p46, a Novel Natural Killer Cell-specific Surface Molecule That Mediates Cell Activation

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Summary

Limited information is available on the surface molecules that are involved in natural killer (NK) cell triggering. In this study, we selected the BAB281 monoclonal antibody (mAb) on the basis of its ability to trigger NK-mediated target cell lysis. BAB281 identified a novel NK cell-specific surface molecule of 46 kD (p46) that is expressed by all resting or activated NK cells. Importantly, unlike the NK cell antigens identified so far, the expression of p46 was strictly confined to NK cells. Upon mAb-mediated cross-linking, p46 molecules induced strong cell triggering leading to [Ca\(^{2+}\)]i increases, lymphokine production, and cytolytic activity both in resting NK cells and NK cell clones. The p46-mediated induction of Ca\(^{2+}\) increases or triggering of cytolytic activity was downregulated by the simultaneous engagement of inhibitory receptors including p58, p70, and CD94/NKG2A. Both the unique cellular distribution and functional capability of p46 molecules suggest a possible role in the mechanisms of non-major histocompatibility complex–restricted cytolyis mediated by human NK cells.

Materials and Methods

Antibodies. mAb JT3A (IgG\(_{2a}\) anti-CD3), GL183 (IgG\(_{1}\) anti-p58.2), Y249 (IgM anti-p58.2), EB6 (IgG\(_{1}\) anti-p58.1), XA-141 (IgM anti-p58.1), PAX250 (IgG\(_{1}\) anti-p50.3), FES 172 (IgG\(_{1}\) anti-p50.3), Z27 (IgG\(_{1}\) anti-p70), XA-185 (IgG\(_{1}\) anti-CD94), Y9 (IgM anti-CD94), Z199 and Z270 (IgG\(_{b}\) and IgG\(_{3}\), respectively, anti-NKG2A), KD1 (IgG\(_{a}\) anti-CD16), c127 (IgG\(_{a}\) anti-CD16), c218 (IgG\(_{a}\) anti-CD56), and A6-220 (IgM anti-CD56) mAbs 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).
Production of mAb and Flow Cytometric Analysis. BAB281 mAb (IgG1) was obtained by immunizing a 5-wk-old Balb/C mouse with the NK cell clone SE192. Analysis of the cell distribution of p46 molecules was performed by using a FACScan® one- or two-color fluorescence cytofluorometric analysis as previously described (17).

Cytolytic Activity and Lymphokine Production by NK Cell Clones. T or NK cell clones were obtained by limiting dilution, as previously described (18). The cytolytic activity was assessed in a 4 h 51Cr-release assay (18) in which effector cells were tested against the K562 or the P815 cell lines. The quantitative determination of TNF-α and IFN-γ production (19) by NK cell clones was performed by an enzyme immunoassay purchased from Genzyme Corp. (Cambridge, MA). The sensitivity of the two tests ranged from 15 to 1,200 and from 16 to 1,024 pg/ml, respectively.

Determination of Intracellular Free Calcium ([Ca2+]i) Increases. Determination of [Ca2+]i was performed as previously described (19). Triggering of NK cell clones was achieved by adding into the cuvette 5 μg/ml of mAb followed by the addition of 20 μg/ml polyclonal isotype-specific goat anti–mouse (Southern Biotechnology Associated, Birmingham, AL).

Biochemical Characterization of the p46 Molecules. Cyanogen bromide sepharose (Pharmacia Biotech Inc., Piscataway, NJ)-coupled BAB281 or Sepharose PA-coupled KD1 mAbs were used to immunoprecipitate specific molecules from 1% NP-40 lysates of cells surface-labeled with 125I (DuPont-New England Nuclear, Boston, MA) as previously described (17). Immunoprecipitates were analyzed by discontinuous SDS-PAGE.

Specific bands were cut out from dried gel and eluted proteins were used for N-glycanase, neuraminidase, or neuraminidase followed by O-glycanase digestion (19). For one-dimensional peptide mapping (19), cut bands were loaded on a 15–20% gradient gel and digestion was performed by adding 500 ng of endoproteinase Glu-C (V8 protease) or papain (Sigma Chemical Co., St. Louis, MO) to each well.

Purification of NK Cell-enriched or NK Cell-depleted Peripheral Blood Populations. To obtain an NK cell-enriched lymphocyte population, PBMCs were depleted of plastic-adherent cells and subsequently incubated with anti-CD3 (JT3A) mAb for 30 min at 4°C followed by treatment with goat anti–mouse-coated Dynal beads (Dynal, Oslo, Norway) for 30 min at 4°C. The resulting CD3− lymphocyte populations contained ~1% CD3+ cells, 20% HLA-DR+ cells, and 80% CD56+ cells. These cells were used for FACS® analysis of CD3-depleted populations (see Fig. 3) and for cytolytic activity against K562 and P815 target cells (see Fig. 6 a). In another set of experiments, PBMCs were stained for both anti-CD3 and anti-p46 mAb and subsequently cells lacking the expression of p46 or CD3 or both markers (CD3−, p46−) were sorted and analyzed for cytolytic activity against K562 target cells (see Fig. 6 b).

Results

Selection of mAb Identifying a 46-kD Surface Molecule Involved in Triggering of NK Cell-mediated Cytolytic Activity. Mice were immunized with the CD3−, 56+ NK cell clone SE192 which did not express p58, p70, or p140 NK cell receptor, while it expressed the CD94/NKG2A inhibitory receptor complex identified by the Z199 and Z270 mAbs (11, 12). mAbs were selected on the basis of the ability to modulate the cytolytic activity of the immunizing clone in a redirected killing assay against murine P815 target cells. The selected mAb (BAB281) induced a sharp increase of the cytolytic activity of clone SE192 (Fig. 1 a) as well as that of a panel of NK cell clones expressing one or more NKR. The magnitude of NK cell triggering induced by BAB281 mAb was comparable to that induced by anti-CD16 mAb under the same experimental conditions (Fig. 1 a). On the other hand, a negative control represented by the c218 (isotype-matched, IgG1, anti-CD56) mAb that binds to the NK cell clone, ruled out a nonspecific effect of BAB281 mAb. Surface molecules recognized by BAB281 mAb were analyzed by SDS-PAGE after immunoprecipitation from cell lysates derived from 125I-labeled NK cell populations or clones. As shown in Fig. 1 b, BAB281 mAb

Figure 1. Triggering of cytolytic activity and molecular characteristics of the novel 46-kD NK cell surface molecule. (a) The NK cell clone SE192, used for mice immunization, was analyzed in a redirected killing assay against P815 target cells in the presence of graded amounts of BAB281 (▲), c127 (anti-CD16) (○), or c218 (anti-CD56) (■) mAbs. All these mAbs are IgG1. (b) NK cell populations derived from donor E.C. (lanes A and D) or donor L.M. (lanes B and E) and the NK cell clone 211 derived from donor F.G. (lanes C and F) were surface labeled with 125I and immunoprecipitated with BAB281 mAb. Samples were analyzed in a 8% SDS-PAGE under nonreducing (lanes A–C) or reducing (lanes D–F) conditions. All the mAbs used here were of the IgG1 isotype.
suggests that, unlike from CD16 (20), p46 molecules do not stained by BAB281 mAb. In addition, all the NK cell clones NK cell populations analyzed (or T cell-enriched populations or clones. All the polyclonal immunoprecipitated a single protein of ~46 kD under both reducing and nonreducing conditions (hereafter termed p46). The comparative analysis of the effect of deglycosylation of p46 or CD16 molecules revealed important differences. As shown in Fig. 2 a, the mobility of p46 molecules was not modified by treatment with N - glycanase, whereas only a slight decrement in molecular weight was induced by neuraminidase followed by O -glycanase treatment. This suggests that, unlike from CD16 (20), p46 molecules do not contain N -linked sugars (whereas O -linked sugars may be present). Further comparative analysis of p46 and CD16 molecules in one-dimensional peptide mapping revealed that the pattern of digestion of the two molecules with either V8 protease or papain was clearly different (Fig. 2 b). Although not shown, the molecular characteristics of p46 molecules were also clearly distinct from other surface molecules mediating NK cell activation, including CD69 and molecules (Fig. 3 a). In particular, because of the similar molecular size, p50 and p46 were further compared. First, anti-p46 mAb did not react with cell transfectants expressing one or another member of the p50 family (19, 21); second, p46 molecules did not contain N -linked sugars (Fig. 2 a) whereas p50 molecules did (19, 21). Finally, preclearing experiments on an NK cell clone displaying the p50; p46 phenotype indicated that after removal of p46 molecules p50.3 molecules could still be immunoprecipitated and vice versa (not shown). Therefore, it appears that BAB281 mAb identifies a novel surface molecule involved in triggering of NK cell-mediated cytotoxicity.

Cell Surface Distribution of p46 Molecules. A panel of >100 NK cell clones were assessed for cytolytic activity in the presence of BAB281 or anti-CD16 (c127) mAbs in a redirected killing assay against the FcγR + P815 target cells. Some of these clones were also analyzed for lymphokine production in the presence of plastic adherent BAB281 mAb. The cytolytic activity of 10 representative NK cell clones is shown in Fig. 4 a. It can be seen that most NK cell clones displayed strong cytolytic responses to both anti-p46 and anti-CD16 mAbs, whereas no increments of cytolytic activity were observed in the presence of the isotype-matched (anti-CD56, IgG1) c218 mAb. Anti-p46 mAb also triggered two clones (B1 and B2), which did not respond to anti-CD16 mAb. These two clones are representative of the rare peripheral blood NK cells displaying the CD3 -56 -16 - phenotype. Some differences in the staining intensity with anti-p46 mAb was observed among different NK cell clones (not shown). In-

Figure 2. Deglycosylation and proteolytic digestion of p46 molecules. (a) CD16 (lanes A - D) or p46 (lanes E - H) molecules isolated from a surface labeled NK cell population were treated with neuraminidase (lanes B and F), neuraminidase plus O -glycanase (lanes C and G), or N -glycanase (lanes D and H). Untreated molecules are shown in lanes A and E. Samples were run in an 11% SDS-PAGE under reducing conditions. (b) p46 and CD16 molecules immunoprecipitated from a surface labeled NK cell population were analyzed by SDS-PAGE. Bands corresponding to p46 (lanes A, C, E, and G) or CD16 (lanes B, D, F, and H) molecules were excised from the gel and analyzed by SDS-PAGE on a 15-20% gradient gel after digestion with V8 protease (lanes C and D) or papain (lanes G and H). In lanes A, B, E, and F untreated molecules are shown.

and G) or CD16 (lanes B, D, F, and H) molecules were excised from the gel and analyzed by SDS-PAGE on a 15-20% gradient gel after digestion with V8 protease (lanes C and D) or papain (lanes G and H). In lanes A, B, E, and F untreated molecules are shown.
Interestingly, this appears to correlate, at least partly, with the degree of anti-p46-induced cytolytic activity as well as with the spontaneous cytolytic activity of the different NK cell clones. Although not shown, the p46-mediated NK cell triggering was not induced by the (Fab')2 fragment of anti-p46 mAb. These data indicate that cross-linking of p46 molecules is required in order to induce triggering of NK cell-mediated cytolytic activity. Indeed, lysis of FcyR- target cells was not incremented by anti-p46 mAbs. On the other hand, a partial inhibition mediated by anti-p46 mAb could be detected against some FcyR- target cells (including C1R and IGRÖV cell lines). At present it is not clear whether this inhibition reflects the interference with the interaction between p46 and its putative ligand expressed by these target cells.

Experiments of lymphokine production showed that plastic-adherent BAB281 mAb induced strong production of both IFN-γ and TNF-α in all the NK cell clones analyzed (Fig. 4, b and c).

As expected, cytolytic T cell clones expressing either TCR-α/β or -γ/δ were not responsive to anti-p46 mAb (they were p46-), whereas they were efficiently triggered by anti-CD3 mAb. Remarkably, among the T cell clones analyzed, some displayed NK cell-like activity and expressed HLA class I-specific NK cell receptors. In spite of their phenotypic and functional features they did not express p46 molecules.

Figure 3. Analysis of the expression of p46 molecules in peripheral blood NK cells. In these experiments freshly isolated PB-MCs either unfractionated (a-d) or depleted of both adherent cells and CD3+ lymphocytes (e-g) were analyzed by two-color immunofluorescence and FACS analysis for the expression of p46 surface molecules in combination with the indicated surface markers. Cells were stained with mAbs to the indicated molecules followed by FITC or PE-conjugated isotype-specific goat anti-mouse second reagent. 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).

Figure 4. All human NK cell clones can be triggered via p46 molecule. (a) 10 NK cell clones (representative of >100 clones analyzed) were assessed for cytolytic activity against the FcγR- P815 target cells in the absence (□) or presence of anti-p46 mAb (■), anti-CD16 mAb (□), or anti-CD56 mAb (□). Two clones (B1 and B2) representative of the infrequent CD56-16+ NK cell clones, were triggered only by anti-p46 mAb. (b and c) Three representative NK cell clones were analyzed for IFN-γ or TNF-α production in the absence (□) or presence of anti-p46 mAb (■) or anti-CD56 mAb (□).
Cross-linking of Inhibitory NKR Downregulates NK Cell Triggering via p46. As shown above, BAB281 mAb triggered all the NK cell clones (including those expressing p58 or p70 NKR). Cross-linking of p58 (23) or p70 (24, 25) NKR results in inhibition of the CD16-mediated NK cell triggering. We therefore analyzed whether NK cell triggering via p46 could also be downregulated by cross-linking of the inhibitory NKR s. To this end, a series of NK cell clones expressing inhibitory NKR s were analyzed for their cytolytic activity in the presence of both anti-p46 and anti-NKR mAbs (Fig. 5). For example, clone KK26 expressed the p58.1 (EB6) receptors; it is evident that triggering of target cell lysis induced by BAB281 mAb was strongly inhibited in the presence of anti-p58.1 mAbs (but not by an isotype-matched anti-CD56 mAb). A similar effect was exerted by mAb-mediated cross-linking of other inhibitory receptors (see the p70+ clone LO1 and the CD94/NKG2A+ clone KK4).

Figure 5. The p46-mediated NK cell triggering is downregulated by the cross-linking of HLA-class I specific inhibitory receptors. Three representative NK cell clones expressing different HLA-class I specific inhibitory receptors were analyzed for cytolytic activity in a redirected killing assay against the Fc γ R− P815 target cells in the absence () or presence of anti-NKR ( ), anti-p46 ( ), both anti-p46 and anti-NKR ( ), or both anti-p46 and anti-CD56 ( ) mAbs. The anti-NKR mAbs were represented by: EB6 (anti-p58.1) in clone KK26; Z139 (anti-CD94/NKG2A complex) in clone KK4; and Z27 (anti-p70) in clone LO1.

Moreover, the anti-p46–induced [Ca 2+]i increase did not occur upon engagement of inhibitory NKR expressed on the same cells. For example, the p46-dependent [Ca 2+]i increase in clone KK26 was completely inhibited by cross-linking of the p58.1 inhibitory NKR (Fig. 7 a). As shown in Fig. 7 a, a sharp [Ca 2+]i increase could be detected in the representative clone KK26 (p46+, p58.1+, p50.3+) after stimulation with anti-p46 mAb. However, [Ca 2+]i increments occurred only in the presence of a goat anti–mouse second reagent (i.e., in the presence of efficient cross-linking of P46 molecules).

Induction of [Ca 2+]i Mobilization by Anti-p46 mAb and p58-mediated Inhibition. In these experiments we analyzed whether NK cell triggering via p46 involved [Ca 2+]i increases, similar to other triggering surface molecules expressed by NK cells (6). As shown in Fig. 7 a, a sharp [Ca 2+]i increase did not occur upon engagement of inhibitory NKR expressed on the same cells. For example, the p46-dependent [Ca 2+]i increase in clone KK26 was completely inhibited by cross-linking of the p58.1 inhibitory NKR (Fig. 7 b). This effect occurs only when both anti-p46 and anti-p58.1 mAbs of IgG1 isotype are co–cross-linked by an IgG2-specific sec-

Figure 6. p46-mediated triggering of cytolytic activity in fresh peripheral blood NK cells (a) Freshly isolated peripheral blood NK cells were analyzed for their ability to kill the indicated FcγR+ target cells in the absence of mAb (□) or presence of anti-CD16 (■), BAB281 ( ), or anti-CD56 mAb ( ). The effector target ratio was 20:1 against P815 target cells and 10:1 against K562 target cells. (b) Freshly isolated PBL were sorted according to the lack of expression of p46 (□), of CD3 ( ■) or both p46 and CD3 (*) and compared to unfractionated cells ( ) for cytolytic activity against 51Cr-labeled K562 target cells in the absence of added mAbs.
Discussion

In this study, we identified and characterized a novel 46-kD surface molecule. This p46 molecule is specifically expressed by human NK cells and mediates efficient NK cell triggering leading to [Ca^{2+}] mobilization, cytolytic activity, and cytokine production.

The precise identification of NK cells has long been hampered by the lack of specific markers. Thus, the lack of CD3/TCR in cells expressing CD16 and CD56 is generally considered a reliable surface phenotype for defining NK cells (26). However, a small fraction of peripheral blood NK cells (CD56^{bright}) do not express CD16 (22). Remarkably, this CD16^-NK cell population is more frequent in tissues (1). P46 was found to be expressed not only by conventional CD56^-CD16^- NK cells but also by CD56^{bright}CD16^- cells. Typical NK cell markers including CD56, CD16, and NKR-P1 are not unique to NK cells but can be expressed by subsets of peripheral T cells. In addition, a variable fraction of T cells has been found to express HLA class I-specific inhibitory or activating receptors typical of NK cells (i.e., p58, p70, p140, and CD94/NKG2A) (27-29). Both fresh T cell populations or T cell clones expressing these markers and/or one or more HLA class I-specific NKR did not react with anti-p46 mAb. Therefore, p46 appears to identify NK cells more precisely than any of the other NK cell markers available so far.

mAb-induced ligation of p46 molecules strongly triggered the NK cell-mediated cytolytic activity. This functional effect was particularly evident in NK cell clones but could also be detected in freshly isolated NK lymphocytes. Among NK cell clones, both the CD56^-CD16^- and the CD56^-CD16^- clones were activated by anti-p46 mAb. Anti-p46-induced triggering was inhibited by the simultaneous cross-linking of inhibitory NK receptors for HLA class I molecules including p58, p70, and CD94/NKG2A. We show that the independent cross-linking of inhibitory receptors and p46 is not sufficient for inhibiting the function of p46 and that inhibition was achieved only when the two molecules were co-aggregated at the NK cell surface. Thus the cross-talk between inhibitory and activating receptors takes place when they are co-polarized as previously suggested by Blyer et al. (30). Indeed these authors showed that the FcεRI-dependent degranulation of RBL-2H3 cells transfected with the p58.2 molecules was inhibited when the FcεRI and the p58.2 receptors were co-aggregated. Although there is now a better understanding on the role of MHC molecules in NK cell functions and on the role of the MHC-specific inhibitory receptors, limited information exists on the molecules and the mechanisms of NK cell triggering. A number of surface molecules is likely to play an activating role in the process of NK cell-mediated lysis upon interaction with ligands expressed on target cells. Activating receptors for HLA-C molecules (p50) have recently been described (21, 31). Although their role may be physiologically relevant and they may substantially contribute to NK cell triggering in the case of HLA-C^+ target cells, it should be stressed that a major characteristic of NK cells (including those expressing the p50 receptors) is their ability to lyse MHC class I-negative target cells. Thus, activating receptors specific for non-MHC ligands must play a fundamental role in the NK-mediated target cell lysis. However, the surface molecules that are primarily responsible for the MHC-independent activation of NK cells have not been defined. It is of note that, in most instances, molecules which mediate NK cell triggering are also expressed by other cell types. For example, CD2 (32) and CD69 (33) and the molecules recognized by the PP35 (34) and CI.7 (35) mAbs are expressed by variable proportions of T lymphocytes. Other molecules mediating strong NK cell activation, such as CD16 (15), are triggered upon interaction with immune complexes and do not appear to play a role in the NK cell-mediated natural cytotoxicity. Also, in rodents a number of molecules involved in NK cell triggering has been described. These include the NKR-P1 molecule,
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