NKp44, a Novel Triggering Surface Molecule Specifically Expressed by Activated Natural Killer Cells, Is Involved in Non–Major Histocompatibility Complex-restricted Tumor Cell Lysis

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Summary

After culture in interleukin (IL)-2, natural killer (NK) cells acquire an increased capability of mediating non–major histocompatibility complex (MHC)-restricted tumor cell lysis. This may reflect, at least in part, the de novo expression by NK cells of triggering receptors involved in cytolysis. In this study we identified a novel 44-kD surface molecule (NKp44) that is absent in freshly isolated peripheral blood lymphocytes but is progressively expressed by all NK cells in vitro after culture in IL-2. Different from other markers of cell activation such as CD69 or VLA.2, NKp44 is absent in activated T lymphocytes or T cell clones. Since NKp44 was not detected in any of the other cell lineages analyzed, it appears as the first marker specific for activated human NK cells. Monoclonal antibody (mAb)-mediated cross-linking of NKp44 in cloned NK cells resulted in strong activation of target cell lysis in a redirected killing assay. This data indicated that NKp44 can mediate triggering of NK cell cytotoxicity. mAb-mediated masking of NKp44 resulted in partial inhibition of cytolytic activity against certain (FcγR-negative) NK-susceptible target cells. This inhibition was greatly increased by the simultaneous masking of p46, another recently identified NK-specific triggering surface molecule. These data strongly suggest that NKp44 functions as a triggering receptor selectively expressed by activated NK cells that, together with p46, may be involved in the process of non-MHC-restricted lysis. Finally, we show that p46 and NKp44 are coupled to the intracytoplasmic transduction machinery via the association with CD3ζ or KARAP/DAP12, respectively; these associated molecules are tyrosine phosphorylated upon NK cell stimulation.

Key words: natural killer cells • cell-mediated cytotoxicity • activating receptors • subset-specific markers • tumor target cells
masking of p46 molecules resulted in a low degree inhibition of NK cell–mediated lysis of some HLA-negative target cells (14). A possible interpretation of these data is that p46 may contribute to the process of tumor cell lysis, which, however, would require the cooperation of additional receptor–ligand interactions.

In this study, we describe a novel NK-specific molecule termed NKp44 that, similar to p46, mediates triggering of all NK cell clones. However, different from p46, NKp44 is only expressed by activated NK cells, thus representing a useful marker for their identification. More importantly, NKp44 appears to function as a receptor initiating a pathway of NK cell triggering during the process of non-MHC-restricted cytotoxicity by activated NK cells.

Materials and Methods

Antibodies. The following mAbs were produced in our lab: JT3A (IgG1, anti-CD3); c127 (IgG1, anti-CD6); KD1 (IgG1, anti-CD16); c218 (IgG1, anti-CD56); A6-220 (IgM, anti-CD56); c227 (IgG1, anti-CD69); BAB281 (IgG1, anti-p46); and FES172 (IgG1, anti-p50.3). mAbs D1.12 (IgG1, anti-aCD4), and B9.4 (IgG3, anti-CD8) were provided by J. Trepels (Centre de Immunologie INSERM/CNRS, Marseille, France). The following mAbs were produced in our lab: a21 (IgG1, anti-CD4), and B9.4 (IgG3, anti-CD8) were provided by J. Trepels (Centre de Immunologie INSERM/CNRS, Marseille, France). The 24-31 mAb (IgG1, anti-CD154/CD40L) was purchased from Ancell (Bayport, MN), and the Gi9 mAb (IgG1, anti-CD49b/CD29) was purchased from Immunotech (Marseille, France). The 24-31 mAb (IgG1, anti-CD154/CD40L) was purchased from Ancell (Bayport, MN), and the Gi9 mAb (IgG1, anti-CD49b/CD29) was purchased from Immunotech (Marseille, France).

Purification of Polyclonal or Clonal NK or T Cell Populations and Cytolytic Activity. PBMCs were depleted of plastic-adherent cells to obtain NK cell–enriched lymphocyte populations. PBMCs were incubated with anti-CD3 (JT3A) and anti-CD4 (HP2.6) mAbs for 30 min at 4°C followed by treatment with goat anti-mouse-coated Dynabeads (Dynal, Oso, Norway) for 30 min at 4°C. The resulting CD3–lymphocyte populations containing ~1% CD3+ cells, 20–30% HLA-DR+ cells, and 70–80% CD56–cells, were cultured in rIL-2 (Cetus Corp., Emeryville, CA). To obtain polyclonally activated T cell–enriched lymphocyte populations, PBMCs were stimulated with 0.1% (vol/vol) PHA (GIBCO Ltd., Paisley, Scotland) for 24 h and then cultured in rIL-2. T or NK clones were obtained by limiting dilution, as previously described (15). Polyclonally activated cell cultures containing both NK and T cells, obtained by culturing PBMCs in the presence of irradiated H9 cell line and rIL-2, were used for the analysis of NKp44 expression in resting or activated CD3+ and CD3–cells at various time intervals. The cytolytic activity was assessed in a 4-h 51Cr-release assay (15) in which effector cells were tested against the following (FcγR+) human cell lines: A549 (lung adenocarcinoma), LCL 721.221 (B-EBV), RPMI 8866 (B-EBV), and IGR0V (ovarian carcinoma) at the indicated E/T ratio. In redirected killing assays, target cells were represented by the (FcγR+) murine mastocytoma P815 cell line.

Biochemical characterization of the NKp44 molecules. Cyanogen bro-

Production of Z231 mAb and Flow Cytomfluometric Analysis. Z231 mAb (IgG1) was obtained by immunizing a 5-wk-old BALB/c mouse with the NK clone SA260. Analysis of the cell distribution of NKp44 molecules was performed by using one- or two-color fluorescence cytomfluometric analysis (FACSscan®, Becton Dickinson, San Jose, CA), as previously described (15).

Figure 1. Functional and molecular characteristics of NKp44 surface molecule. (a) Triggering of cytolytic activity. Four representative NK clones, derived from two donors, were analyzed in a redirected killing assay against the FcγR+ P815 target cells either in the absence (□) or presence of Z231 (■), anti-CD16 ( ), anti-p46 ( ), anti-CD56 ( ), anti-CD69 ( ), or anti-CD40L ( ) mAbs at a 5:1 E/T ratio. All these mAbs are IgG1. Clones MA4 and MA5 were CD40L+, whereas clones MX340 and MX310 were CD40L−. (b) Biochemical characteristics. A 2D11-surface labeled polyclonal NK population, derived from donor LM, was immunoprecipitated with Z231 (lanes A and E), anti-CD69 (lanes B and F), anti-CD16 (lanes C and G) or BAB281 (lanes D and H) mAbs. Samples were analyzed in an 11% SDS-PAGE under nonreducing (lanes A–D) or reducing (lanes E–H) conditions. (c) N-glycosidase F digestion. NKp44 (lanes A and B), CD69 (lanes C and D), CD16 (lanes E and F), or p46 (lanes G and H) molecules isolated from the surface-labeled NK cell population shown in b were treated or not with N-glycosidase F. Samples were run in an 11% SDS-PAGE under reducing conditions.
mide sepharose (Pharmacia Biotech, Piscataway, NJ)-coupled Z231 or BAB281 mAbs or Sepharose–protein A–coupled K01 and C227 mAbs were used to immunoprecipitate specific molecules from 1% NP40 lysates of cells surface-labeled with 125I (Du-Pont-NEN, Waltham, MA) as previously described (5, 15).

Immunoprecipitates were analyzed by discontinuous SDS-PAGE. Specific bands were cut out from dried gel and eluted proteins were used for N-glycosidase F digestion (Boehringer Mannheim, GmbH, Mannheim, Germany) (5, 16). For two-dimen-
sional peptide mapping analysis, the purified proteins were di-
gested with pepsin and peptides were analyzed by electrophoresis in the first dimension [M ultriphor II, Pharmacia Biotech], fol-
lowed by chromatography in the second dimension (5, 16).

Biochemical characterization of the p46- and NKp44-associated Molecules. 106 NK cells were stimulated or not with 100 μM Nα-pervanadate (Nαv, 107, 37°C), washed twice in cold PBS, and lysed in PLB (50 mM Tris-HCL, pH 8, 15 mM NaCl, 1% Digitonin, and phosphatase and protease inhibitors) (45, 1°C). Lysates were immunoprecipitated with various mAbs for 1 h at 4°C. Immunoprecipitates were washed twice in PLB and eluted samples were analyzed in 15% discontinuous SDS-PAGE under reducing conditions. Proteins were transferred to Immobilon P (Millipore Corp., Bedford, MA), and the membranes were saturated with 5% BSA and probed with Tia/2 (anti-C3; mAb, provided by Dr. B. Malissen), followed by goat anti-mouse-horseradish peroxidase (HRPO; DAKO A/S, Glostrup, Denmark) or with PY20-HRPO (antiphosphotyrosine [anti-PTyr] mAb; Transduction Laboratories, Lexington, KY). The Renaissance Chemiluminescence Kit (New England Nuclear, Boston, MA) was used for detection.

Results and Discussion

Identification of a Novel 44-kD Triggering Surface Molecule Selectively Expressed by Activated NK Cells. Mice were im-
munized with the NK cell clone SA260 (surface pheno-
type: CD3+, CD16+, p46+, p70+, NKG2A−). After cell fu-
sion, an mAb termed Z231 was selected on the basis of its abil-
ity to induce lysis of the FcγR+ P815 target cells by a panel of NK cell clones. The magnitude of cytolysis induced
by Z231 mAb in this redirected killing assay was comparable to that induced by anti-C3β or the anti-p46 mAb BAB281 (Fig. 1 a). Cell lysates, obtained from IL-2–cultured, polyclonal NK cells that had been surface labeled with 125I, were immunoprecipitated with Z231 mAb and analyzed by SDS-PAGE. A broad band of ~44-kD could be identified both under reducing and nonreducing condi-
tions (Fig. 1 b). The mobility of this molecule (hereafter termed NKp44) was slightly modified by treatment with N-glycosidase F (Fig. 1 c). On the other hand, treatment with neuraminidase followed by β-galactosidase had no effect (data not shown). These data suggest that NKp44 may represent a molecule with a low degree of glycosyla-
tion.

Cytofluorimetric analysis revealed that NKp44 was ex-
pressed on virtually 100% of cells in activated polyclonal
NK cell populations (Fig. 2, box A). Moreover, it was ex-
pressed in all NK cell clones analyzed. Fig. 2 (boxes B–E) shows four representative clones that illustrate the hetero-
genrety in the levels of NKp44 expression among different clones. On the other hand, none of the numerous T cell
clones or polyclonal T cell populations analyzed expressed
NKp44 (Fig. 2, boxes F–I). This was also true for selected
T cell clones (either TCR-α/β+ or -γδ+) expressing NK
cell markers including CD56, CD16, and KIRs (p58, p70,
or CD94/NKG2A). In addition, Z231 mAb did not stain fresh
BMCs (isolated from different donors), granulocytes,
monocyte/macrophages, enriched T, B, or NK cells iso-
lated from peripheral blood, EBV-induced B cell lines, or
various nonhematopoietic cell lines (data not shown).

These data indicate that NKp44 is specifically expressed
by activated NK cells, thus differing from other molecules
such as p46 or CD16, which are expressed also in resting
NK cells. We next analyzed the surface expression
of NKp44 in unfractionated PBMCs cultured in IL-2. The
expression of NKp44 or other informative markers includ-
ing CD56, p46, CD69, and VLA-2 was analyzed at various
time intervals by double fluorescence against CD3. As
shown in Fig. 2 (day 0), at the onset of the culture NKp44
molecules were undetectable in both CD3+ and CD3−
cells. After 10 d of culture, NKp44 was expressed by a
portion of CD3− cells, whereas after 20 d, virtually all CD3−
cells were NKp44+. No expression of NKp44 could be
detected in CD3+ T cells even after 30 d of culture (data
not shown). These data support the notion that NKp44
represents a novel marker of cell activation specifically
expressed by NK cells. In agreement with previous data, the
other markers of cell activation, used for comparison, were
not confined to NK cells (17, 18). Thus, CD69 (Fig. 2) and
VLA-2 (data not shown) were expressed both in CD3+ and
CD3− cultured cells. In addition, CD69 reached maximal
expression after 24–48 h of culture (17), whereas expres-
sion of NKp44 only started after 3 d and progressively in-
creased thereafter (data not shown). In agreement with
the data that NKp44 is not expressed in fresh NK cells, mAb
Z231 failed to trigger target cell lysis by fresh CD3− cells
in a redirected killing assay. Increments in cytolitic activity
induced by Z231 mAb paralleled the progressive expres-
sion of NKp44 in cultured NK cells. Moreover, triggering
of cytolitic activity in a redirected killing assay was detect-
able only by using the intact mAb and not the F(ab′)2 frag-
ment (data not shown). This data indicates that NKp44
requires efficient cross-linking to induce NK cell triggering.

Comparative analysis between NKp44 and p46 Molecules. Both the finding that NKp44 mediates triggering of NK
cell cytotoxicity and the finding that its expression is con-
fined to NK cells are reminiscent of the previously de-
scribed p46 molecule. The two molecules also display a
similar molecular mass (44 and 46 kD, respectively). In
addition, as with p46, cross-linking of NKp44 led to Ca2+
mobilization and lymphokine (TNF-α and IFN-γ) release
in cloned NK cells (data not shown). A major difference
between the two molecules is that NKp44 is expressed only
upon NK cell activation. However, the possibility exists
that the epitope recognized by the Z231 mAb, although
carried by p46 molecules, may be exposed only upon cell
activation. To verify this possibility, 125I-labeled cell lysates
were prepared from either polyclonal or clonal (IL-2-cultured) NK cell
populations were precleared with anti-p46 mAb followed by immunoprecipitation with anti-NKp44 mAb (and vice versa). These experiments clearly indicated that the two molecules were distinct (Fig. 3 a). These data were further confirmed by two-dimensional peptide map analysis as shown in Fig. 3 b, none of the spots visualized in the NKp44 map correspond to those of the p46 map and vice versa.

NKp44 and p46 Are Associated with Different Signal-transducing Molecules. In human NK cells, triggering receptors such as CD16 or p50 (i.e., the activating forms of the HLA-C-specific receptors; references 5, 6, 16) are coupled...
to the intracytoplasmic transduction machinery via the association with polypeptide subunits characterized by the presence of immunoreceptor tyrosine-based activation motifs (ITAMs). CD16 associates with CD3ζ/FcεRIγ polypeptides (19, 20), whereas p50 molecules have recently been shown to be part of a multimeric complex formed by the association of these receptors with a newly identified transducing molecule, termed KARAP/DAP12 (21, 22). Both CD3ζ and KARAP/DAP12 molecules are tyrosine phosphorylated after engagement of the associated receptors. We analyzed whether NKp44 and p46 molecules were associated with one or another signal-transducing molecules. Cell lysates derived from NK bulk populations, stimulated or not with Na-pervanadate, were immunoprecipitated with anti-NKp44, anti-p46, anti-p50.3, or anti-CD16 mAbs. Samples were analyzed in SDS-PAGE, transferred to PVDF membranes, and then immunoblotted with anti-CD3ζ (Fig. 4a) or anti-PTyr (Fig. 4b) mAbs. No tyrosine phosphorylation of either p46 or NKp44 molecules could be detected under these experimental conditions (data not shown). On the other hand, as shown in Fig. 4a, p46, like CD16, was associated with CD3ζ molecules that became tyrosine phosphorylated upon cell stimulation (Fig. 4b). On the contrary, NKp44 did not associate with CD3ζ molecules (Fig. 4a). However, after stimulation, tyrosine phosphorylated molecules displaying a molecular mass of ~14–16 kD could be detected in NKp44 immunoprecipitates (Fig. 4b). These molecules displayed a molecular mass similar to the p50.3-associated KARAP/DAP12 molecules (Fig. 4b). In addition, the 14–16-kD NKp44-associated molecules were still detectable after extensive preclearing with anti-CD3ζ/FcεRIγ Abs. This experiment ruled out the possibility that the NKp44-associated 14–16-kD tyrosine phosphorylated molecules were either CD3ζ or FcεRIγ, and suggested that they correspond to the recently identified KARAP/DAP12 molecules.

NKp44 Is Involved in NK Cell Activation during the Process of Non-MHC-restricted Cytotoxicity. Since NKp44 represents a triggering surface molecule that is selectively expressed by activated NK cells, it is possible that it represents a receptor involved in the process of non-MHC-restricted tumor cell lysis by activated NK cells. To analyze this possibility, (NKp44+) NK cell clones were selected for their ability to kill a panel of (FcγR2) human target cell lines. Selected clones were next assessed for cytotoxicity either in the presence or absence of Z231 mAb. Although in the absence of mAb all targets were efficiently lysed, in the presence of Z231 mAb a minor inhibitory effect was detectable against the A549 (lung adenocarcinoma), RPMI 8866, and LCL 721.221 (EBV-transformed B cell lines) target cells.
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(Fig. 5). A similar inhibitory effect was obtained with the anti-p46 mAb BAB281. These data suggested that NKp44 and p46 molecules could cooperate in the process of NK cell triggering, resulting in killing of A549, 221, and 8866 cells. To assess this possibility, anti-NKp44 and anti-p46 mAbs were used in combination. Indeed, a sharp inhibition of target cell lysis was obtained (Fig. 5), thus suggesting that the two molecules may function as receptors that act synergistically in the process of non-MHC-restricted lysis by activated NK cells. No inhibition of cytolysis was detected in the presence of a control, isotype-matched antibody (CD56, IgG1; data not shown) or in the presence of an antibody directed to another triggering molecule expressed by NK cells after activation (anti-CD69, IgG1). In addition, the inhibitory effects of anti-NKp44 or p46 mAb were not increased when used in combination with anti-CD69 mAb (Fig. 5).

Remarkably, killing of IGROV cells was not affected by anti-p46 and anti-NKp44 mAbs even when used in combination. This may be explained as follows. (a) IGROV cells do not express, or express in low density, the ligands recognized by NKp44 or p46 receptors. (b) IGROV cells do express the p46 and NKp44 ligands; however, their recognition by the corresponding NK receptors does not contribute significantly to NK cell triggering. In any case, it is conceivable that IGROV cells express additional ligands for still undefined triggering NK receptors. Interactions between these receptors and their ligands would play a predominant role in the NK-mediated lysis of these target cells. These data are consistent with the idea that NK cell triggering may be mediated by several different receptor-ligand interactions (23). Expression, lack of expression, or even different levels of expression of given ligands for triggering receptors may influence the susceptibility of tumor cells to NK cell-mediated killing. In this context, the use of target cells such as A549, 8866, and 221 gave useful results in this study in identifying the role of NKp44 molecule as a triggering receptor in the NK-target cell interactions.

Regarding the still undefined ligand for NKp44, the use as target cells of the HLA class I-221 cell line transfected or not with different HLA class I alleles ruled out the possibility that HLA class I molecules could trigger NK cells via NKp44 (data not shown).

The NK-mediated target cell lysis may also depend upon the type (and density) of receptors on different effector cells. For example, since NKp44 molecule is selectively expressed by activated NK cells, it is conceivable that it may play at least a partial role in the enhanced cytolytic activity of these cells. Although both NKp44 and p46 are homogeneously expressed on activated NK cells, it is possible that other triggering receptors may be expressed selectively by subsets of NK cells (as is the case for the HLA-C-specific p50 molecules).

In humans, other surface molecules, including CD69 (24) and CD40L (25), have been reported to be involved in triggering of cytotoxicity mediated by in vitro activated NK cells. However, these molecules are not NK-specific and do not appear to interfere with non-MHC-restricted target cell lysis. Regarding the CD40L, we found that it is expressed only by some NK cell clones and we failed to induce any substantial NK cell triggering in redirected killing assays (Fig. 1a). Gp42, a 42-kD, NK-specific rat molecule, is reminiscent of NKp44 (26, 27). Indeed, it was detected in IL-2-induced NK cells and was shown to trigger cytotoxicity mediated by the RNK-16 rat NK cell line. However, Gp42 failed to activate IL-2-induced NK cells. In addition, Gp42 is a glycosylphosphatidylinositol-anchored surface molecule, which is not the case for GPI NKp44 since its surface expression is not modified by cell treatment with phosphatidylinositol-specific phospholipase (data not shown).

In conclusion, this study revealed a novel surface molecule selectively expressed by human activated NK cells that appears to function as a triggering receptor involved in non-MHC-restricted cytotoxicity by activated NK cells. The identification of NKp44 as a putative NK receptor may lead to a more precise definition of the nature of the receptor-ligand interactions occurring in the NK-mediated lysis of tumor cells.
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