

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

By Massimo Vitale,\* Cristina Bottino,\* Simona Sivori,†  
Lorenza Sanseverino,† Roberta Castriconi,† Emanuela Marcenaro,†  
Raffaella Augugliaro,† Lorenzo Moretta,† and Alessandro Moretta‡

From the \*Istituto Nazionale per la Ricerca sul Cancro and Centro Biotecnologie Avanzate, 16132 Genova, Italy; and the †Dipartimento di Medicina Sperimentale, Università degli Studi di Genova, 16132 Genova, Italy

### 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

**H**uman NK cells express a series of clonally distributed HLA class I-specific killer inhibitory receptors (KIR) that suppress the NK cytotoxicity upon engagement with self HLA class I alleles (1–4). At least in the case of HLA-C-specific receptors, an activating form (termed p50) has also been identified. Upon interaction with appropriate HLA-C alleles, p50 triggers the NK-mediated cytolytic activity (5, 6). Therefore, HLA class I-specific receptors may regulate the NK cell function both in a negative and a positive fashion. However, NK cells are known to efficiently kill target cells that do not express HLA class I molecules, thus implying the existence of triggering receptors for non-HLA ligands. Since all NK cell clones kill HLA-negative target cells (1–4, 7, 8), it is conceivable that triggering re-

ceptors for non-HLA ligands are broadly expressed in NK cells. However, major variability exists in the susceptibility of different HLA class I-negative target cells to NK-mediated lysis (9, 10). This phenomenon may reflect a variable expression of different non-HLA ligands. Moreover, culture in exogenous IL-2 greatly enhances the cytolytic activity of NK cells against HLA-negative target cells (11–13). A possible explanation could be the de novo expression in NK cells of triggering receptors specific for additional non-HLA ligands. At present, little is known on the nature of the triggering NK receptors or of their non-HLA ligands. Along this line, we recently identified a novel triggering surface molecule, termed p46, that is specifically expressed on all resting and activated human NK cells (14). mAb-mediated

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.

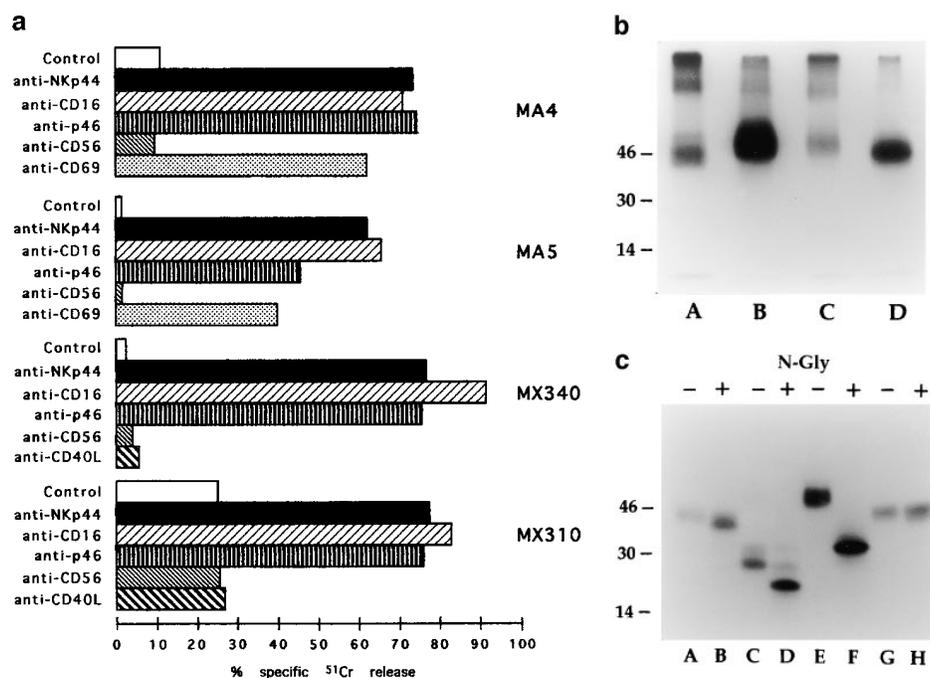
## Materials and Methods

**Antibodies.** The following mAbs were produced in our lab: JT3A (IgG<sub>2a</sub> anti-CD3); c127 (IgG<sub>1</sub> anti-CD16); KD1 (IgG<sub>2a</sub> anti-CD16); c218 (IgG<sub>1</sub> anti-CD56); A6-220 (IgM anti-CD56); c227 (IgG<sub>1</sub> anti-CD69); BAB281 (IgG<sub>1</sub> anti-p46); and FES172 (IgG<sub>2a</sub> anti-p50.3). mAbs D1.12 (IgG<sub>2a</sub> anti-HLA-DR), HP2.6 (IgG<sub>2a</sub> anti-CD4), and B9.4 (IgG<sub>2b</sub> anti-CD8) were provided by Drs. R.S. Accolla (Università di Pavia, Pavia, Italy), P. Sanchez-Madrid (Hospital de la Princesa, Madrid, Spain), and B. Malissen (Centre de Immunologie INSERM/CNRS, Marseille, France). The 24-31 mAb (IgG<sub>1</sub> anti-CD154/CD40L) was purchased from Ancell (Bayport, MN), and the Gi9 mAb (IgG<sub>1</sub> anti-CD49b/VLA.2) was purchased from Immunotech (Marseille, France).

**Production of Z231 mAb and Flow Cytofluorometric 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 cytofluorometric analysis (FACScan<sup>®</sup>, Becton Dickinson, San Jose, CA), as previously described (15).

**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 (Dyna, Oslo, Norway) for 30 min at 4°C. The resulting CD3<sup>-</sup> lymphocyte populations, containing ~1% CD3<sup>+</sup> cells, 20–30% HLA-DR<sup>+</sup> cells, and 70–80% CD56<sup>+</sup> 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<sup>+</sup> and CD3<sup>-</sup> cells at various time intervals. The cytolytic activity was assessed in a 4-h <sup>51</sup>Cr-release assay (15) in which effector cells were tested against the following (FcγR<sup>-</sup>) human cell lines: A549 (lung adenocarcinoma), LCL 721.221 (B-EBV), RPMI 8866 (B-EBV), and IGROV (ovarian carcinoma) at the indicated E/T ratio. In redirected killing assays, target cells were represented by the (FcγR<sup>+</sup>) murine mastocytoma P815 cell line.

**Biochemical Characterization of the NKp44 Molecules.** Cyanogen bro-



**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<sup>+</sup> 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<sup>-</sup>, whereas clones MX340 and MX310 were CD40L<sup>+</sup>. (b) Biochemical characteristics. A <sup>125</sup>I-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.

mid-sepharose (Pharmacia Biotech, Piscataway, NJ)-coupled Z231 or BAB281 mAbs or Sepharose-protein A-coupled KD1 and C227 mAbs were used to immunoprecipitate specific molecules from 1% NP40 lysates of cells surface-labeled with  $^{125}\text{I}$  (DuPont-NEN, Wilmington, DE) 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-dimensional peptide mapping analysis, the purified proteins were digested with pepsin and peptides were analyzed by electrophoresis in the first dimension (Multiphor II, Pharmacia Biotech), followed by chromatography in the second dimension (5, 16).

**Biochemical Characterization of the p46- and NKp44-associated Molecules.**  $10^8$  NK cells were stimulated or not with 100  $\mu\text{M}$  Na-pervanadate (NaV, 10', 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', 4°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-CD3 $\zeta$  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.

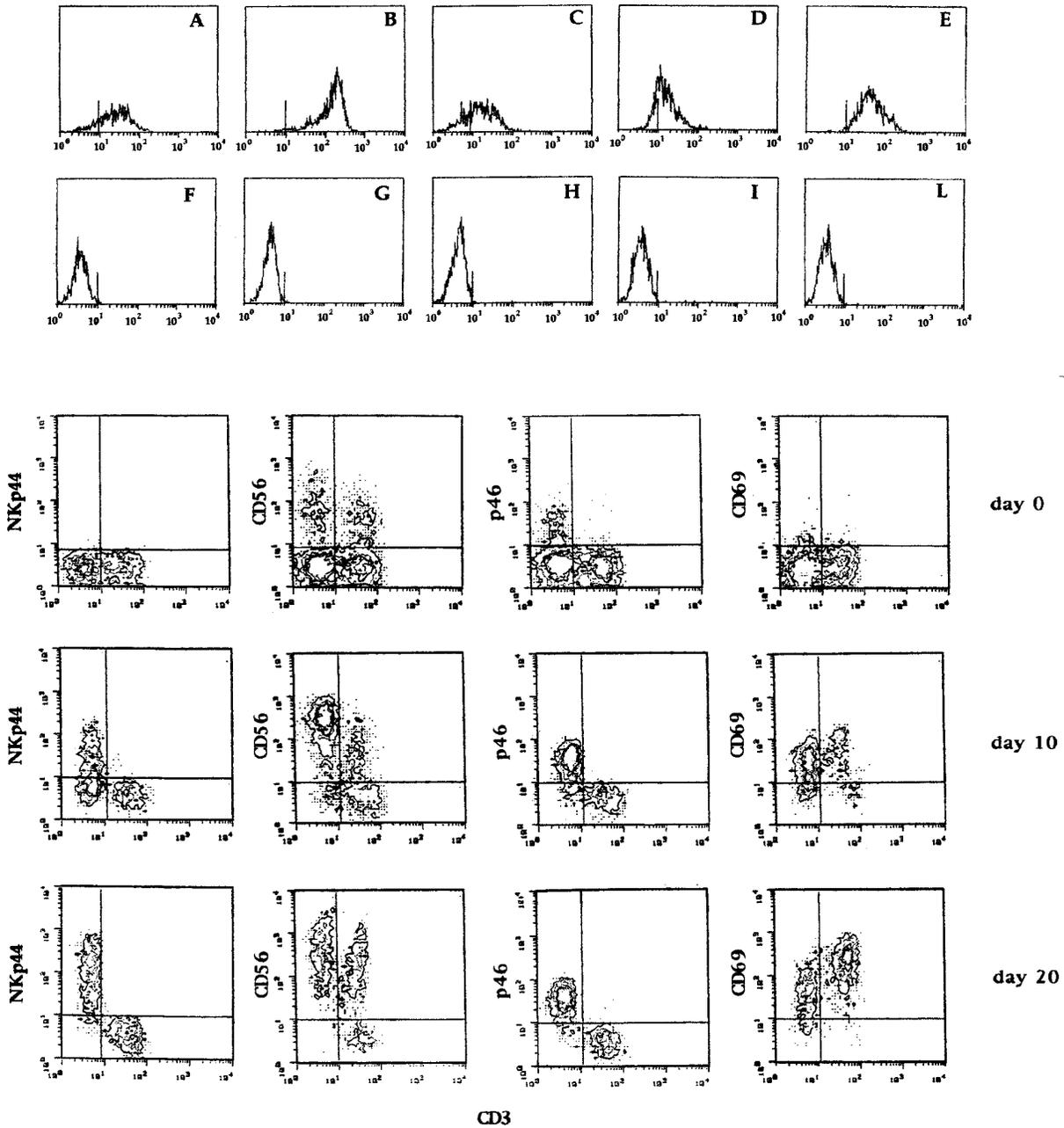
## Results and Discussion

**Identification of a Novel 44-kD Triggering Surface Molecule Selectively Expressed by Activated NK Cells.** Mice were immunized with the NK cell clone SA260 (surface phenotype: CD3 $^-$ , CD16 $^+$ , p46 $^+$ , p70 $^+$ , NKG2A $^+$ ). After cell fusion, an mAb termed Z231 was selected on the basis of its ability to induce lysis of the Fc $\gamma$ R $^+$  P815 target cells by a panel of NK cell clones. The magnitude of cytotoxicity induced by Z231 mAb in this redirected killing assay was comparable to that induced by anti-CD16 or by the anti-p46 mAb BAB281 (Fig. 1 a). Cell lysates, obtained from IL-2-cultured, polyclonal NK cells that had been surface labeled with  $^{125}\text{I}$ , were immunoprecipitated with Z231 mAb and analyzed by SDS-PAGE. A broad band of ~44-kD could be identified both under reducing and nonreducing conditions (Fig. 1 b). The mobility of this molecule (hereafter termed NKp44) was slightly modified by treatment with *N*-glycosidase F (Fig. 1 d). On the other hand, treatment with neuraminidase followed by *O*-glycanase had no effect (data not shown). These data suggest that NKp44 may represent a molecule with a low degree of glycosylation.

Cytofluorimetric analysis revealed that NKp44 was expressed on virtually 100% of cells in activated polyclonal NK cell populations (Fig. 2, box A). Moreover, it was expressed in all NK cell clones analyzed. Fig. 2 (boxes B-E) shows four representative clones that illustrate the heterogeneity 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-L). This was also true for selected T cell clones (either TCR- $\alpha/\beta^+$  or  $-\gamma/\delta^+$ ) expressing NK cell markers including CD56, CD16, and KIRs (p58, p70, or CD94/NKG2A). In addition, Z231 mAb did not stain fresh PBMCs (isolated from different donors), granulocytes, monocyte/macrophages, enriched T, B, or NK cells isolated 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 including 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 expression of NKp44 only started after 3 d and progressively increased 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 cytolytic activity induced by Z231 mAb paralleled the progressive expression of NKp44 in cultured NK cells. Moreover, triggering of cytolytic activity in a redirected killing assay was detectable only by using the intact mAb and not the F(ab') $_2$  fragment (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 confined to NK cells are reminiscent of the previously described 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 Ca $^{2+}$  mobilization and lymphokine (TNF- $\alpha$  and IFN- $\gamma$ ) 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,  $^{125}\text{I}$ -labeled cell lysates from either polyclonal or clonal (IL-2-cultured) NK cell

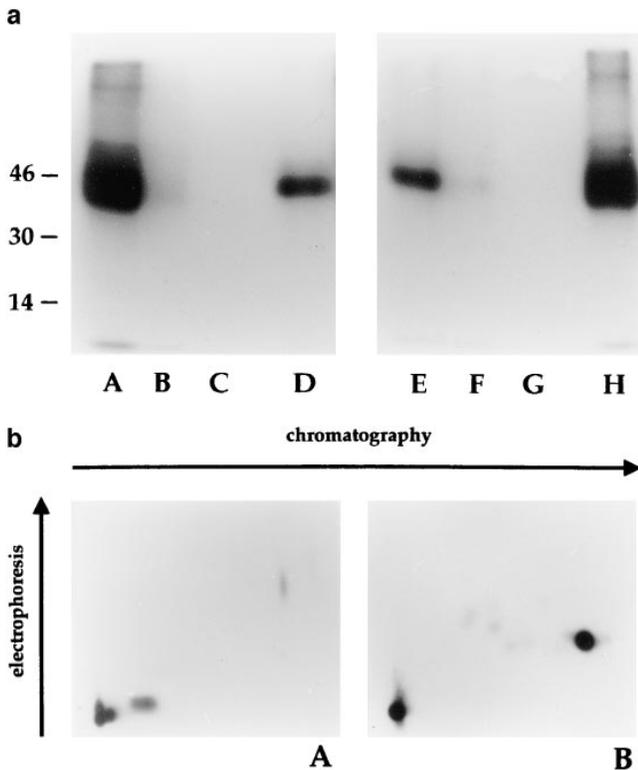


**Figure 2.** Expression of NKp44 molecules in freshly isolated or cultured polyclonal and clonal NK or T cell populations. (top) A polyclonal NK cell population (A), NK clones (B-E), a polyclonal T population (F), TCR- $\gamma/\delta^+$  (G), or TCR- $\alpha/\beta^+$  (H) T cell clones or TCR- $\gamma/\delta^+$  p58.2 $^+$  (I) and TCR- $\alpha/\beta^+$  p70 $^+$  (L) T cell clones were analyzed by immunofluorescence and FACS<sup>®</sup> analysis for surface expression of NKp44 molecules. (bottom) Freshly isolated PBMCs were cultured in the presence of the irradiated H9 cell line and rIL-2 and analyzed by two-color immunofluorescence and FACS<sup>®</sup> analysis at various time intervals for the expression of NKp44, CD56, p46, and CD69 surface molecules in combination with CD3 molecules. Cells were stained with mAbs to the indicated molecules followed by FITC- or PE-conjugated isotype-specific goat anti-mouse second reagents. The contour plots were divided into quadrants representing unstained cells (lower left), cells with only red fluorescence (upper left), cells with red and green fluorescence (upper right), and cells with only green fluorescence (lower right).

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

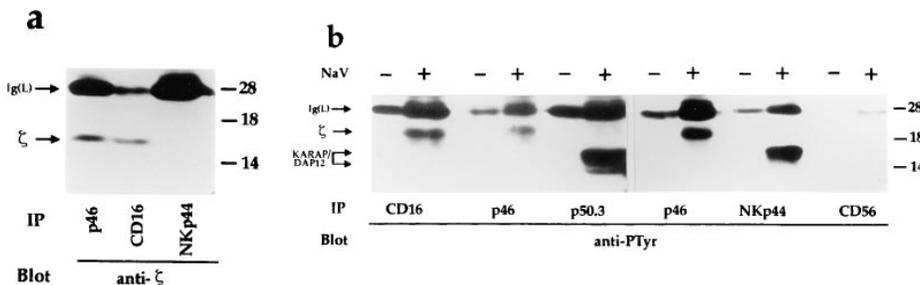


**Figure 3.** Comparison between NKp44 and p46 molecules. (a) Pre-clearing experiments. The  $^{125}\text{I}$ -surface labeled NK cell population derived from donor KK was immunoprecipitated three times with Z231 (lanes A–C) or BAB281 (lanes E–G). The NKp44 or p46 precleared lysates were then immunoprecipitated with BAB281 (lane D) or Z231 (lane H) mAbs, respectively. Samples were analyzed in an 11% SDS-PAGE under reducing conditions. (b) Peptide mapping analysis of NKp44 and p46 molecules.  $^{125}\text{I}$ -labeled NKp44 (A) or p46 (B) molecules were purified from the polyclonal NK cell population KK. Proteins were digested with pepsin and peptides were analyzed by electrophoresis in the first dimension followed by chromatography in the second dimension.

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 CD3 $\zeta$ /Fc $\epsilon$ RI $\gamma$  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 CD3 $\zeta$  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-CD3 $\zeta$  (Fig. 4 a) or anti-PTyr (Fig. 4 b) 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. 4 a, p46, like CD16, was associated with CD3 $\zeta$  molecules that became tyrosine phosphorylated upon cell stimulation (Fig. 4 b). On the contrary, NKp44 did not associate with CD3 $\zeta$  molecules (Fig. 4 a). However, after stimulation, tyrosine phosphorylated molecules displaying a molecular mass of  $\sim$ 14–16 kD could be detected in NKp44 immunoprecipitates (Fig. 4 b). These molecules displayed a molecular mass similar to the p50.3-associated KARAP/DAP12 molecules (Fig. 4 b). In addition, the 14–16-kD NKp44-associated molecules were still detectable after extensive preclearing with anti-CD3 $\zeta$ /Fc $\epsilon$ RI $\gamma$  Abs. This experiment ruled out the possibility that the NKp44-associated 14–16-kD tyrosine phosphorylated molecules were either CD3 $\zeta$  or Fc $\epsilon$ RI $\gamma$ , 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 (Fc $\gamma$ R $^{-}$ ) 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



**Figure 4.** Association of p46 and NKp44 with immunoreceptor tyrosine-based activation motif-bearing molecules. (a) Cell lysates derived from a polyclonal NK cell population were immunoprecipitated with mAbs to the indicated molecules. Samples were run in a 15% SDS-PAGE under reducing conditions, transferred to PVDF membranes, and probed with anti- $\zeta$  mAb. (b) Cell lysates derived from a polyclonal NK cell population unstimulated (NaV $^{-}$ )

or stimulated (NaV $^{+}$ ) with 100  $\mu\text{M}$  Na-pervanadate, were immunoprecipitated with mAbs to the indicated molecules. Samples were analyzed in a 15% SDS-PAGE under reducing conditions, transferred to PVDF membranes, and probed with anti-PTyr mAb.

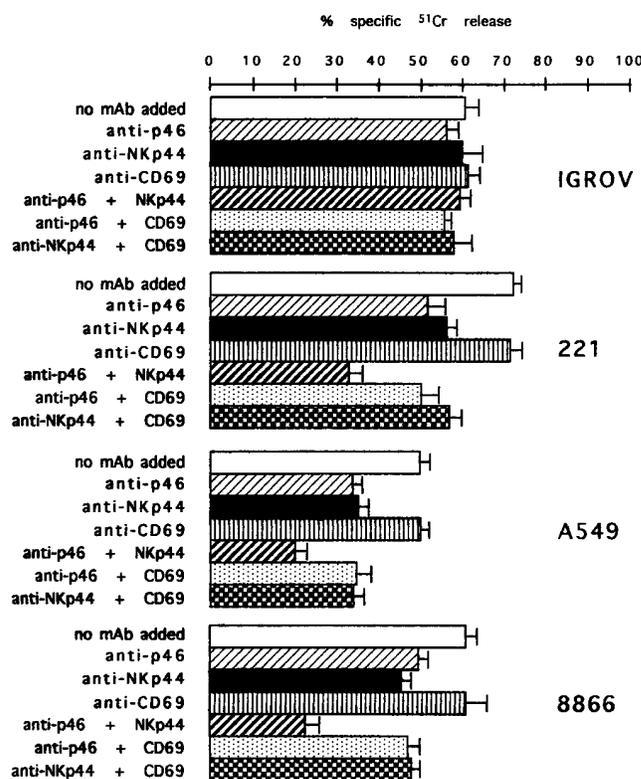
(Fig. 5). A similar inhibitory effect was obtained with the anti-p46 mAb BAB281. These data suggested that NKp44 and p46 molecules could cooperate in the process of NK cell triggering, resulting in killing of A548, 221, and 8866 cells. To assess this possibility, anti-NKp44 and anti-p46 mAbs were used 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 cytotoxicity 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<sup>-</sup> 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



**Figure 5.** Inhibition of non-MHC-restricted cytotoxicity by mAb-mediated masking of NKp44 and p46 molecules. The representative NK clone MID1 was analyzed for cytolytic activity against the indicated FcγR<sup>-</sup> target cell lines either in the absence or presence of anti-p46, anti-NKp44, anti-CD69, or a combination of anti-p46 + anti-NKp44, anti-p46 + anti-CD69, or anti-NKp44 + anti-CD69 mAbs. Percentage of lysis ± SD at the 8:1 E/T ratio is shown. Each bar represents the mean of five different experiments.

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. 1 a).

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.

We thank Mrs. Cinzia Miriello for typing the manuscript.

This work was supported by grants awarded by the Associazione Italiana per la Ricerca sul Cancro (A.I.R.C.), Istituto Superiore di Sanità (I.S.S.), Consiglio Nazionale delle Ricerche (C.N.R.), and Progetto Finalizzato Applicazioni Cliniche per la Ricerca Oncologica.

Address correspondence to Lorenzo Moretta, Centro Biotecnologie Avanzate, Largo R. Benzi 10, 16132 Genova, Italy. Phone: 39-10-57-37-217; FAX: 39-10-35-41-23; E-mail: miriello@sirio.cba.unige.it

Received for publication 3 March 1998 and in revised form 8 April 1998.

## References

1. Moretta, A., C. Bottino, M. Vitale, D. Pende, R. Biassoni, M.C. Mingari, and L. Moretta. 1996. Receptors for HLA-class I molecules in human natural killer cells. *Annu. Rev. Immunol.* 14:619-648.
2. Moretta, A., R. Biassoni, C. Bottino, D. Pende, M. Vitale, A. Poggi, M.C. Mingari, and L. Moretta. 1997. Major histocompatibility complex class I-specific receptors on human natural killer and T lymphocytes. *Immunol. Rev.* 155:105-117.
3. Lanier, L.L. 1997. NK cells: from no receptors to too many. *Immunity.* 6:371-378.
4. Valiante, N.M., K. Lienert, H.G. Shilling, B.J. Smits, and P. Parham. 1997. Killer cell receptors: keeping pace with MHC class I evolution. *Immunol. Rev.* 155:155-164.
5. Moretta, A., S. Sivori, M. Vitale, D. Pende, L. Morelli, R. Augugliaro, C. Bottino, and L. Moretta. 1995. Existence of both inhibitory (p58) and activatory (p50) receptors for HLA-C molecules in human natural killer cells. *J. Exp. Med.* 182:875-884.
6. Biassoni, R., C. Cantoni, M. Falco, S. Verdiani, C. Bottino, M. Vitale, R. Conte, A. Poggi, A. Moretta, and L. Moretta. 1996. The human leukocyte antigen (HLA)-C-specific "activatory" or "inhibitory" natural killer cell receptors display highly homologous extracellular domains but differ in their transmembrane and intracytoplasmic portions. *J. Exp. Med.* 183:645-650.
7. Ciccone, E., D. Pende, M. Vitale, L. Nanni, C. Di Donato, C. Bottino, L. Morelli, O. Viale, A. Amoroso, A. Moretta, and L. Moretta. 1994. Self class I molecules protect normal cells from lysis mediated by autologous natural killer cells. *Eur. J. Immunol.* 24:1003-1006.
8. Valiante, N.M., M. Uhrberg, H.G. Shilling, K. Lienert-Weidenbach, K.L. Arnett, A. D'Andrea, J.H. Phillips, L.L. Lanier, and P. Parham. 1997. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. *Immunity.* 7:739-751.
9. Garrido, F., F. Ruiz-Cabello, T. Cabrera, J.J. Perez-Villar, M. Lopez-Botet, M. Duggan-Keen, and P.L. Stern. 1997. Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. *Immunol. Today.* 18:89-95.
10. Höglund, P., C. Ohlen, E. Carbone, L. Franksson, H.G. Ljunggren, A. Latour, B. Koller, and K. Kärre. 1991. Recognition of  $\beta$ 2-microglobulin-negative ( $\beta$ 2m<sup>-</sup>) T cell blasts by natural killer cells from normal but not from  $\beta$ 2m<sup>-</sup> mice: nonresponsiveness controlled by  $\beta$ 2m-bone marrow in chimeric mice. *Proc. Natl. Acad. Sci. USA.* 88:10332-10336.
11. Robertson, M.J., and J. Ritz. 1992. Role of IL-2 receptors in NK cell activation and proliferation. In *NK Cell Mediated Cytotoxicity: Receptors, Signaling and Mechanism*. E. Lotzova and R.B. Herberman, editors. CRC Press, Boca Raton, FL. 183-206.
12. Ferrini, S., S. Miescher, M.R. Zocchi, V. Von Flidner, and A. Moretta. 1987. Phenotypic and functional characterization of recombinant interleukin-2 (rIL-2)-induced activated killer cells. Analysis at the population and clonal levels. *J. Immunol.* 138:1297-1302.
13. Trinchieri, G. 1989. Biology of natural killer cells. *Adv. Immunol.* 47:187-376.
14. Sivori, S., M. Vitale, L. Morelli, L. Sanseverino, R. Augugliaro, C. Bottino, L. Moretta, and A. Moretta. 1997. p46, a novel natural killer cell-specific surface molecule that mediates cell activation. *J. Exp. Med.* 186:1129-1136.
15. Moretta, A., G. Tambussi, C. Bottino, G. Tripodi, A. Merli, E. Ciccone, G. Pantaleo, and L. Moretta. 1990. A novel surface antigen expressed by a subset of human CD3-CD16<sup>+</sup> natural killer cells. Role in cell activation and regulation of cytolytic function. *J. Exp. Med.* 171:695-714.
16. Bottino, C., S. Sivori, M. Vitale, C. Cantoni, M. Falco, D. Pende, L. Morelli, R. Augugliaro, G. Semenzato, R. Biassoni, et al. 1996. A novel surface molecule homologous to the p58/p50 family of receptors is selectively expressed on a subset of human natural killer cells and induces both triggering of cell functions and proliferation. *Eur. J. Immunol.* 26:1816-1824.
17. Cebrián, M., E. Yague, M. Rincón, M. López-Botet, M.O. De Landazuri, and F. Sánchez-Madrid. 1988. Triggering of T cell proliferation through AIM, an activation inducer molecule expressed on activated human lymphocytes. *J. Exp. Med.* 168:1621-1637.
18. Hemler, M.E. 1990. VLA proteins in the integrin family: structure, functions and their role on leukocytes. *Annu. Rev. Immunol.* 8:365-400.
19. Lanier, L.L., G. Yu, and J.H. Phillips. 1989. Co-association of CD3 $\zeta$  with a receptor (CD16) for IgG Fc on human natural killer cells. *Nature.* 342:803-805.
20. Vivier, E., N. Rochet, J.P. Kochan, D.H. Presky, S.F. Schlossman, and P. Anderson. 1991. Structural similarities between Fc receptors and T cell receptors: expression of the  $\gamma$  subunit of the Fc $\epsilon$ RI in human T cells, NK cells and thymocytes. *J. Immunol.* 147:4263-4270.
21. Olcese, L., A. Cambiaggi, G. Semenzato, C. Bottino, A. Moretta, and E. Vivier. 1997. Human killer cell activatory receptors for MHC class I molecules are included in a multimeric complex expressed by natural killer cells. *J. Immunol.* 158:5083-5086.
22. Lanier, L.L., B.C. Corliss, W. Jun, C. Leong, and J.H. Phillips. 1998. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. *Nature.* 391:703-707.

23. Lanier, L.L., B. Corliss, and J.H. Phillips. 1997. Arousal and inhibition of human NK cells. *Immunol. Rev.* 155:145–154.
24. Moretta, A., A. Poggi, D. Pende, G. Tripodi, A.M. Orengo, N. Pella, R. Augugliaro, C. Bottino, E. Ciccone, and L. Moretta. 1991. CD69-mediated pathway of lymphocyte activation. Anti-CD69 mAbs trigger the cytolytic activity of different lymphoid effector cells with the exception of cytolytic T lymphocytes expressing TCR  $\alpha/\beta$ . *J. Exp. Med.* 174: 1393–1398.
25. Carbone, E., G. Ruggiero, G. Terrazzano, C. Palomba, C. Manzo, S. Fontana, H. Spitz, K. Karre, and S. Zappacosta. 1997. A new mechanism of NK cell cytotoxicity activation: the CD40–CD40 ligand interaction. *J. Exp. Med.* 185:2053–2060.
26. Imboden, J.B., E.C. Eriksson, M. McCutcheon, C.W. Reynolds, and W.E. Seaman. 1989. Identification and characterization of a cell-surface molecule that is selectively induced on rat lymphokine-activated killer cells. *J. Immunol.* 143: 3100–3103.
27. Seaman, W.E., E.C. Niemi, M.R. Stark, R.D. Goldfien, A.S. Pollock, and J.B. Imboden. 1991. Molecular cloning of gp42, a cell-surface molecule that is selectively induced on rat natural killer cells by interleukin 2: glycolipid membrane anchoring and capacity for transmembrane signaling. *J. Exp. Med.* 173:251–260.