is shown and expressed as pixel number (a.u.). Superimposable results were obtained with two additional NK cell populations (NK2, NK3). B, FasL present in culture SN of NK cells was evaluated by ELISA. The bulk NK cell population NK1 was stained with the indicated mAbs (3 μg/ml). Mix anti-NCR: NK cells stained with anti-NKp30, NKp44, and NKp46 mAbs (3 μg/ml for each mAb). Anti-ICAM-1 mAb was used as isotype-matched control, while anti-CD16 mAb was used as a positive control in inducing FasL release by NK cells. None, FasL release by NK cells incubated with medium alone. Cells were incubated for 36 h in medium alone (none, FasL release by NK cells incubated with medium alone. Cells were incubated for 36 h in medium alone (none, Anti-Fas mAb (CH11, IgM) was used as positive control of caspase 3 activation. Results are expressed as percentage of increase above the basal level, that is, caspase 3 activation in NK cells cultured in medium alone. Superimposable results were obtained with three additional NK cell populations (NK2, NK3, and NK6).

Supporting the hypothesis that the stronger the activating signal delivered through these NCR, the more powerful the NK cell-activated lytic effect and the more evident the NCR-mediated NK cell apoptosis.

**FIGURE 5.** The engagement of NCR NKp30, NKp44, or NKp46 induces FasL mRNA up-regulation, FasL protein release, and activation of caspase 3. A, Upper, Up-regulation of FasL mRNA by the engagement of NKp30, NKp44, or NKp46. The bulk NK cell population NK1 was incubated with the specific mAbs (3 μg/ml) to the indicated surface receptors, followed by GAM-coated beads (four beads for each NK cell) for 3 h. The engagement of ICAM-1 with an isotype-matched mAb is shown for comparison. Total mRNA was isolated and subjected to PCR with primers for FasL or β-actin. PCR products were size fractionated by agarose electrophoresis and normalized according to the amount of β-actin detected in the same mRNA sample. A, Densitometric analysis of each band is shown and expressed as pixel number (a.u.). Superimposable results were obtained with two additional NK cell populations (NK2, NK3). B, FasL present in culture SN of NK cells was evaluated by ELISA. The bulk NK cell population NK1 was stained with the indicated mAbs (3 μg/ml). Mix anti-NCR: NK cells stained with anti-NKp30, NKp44, and NKp46 mAbs (3 μg/ml for each mAb). Anti-ICAM-1 mAb was used as isotype-matched control, while anti-CD16 mAb was used as a positive control in inducing FasL release by NK cells. None, FasL release by NK cells incubated with medium alone. Cells were incubated for 36 h in medium alone (none, Anti-Fas mAb (CH11, IgM) was used as positive control of caspase 3 activation. Results are expressed as percentage of increase above the basal level, that is, caspase 3 activation in NK cells cultured in medium alone. Superimposable results were obtained with three additional NK cell populations (NK2, NK3, and NK6).
Cyclosporin A (CsA) down-regulates target-induced NK cell apoptosis

It has been reported that FasL synthesis is dependent on the activation of calcineurin NF-AT, which is inhibited by CsA (22). Accordingly, we found that NK cell treatment with CsA inhibited, in a dose-dependent manner, the NK cell apoptosis consequent to interaction with target cells. This inhibitory effect was maximal at 500 ng/ml CsA ($p < 0.001$, $n = 6$, data from six bulk NK cell populations), while it was negligible at 5 ng/ml (Fig. 6A). Furthermore, sFasL was not found in culture SN of NK cells stimulated with anti-NKp30, NKp44, or NKp46, and GAM-coated beads in the presence of CsA (Fig. 6B). However, this drug did not affect NK cell-mediated cytolytic activity of these targets (data not shown). In addition, CsA-treated NK cells could kill tumor targets as efficiently as untreated NK cells upon triggering with anti-NCR mAbs (Fig. 6C). Importantly, we found that the H$^+$-ATPase vacuolar inhibitor concanamycin A (CMA), which is known to inhibit perforin-based cytotoxic activity (23, 24), did not affect target cell-induced apoptosis of NK cells and sFasL release (Fig. 6, A and B) ($p < 0.001$, $n = 14$, data from 14 different NK cell clones obtained from 3 different donors). In contrast, this drug inhibited by 50–70% the cytolytic elicited via NCRs (Fig. 6C). These findings strongly support the idea that CsA can block NK cell apoptosis by avoiding sFasL release without affecting the triggering of NK cell-mediated cytolytic activity.

Discussion

In this study, we have shown that NK cells undergo apoptosis upon interaction with tumor cells. NK cell death follows the killing of targets, and it is initiated by the engagement of NCR. Indeed, the cross-linking of NKp30, NKp44, or NKp46 induces the up-regulation of FasL transcription, synthesis, and release. This, in turn, triggers the apoptosis of NK cells by interacting with Fas on the NK cell surface. Thus, we provide evidence that FasL released by NK cells upon tumor cell contact, besides being a means of killing tumor targets (25), is a mediator of effector cell death.

According to these findings, it is conceivable that tumor-induced effector cell apoptosis functions as a mechanism of tumor escape. Indeed, any time a powerful cytolytic NK lymphocyte encounters a tumor cell, this interaction leads to the death of the target and eventually to its death, but also to NK cell suicide. This event appears to be a consequence of the triggering of cytolytic activity; thus, NK cell suicide might also represent an intrinsic regulatory mechanism involved in switching off cytolytic to block dangerous NK cell-mediated effects on healthy host cells.

Different molecules, known to activate cytolytic in NK cells, including CD16 and CD2, have been demonstrated to be involved in inducing NK cell death (1, 26). Along this line, we reported that the engagement of the activating isoforms of IRS triggers both cytotoxicity and NK cell apoptosis, due to the up-regulation of mRNA, protein synthesis, and release of FasL that binds to Fas expressed by NK cells (16). Other activating molecules, including NKG2D, expressed by cytotoxic lymphocytes and involved in the triggering of cytotoxicity upon recognition of ligands on target cells (27, 28), may play a role in inducing NK cell apoptosis following target cell binding. However, no experimental evidence is reported to date indicating that apoptosis can be elicited via NKG2D.

In the present work, we show that NCR NKp30, NKp44, and NKp46, which are specifically expressed by NK cells and responsible for the killing of tumor targets (14), are directly involved in NK cell suicide consequent to effector-target interaction. Furthermore, NK cells are known to express the inhibitory isoforms of IRS, whose engagement down-regulates NK cell functions, including cytolytic activity (6–8). IRS recognize self HLA-I Ags, and it has been stated that inhibiting signals delivered through IRS can overcome any activating signal initiated via triggering receptors...
(6–8). According to this model, NK cells cannot lyse tumor cells because many cancers express HLA-I Ags. Thus, on one hand, to escape the immune system-mediated control tumors would express those HLA-I Ags that are counterreceptors of IRS at the NK cell surface. In contrast, tumor cells lacking HLA-I can be killed by NK cells, possibly through the engagement of NCR by their putative ligands expressed on tumor targets; however, NK cells cannot survive to the delivery of the lethal hit as they trigger their own suicide through NCR. During NK/tumor target cell interaction, NCR may trigger not only cytolysis, but also production of immunoregulatory cytokines as IFN-γ and TNF-α (6). In turn, these cytokines can activate NK cell cytosis (1) as well as cell death (29). This effect would further down-regulate antitumor NK cell response.

Based on these considerations, we can speculate that antitumor response might be down-regulated by the same molecules whereby it is triggered. This would greatly decrease the efficiency of immunotherapy of tumors, which is largely based on the ability of inducing potent cytolytic effector cells (2–5). In addition, the ratio between NK effector cells and tumor target cells should be taken into consideration. Indeed, in our experimental system, NK cell apoptosis was evident at low E:T ratios, as 2:1 or 1:1, while it was negligible at 10:1. This would imply that, to perform an effective antitumor trial by infusing cytolytic effector cells, one should use not only the most powerful antitumor cells, that is, NK cells activated with high doses of IL-2 (1–5), but also an amount of NK cells 10-fold higher than that of tumor cells.

In this regard, the finding that CsA can selectively inhibit the apoptosis of NK cells, but not their cytolytic function, would provide a potential tool to avoid NK cell death after interaction with tumor targets. Indeed, CsA would prolong both NK cell survival and the antitumor effect exerted by NK cells. If this were true also in vivo, this may allow the usage of low amounts of NK cells for immunotherapy, while the discontinuation of CsA might render NK cells again susceptible to apoptosis after interaction with tumor cells, thus avoiding potentially harmful reactions against healthy tissues.

Disclosures
The authors have no financial conflict of interest.

References