Evidence for the Involvement of CD56 Molecules in Alloantigen-specific Recognition by Human Natural Killer Cells

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Summary

In recent reports we have described the generation of natural killer (NK) lines devoid of CD3/TCR structures but with apparent specificity for allogeneic target cells. Using one such NK line as an immunogen, we now report the generation of two monoclonal antibodies (mAbs), designated 2–13 and 5–38, which bind selectively to the majority of $CD3^-$, $CD16^+$, $CD56^+$ lymphocytes and inhibit the lysis of specific allogeneic target cells by a panel of alloreactive NK lines. By contrast, these mAbs had no effect on classical NK cell mediated lysis of K562 cells or major histocompatability–restricted T cell-mediated cytolysis. Immunoprecipitation of radiolabeled NK lines followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis revealed that the target molecules of both mAbs have a molecular mass of approximately 180 kD. Leu 19, a well-described anti-CD56 mAb, precipitated a 180 kD protein from NK cells, and the binding of Leu 19 to NK cells was blocked by pretreatment with both 2–13 and 5–38. However, in contrast to these mAbs, Leu 19 had no effect on the cytolytic activity of allospecific NK cells. Sequential immunoprecipitation analysis revealed that all three mAbs recognized distinct molecular species of CD56. We interpret these findings as indicating that multiple isoforms of CD56 are differentially expressed on NK lines and play critical roles in the recognition/interaction of these cells with their specific allogeneic targets.

Natural killer (NK) cells constitute up to 25% of PBLs in humans and can be distinguished from other lymphocytes on the basis of their ability to lyse selected tumor lines and virus-infected cells without prior sensitization or restriction by MHC class I or II gene products (1–3). Although a small subpopulation of CD3/TCR-bearing lymphocytes have NK-like activity (4), classical NK cells lack CD3/TCR structures and express cell surface markers such as CD16 and CD56 (5–7). Despite their distinct function and phenotype, however, to date neither target structure(s) (ligands) nor recognition molecules (receptors) of NK cells have been identified.

Recently, we and others have provided evidence for the existence of CD3/TCR independent, alloantigen recognition by NK cells (8–10). In such studies, NK lines and clones were generated which not only killed classical NK-sensitive targets such as K562 cells but, in addition, selectively lysed the allogeneic cells (lymphocytes, lymphoblastoid cell lines (LCL)[1], or endothelial cells) with which fresh peripheral blood derived NK cells had been cocultured. In the present study we used one of these alloantigen-specific NK lines as an immunogen for the purpose of generating mAbs that recognize molecules on the surface of NK cells involved in alloantigen-specific cytolysis. Two mAbs were generated that inhibit alloantigen-specific killing without affecting the lysis of K562 cells. These mAbs appear to recognize different isoforms of CD56, suggesting that this molecule, which is selectively expressed on lymphocytes with NK activity, plays a critical role in alloantigen recognition/cytolysis of NK cells.

Materials and Methods

Generation of Antigen-specific $CD3^-$, $CD56^+$ Natural Killer Cell Line. Procedures for generation of antigen-specific NK lines have been described in detail (8). Briefly, highly purified $CD3^-$, $CD16^+$ cells were incubated on a monolayer of irradiated LCL (ARENT line, HLA A2,2:B38,39:DR6,6 and MSAB line A1,2:B57,57:DR7,7) which had been bound to flat-bottomed microtiter wells with Cell-Tak (Biopolymers, Inc., Farmington, CT). Thereafter, cells adherent to the LCL were recovered and cultured in medium supplemented with IL-2–containing supernatant. Culture medium supplemented with this supernatant was added every 2–3 d, together with the original stimulator LCL. The resulting NK lines express CD56 but not CD3, and lyse the original LCL with substantially greater potency than irrelevant LCL. NK sensitive K562 cells are lysed by all of the NK lines with high potency (8).

[1] *Abbreviations used in this paper:* LCL, lymphoblastoid cell line; N-CAM, neural cell adhesion molecule.

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Production of 2–13 and 5–38 mAbs. 6-wk-old female BALB/c mice were immunized with an NK line (designated NS2) generated as described above. Immunizations were performed using Ribi adjuvant (Ribi Immunochem Research, Inc., Hamilton, MT) according to the manufacturer's protocol. After the third immunization, mice were splenectomized and splenocytes were fused with SP2/0 myeloma cells as described (11). Hybridoma supernatants were screened on the basis of their ability to inhibit the allospecific cytotoxic activity of the immunizing NK line, using ^{51}Cr-labeled ARENT cells as targets in a 4-h ^{51}Cr-release assay (8). This screening procedure led to the identification of two antibody secreting hybridomas that inhibited the cytolytic activity of the antigen-specific NK line against its original stimulator LCL, ARENT. These hybridomas were isolated and subcloned by limiting dilution.

Immunofluorescence Analysis. All mAbs used in this study were produced and purified in this laboratory as described (12) with the exception of Leu 19 (anti-CD56), which was kindly provided by Dr. Lewis Lanier of Becton-Dickinson Corp. (Mountain View, CA). Immunofluorescence staining was performed as described (8, 12). In brief, determination of the distribution of the surface molecule(s) recognized by these mAbs utilized two-color analysis in which cells were stained with mAbs 2–13 or 5–38, followed by FITC-conjugated rat anti-mouse IgG2a Ab (Zymed Laboratory, San Francisco, CA) and one of the following reagents: anti-Leu-2, anti-Leu 3, anti-Leu 4, or anti-Leu 11 mAb directly conjugated to PE. To determine whether mAbs 2–13 and/or 5–38 block the binding of other mAbs, PBL were treated with 2–13 or 5–38 mAbs at 4° C for 30 min, washed and then stained with fluorochrome-conjugated mAb and analyzed in a flow cytometer (12). To study the nature of the epitopes recognized by these mAbs, PBLs were treated with various concentrations of neuraminidase or trypsin (Sigma Chemical Co., St. Louis, MO) at 37° C for 30 min, or were cultured with 1–10 μg/ml tunicamycin (Calbiochem-Behring Corp., San Diego, CA) for 1–3 d. Thereafter, the cells were analyzed by indirect immunofluorescence with mAbs 2–13 and 5–38 as well as mAbs to HLA-DR and Leu 7 (CD57).

Analysis of Immunoprecipitates. NK lines and KG1a5 leukemic cells (kindly provided by Dr. Lewis Lanier of Becton-Dickinson Corp.) (13) were washed five times in cold PBS, and surface-labeled with ^{125}iodine using the lactoperoxidase method or, alternatively, metabolically labeled with [^{35}S]methionine as described elsewhere (14). After radiolabeling, cells were washed three times with PBS and suspended in lysis buffer containing 1% NP-40. The lysates were pre-cleared three times with packed Protein A-Sepharose beads for 2 h under rotation, followed by incubation with hybridoma supernatant for 2 h at 4°C. 20 μl of packed Protein A-Sepharose beads was then added, and samples were incubated overnight at 4°C under rotation. Immunoprecipitates were eluted from Protein A-Sepharose by boiling for 5 min in buffer containing 1% SDS in the presence or absence of 5% 2-ME and analyzed on SDS-polyacrylamide gels (11, 14). Peptide mapping was performed as described (15), using protease XVII from *Staphylococcus aureus* strain V8 (Sigma Chemical Co.) (15).

Cytotoxicity Assays. The alloantigen-specific NK cell line and fresh NK-enriched PBL (obtained by Percoll centrifugation) were tested as effectors in 4-h ^{51}Cr-release cytotoxicity assays as described (8). CD4$^+$ and CD8$^+$ allospecific cytotoxic T cell lines, generated as described (16) were also used as effectors. In experiments designed to explore the role of cell surface molecules in cytotoxicity, mAbs were added at the initiation of the 4-h ^{51}Cr-release assays. In hybridoma lysis assays (17), mAb producing hybridomas were labeled with ^{51}Cr and used as targets in 4-h ^{51}Cr-release assays. All hybridomas expressed surface immunoglobulin as determined by immunofluorescence analysis on a flow cytometer.

Results

Mice were immunized as described in Materials and Methods with an allospecific NK cell line designated NS2

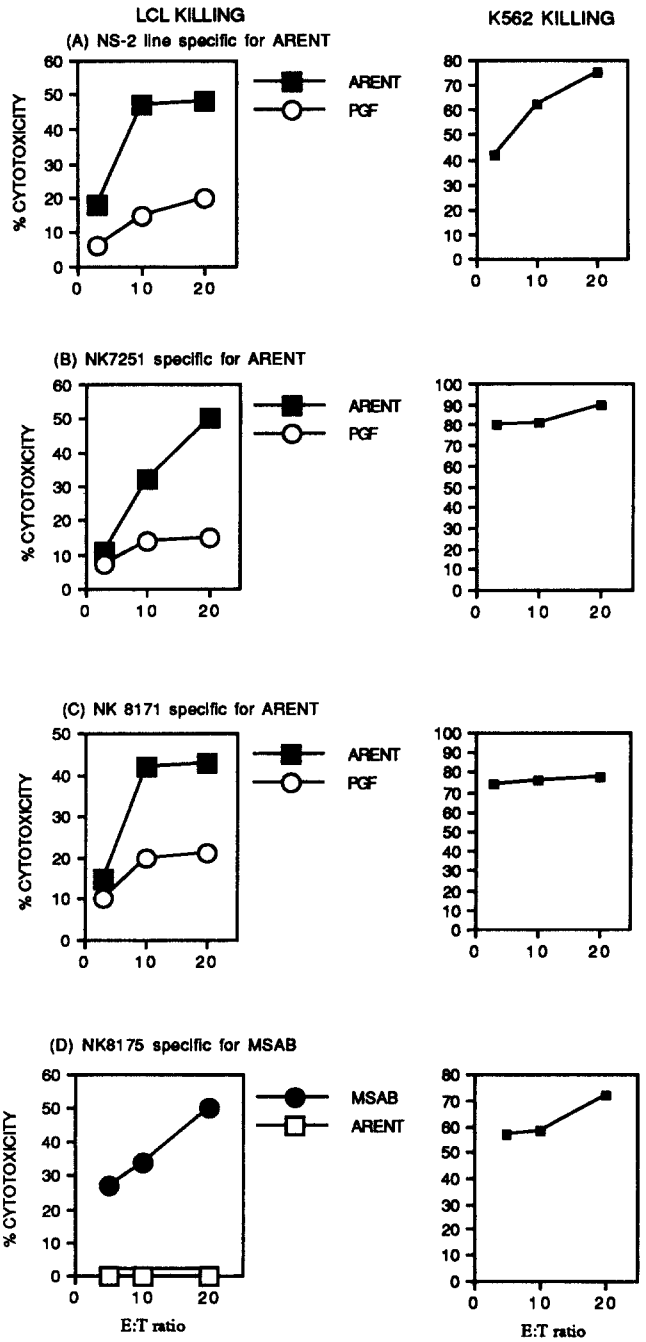


Figure 1. Cytotoxic activity of CD3$^-$ NK cell lines which were used in this study. The lines were generated as described in Materials and Methods and tested for cytotoxic activity against the original stimulator LCL, irrelevant LCL (PGF, HLA A3, -:B7, -:DR2,-), and K562 cells, using a 4-h ^{51}Cr-release assay. Values represent the mean percent killing of triplicate wells.

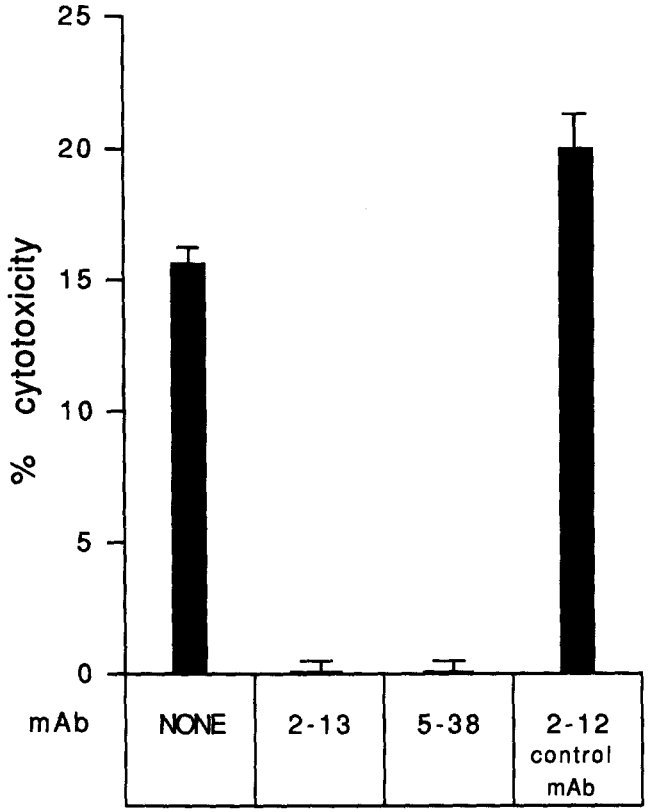


Figure 2. Effects of 2–13 and 5–38 mAbs on alloantigen specific cytotoxic activity. The ARENT specific NK line, NS2 ($CD3^-$, $CD56^+$), which had been used as the immunogen was tested for cytolytic activity against the ARENT line at an E/T of 10:1 in the presence or absence of 50% vol of hybridoma supernatant which was added to the wells at the onset of 4-h ^{51}Cr-release assays. 2–12 mAb which does not affect any known immune responses (data not shown) is included as a negative control.

(surface phenotype $CD3^-$, $CD56^+$) derived from peripheral blood $CD3^-$, $CD16^+$ lymphocytes. Similar to lines previously generated in this laboratory (8), NS2 displayed preferential cytolytic activity against its original stimulator LCL (ARENT) as well as NK-sensitive (K562) target cells (Fig. 1). The supernatants of two hybridomas were identified on the basis of their ability to selectively inhibit the lysis of ARENT cells by the NS2 line (Fig. 2), and these hybridomas were cloned by limiting dilution and the mAbs (designated 2–13 and 5–38) were studied further.

Immunofluorescence analysis of PBL obtained from 10 different subjects revealed that 10–25% of cells were reactive with these mAbs (Fig. 3 *A*). To compare the expression of the target molecules of mAbs 2–13 and 5–38 with that of other lymphocyte surface markers, two-color immunofluorescence analysis was performed in which PBL were stained with both an anti-CD16 mAb (Leu 11) and either 2–13 or 5–38. The results (Fig. 3 *B*) indicate that the majority of cells reactive with 2–13 or 5–38 are also reactive with anti-CD16 mAb. It should be stressed, however, that some $CD16^+$ cells were 2–13 and 5–38 negative, and a small proportion of $CD3^+$ cells were 2–13 and 5–38 positive. Although no 2–13 and 5–38 positive cells coexpressing CD4 would be detected, a subset of 2–13 and 5–38 positive cells consistently expressed the CD8 antigen. Most importantly, Leu 19, a well-characterized anti-CD56 mAb, stained substantially the same PBL as that stained by 2–13 and 5–38 (not shown), and pretreatment of PBL with either 2–13 or 5–38 abolished the reactivity of PE-conjugated Leu 19 (anti-CD56) antibody (Fig. 3 *A*), but not of other mAbs tested, including antibodies to CD2, CD3 (OKT3), CD16, CD45RA, CD45RO, and Leu 7 (Fig. 3 *A*, and data not shown). This suggests that the molecules recognized by 2–13 and 5–38 are identical or closely associated with the CD56 antigen on the cell surface.

Like Leu 19, the 2–13 and 5–38 mAbs did not react with normal B cells, monocytes or granulocytes, and did not bind lymphoblastoid cell lines, including the ARENT line which was the specific stimulus of the NS2 NK line. Among a panel of tumor cell lines analyzed, only the KG1 leukemic line (20% +) and a subclone of that line, designated KG1a5 (90% +) were recognized by 2–13 and 5–38. Leu 19 also stained the KG1a5 clone but failed to stain the parental KG1 cells, indicating that the 2–13 and 5–38 mAbs recognize either a distinct epitope on CD56 or a distinct protein from that recognized by Leu 19. Moreover, when antigen-specific NK lines of different target specificity were stained with these mAbs (Fig. 4), the staining patterns of 5–38 are clearly distinct from that of Leu 19 or 2–13. For example, an NK line with specificity for the ARENT lymphoblastoid cell line (Fig. 4, line B) exhibits uniformly bright staining with 5–38, but both dull and bright populations when stained with 2–13 or Leu 19. In addition, the staining pattern of these mAb on different NK lines also varies, indicating that the expression of the epitopes recognized by these antibodies varies among NK lines. Thus, for example, while the 5–38 antibody stains an anti-ARENT NK line with high, uniform density (Fig. 4, line B), the same antibody exhibits variable staining on the MSAB-specific NK line (Fig. 4, line A). When combined with the results presented earlier, these findings suggest that there are differences between the epitopes recognized by 2–13, 5–38, and Leu 19.

To explore the possibility that the molecules recognized by these mAbs may have a physiologic role in activating or upregulating NK activity, we took advantage of a hybridoma lysis assay, a relatively simple and direct system in which antibody secreting hybridoma cells are ^{51}Cr-labeled and then cultured with cytotoxic effectors to determine their susceptibility to lysis (17). As shown in Fig. 5, both the 2–13 and 5–38 antibody secreting hybridomas were lysed by fresh PBL. Hybridomas secreting antibodies that recognize other molecules expressed by NK cells were studied as controls. An anti-CD16 secreting hybridoma was similarly lysed by fresh PBL, whereas an anti-CD11b expressing hybridoma was resistant to lysis. Identical results were obtained when fresh $CD16^+$ NK cells, enriched from peripheral blood by Percoll gradient centrifugation, were used as effectors (data not shown). These results suggest that the structure(s) recognized by the 2–13 and 5–38 mAbs play a direct role in the cytolytic activity of NK cells.

Despite these findings, however, neither of these mAbs had

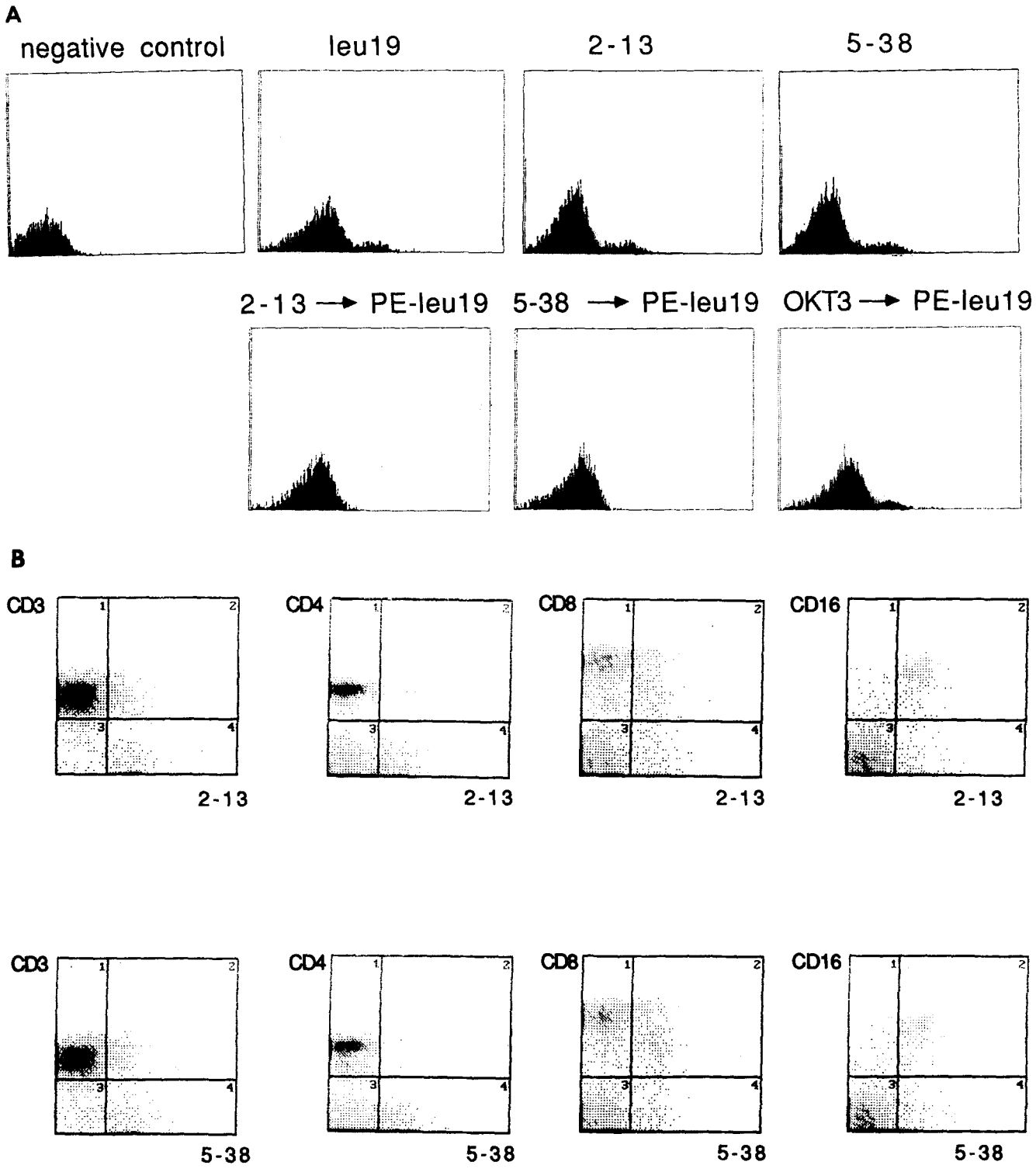


Figure 3. Immunofluorescence analysis of cell surface antigens recognized by 2-13 and 5-38 mAbs. (*A*) The top row depicts normal PBL which were stained with PE-Leu 19 mAb, 2-13 or 5-38 mAb followed by FITC-rat anti-mouse IgG2a antibody. The bottom row depicts antibody blocking of PBL pretreated with 2-13, 5-38, or OKT3 mAb at 4°C for 30 min, washed and stained with PE-Leu 19 mAb. (*B*) PBL were treated with 2-13 or 5-38 mAb followed by FITC-rat anti-mouse IgG2a antibody, and further stained with PE-conjugated anti-CD16, CD4, CD8, or CD3 mAbs. Stained cells were analyzed in a flow cytometer as described in Materials and Methods.

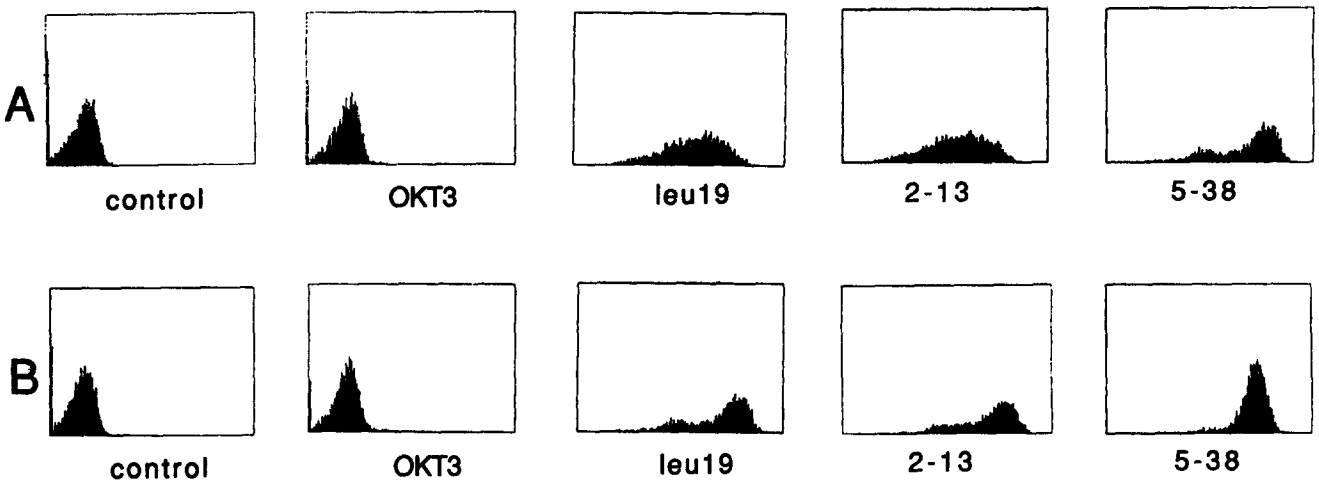


Figure 4. Immunofluorescence analysis of 2–13 and 5–38 antigens on alloantigen-specific NK lines. Two NK lines with different target specificities ([*A*] specific for MSAB; [*B*] specific for ARENT) were separated from feeder cells over a Ficoll-Hypaque gradient and surface antigen expression was analyzed.

any effect on the lysis of K562 cells by the NS2 NK line or fresh PBL (Fig. 6, *A* and *B*). In addition, these mAbs had no inhibitory effect on class I MHC restricted cytolysis mediated by $CD8^+$ T cells or class II MHC restricted cytolysis mediated by $CD4^+$ T cells, even when the ARENT cell line was used as the stimulator and target of such cytolysis (Fig. 6, *C* and *D*). These results are not surprising since neither the T effector cells nor the target ARENT cells are stained by mAbs 2–13 or 5–38.

We next analyzed the effects of these and other mAbs on the cytotoxicity of three additional antigen-specific NK lines (Fig. 7 *A*). Two of these lines, like the NS2 line, were generated against ARENT cells, while a third NK line was generated against an unrelated lymphoblastoid line, MSAB. As shown, the antigen-specific cytotoxicity of all three NK lines

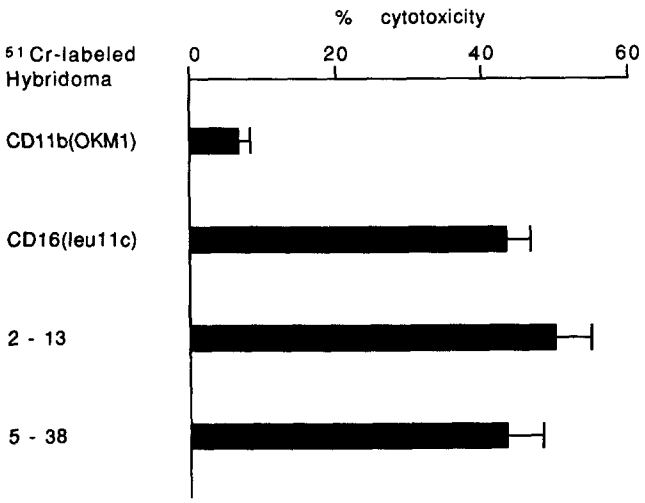


Figure 5. Lysis of hybridomas by PBL. PBL were assayed for cytotoxicity against ^{51}Cr-labeled hybridoma cell line in a 4-h radioisotope release assay. E/T = 10:1.

was completely inhibited by a mAb to LFA-1-α, whereas Leu 19 and Leu 7 mAbs had no effect on cytolysis. mAbs 2–13 and 5–38 inhibited the cytotoxicity of all three NK lines against their specific targets, albeit to a lesser extent than the anti-LFA-1 mAb. As shown in Fig. 7 *B*, these mAbs inhibited allospecific cytotoxicity in a concentration-dependent manner. In combination with our earlier results, which indicate that both 2–13 and 5–38 mAbs bind to the majority of peripheral blood NK cells, these findings are interpreted as suggesting that both mAbs recognize a framework determinant on a molecule or molecules involved in allospecific interactions between NK cells and their targets.

To explore the structural characteristics of the surface antigen(s) recognized by 2–13 and 5–38, the antigen-specific NK line, NS2, and KG1a5 leukemic cell line were surface-labeled with ^{125}I or metabolically labeled with ^{35}S-methionine, followed by immunoprecipitation with 2–13, 5–38, or Leu 19, and analysis of the precipitates by SDS-PAGE. These mAbs precipitated an approximately 180-kD protein from the NS2 line as well as the KG1a5 clone under reducing and nonreducing conditions (Fig. 8), similar to that previously reported for Leu 19 (5, 7, 13). However, the Leu 19 precipitate migrates as a broader band (160–185 kD) than the 2–13 or 5–38 precipitates (170–180 kD). To further analyze the molecules recognized by 2–13, 5–38, and Leu 19 mAbs, sequential immunoprecipitation was performed on a radiolabeled lysate of KG1a5 cells in which the lysate was twice immunoprecipitated with one of the mAbs (until no further band at 180 kD was visible by SDS-PAGE), followed by immunoprecipitation with another of the mAbs. As shown in Fig. 9, the Leu 19 pre-cleared lysate yielded similar but somewhat smaller proteins when immunoprecipitated with either 5–38 or 2–13. Analogous results were obtained in lysates precleared with either 5–38 or Leu 19, followed by immunoprecipitation with the reciprocal antibodies. These results clearly indicate that the target molecule(s), presumably isoforms of CD56, are heterogeneous, and that while some of

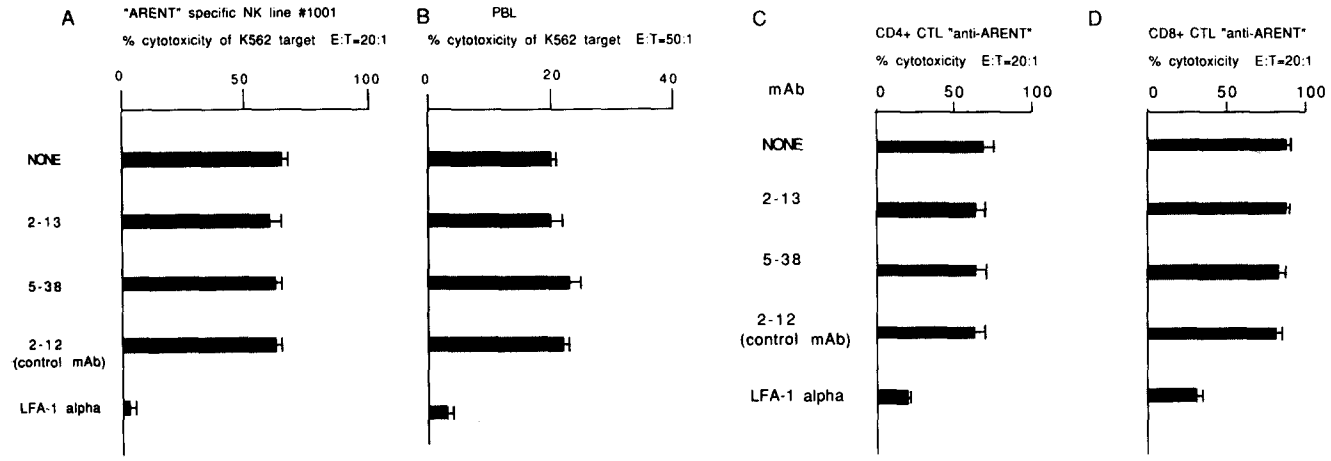
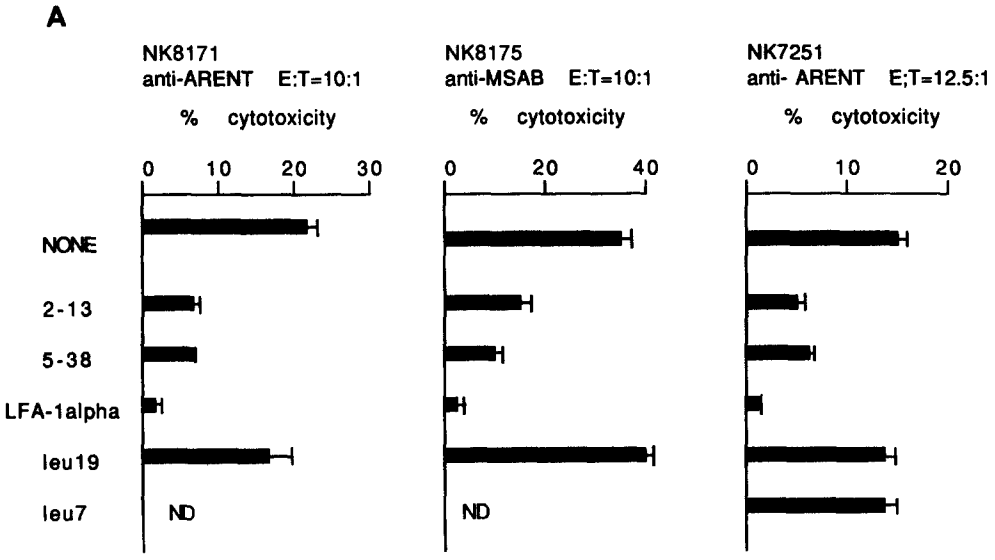


Figure 6. Effects of 2–13 and 5–38 mAb on various cytotoxic effector populations. An alloantigen-specific NK line (*A*) and PBL (*B*) were assayed for cytotoxicity against ^{51}Cr-labeled K562 cells in a 4-h ^{51}Cr-release assay performed in the presence of 50% volume of hybridoma supernatant. E/T = 20:1 for *A* and 50:1 for *B*. CD4$^+$ (*C*) and CD8$^+$ (*D*) CTL lines specific for ARENT, the same stimulator used to generate the NS2 NK cell line, were assayed for cytotoxicity against ARENT cells in a 4-h ^{51}Cr-release assay performed in the presence of 50% volume of hybridoma supernatant. E/T = 20:1 for both *C* and *D*.



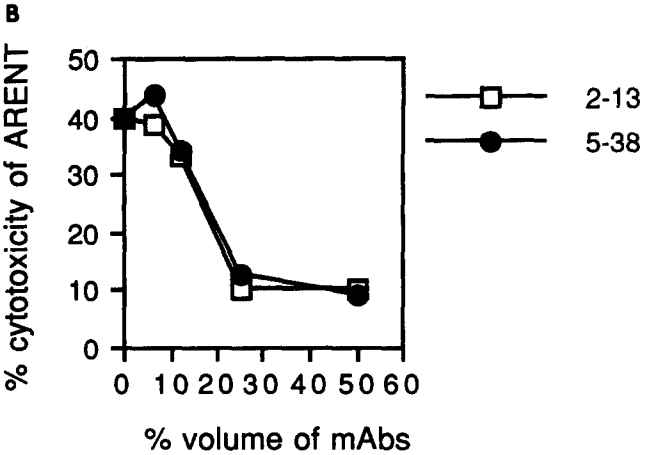


Figure 7. Effects of 2–13 and 5–38 mAbs on the allospecific cytolytic activity of a panel of NK lines. (*A*) Antigen-specific NK lines with two different target specificities were assayed for their cytotoxic activity against their original stimulator LCL in 4-h ^{51}Cr-release assays in the presence of various mAbs; for these assays 2–13 and 5–38 mAbs were present as 50% volume of culture supernatant, while the final concentration of LFA-1α, Leu 19, and Leu 7 mAbs was 25 μg/ml. (*B*) An ARENT-specific NK line (NK8171) was assayed for cytolytic activity against its original stimulator LCL in the presence of varying amounts of 2–13 or 5–38 mAb culture supernatants. E/T ratio = 20:1.

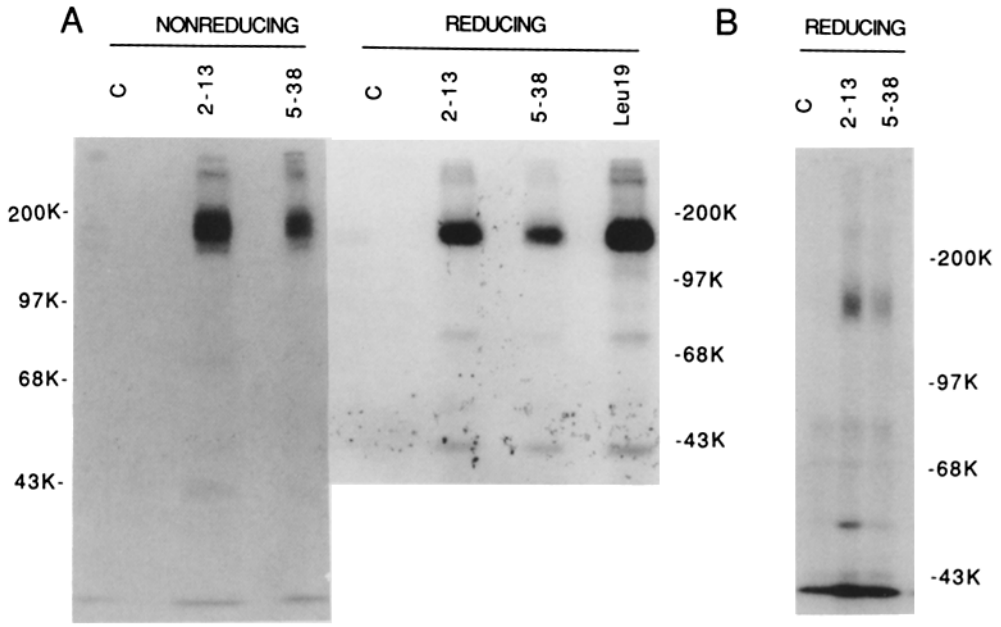


Figure 8. SDS-PAGE analysis of surface molecules immunoprecipitated by 2–13 and 5–38 mAbs. The KG1a5 leukemic cell line (*A*) and an alloantigen specific NK line (*B*) were surface labeled with ^{125}I using the lactoperoxidase technique, and lysed in buffer containing 1% NP-40. Immunoprecipitation was performed with 2–13 mAb and 5–38 mAb coupled to protein A-sepharose beads. SDS-PAGE analysis was performed under reducing and nonreducing conditions, using 8% acrylamide gels for the KG1a5 lysate and 6% acrylamide gels for the NK lysate. The Leu 19 immunoprecipitate was included as a control. (*C*) represents immunoprecipitation with nonimmune mouse serum.

these forms are recognized by all three mAbs, other forms are not.

To further characterize the target molecules of these mAbs, the radiolabeled immunoprecipitates were subjected to digestion with V8 protease, and the cleaved products analyzed by SDS-PAGE. As shown in Fig. 10 *A*, the V8 cleaved products of the three immunoprecipitates are similar. However, under the milder conditions of Fig. 10 *B*, 50-kD and 47-kD bands were visible in the 5–38 digest (indicated by arrows), whereas in the Leu 19 digest only the 50-kD band can be seen (indicated by an arrow). The 47-kD band was reproducibly present in 5–38 digests but not in Leu 19 digests, strongly suggesting that there are minor amino acid sequence differences between some of the CD56 proteins recognized by the three mAbs under study. When combined with the earlier analysis of sequential immunoprecipitates (Fig. 9), it is apparent that these mAbs recognize several proteins, some of which share epitopes and some of which do not.

In a final series of experiments designed to determine whether the epitopes recognized by 2–13 and 5–38 contain carbohydrate, PBLs were treated with neuraminidase to remove sialic acid or cultured with tunicamycin to prevent incorporation of N-linked carbohydrate (13, 18, 19). The binding of 2–13 and 5–38, as well as Leu 19, to NK cells was not reduced by either procedure, whereas the binding of anti-HLA-DR antibody included as a positive control, was significantly reduced as previously reported (19) (data not shown). By contrast, treatment of NK cells with trypsin

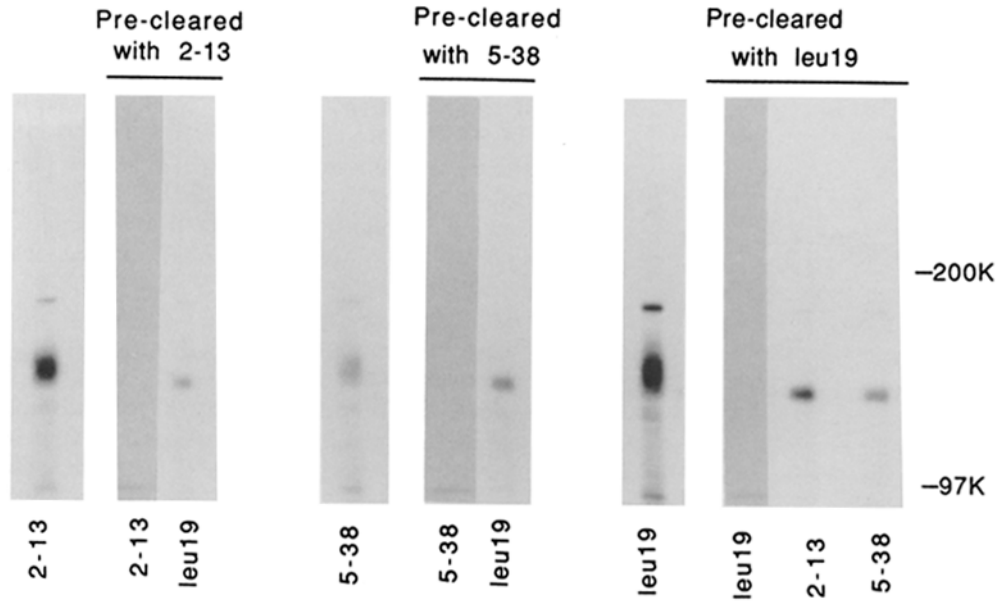
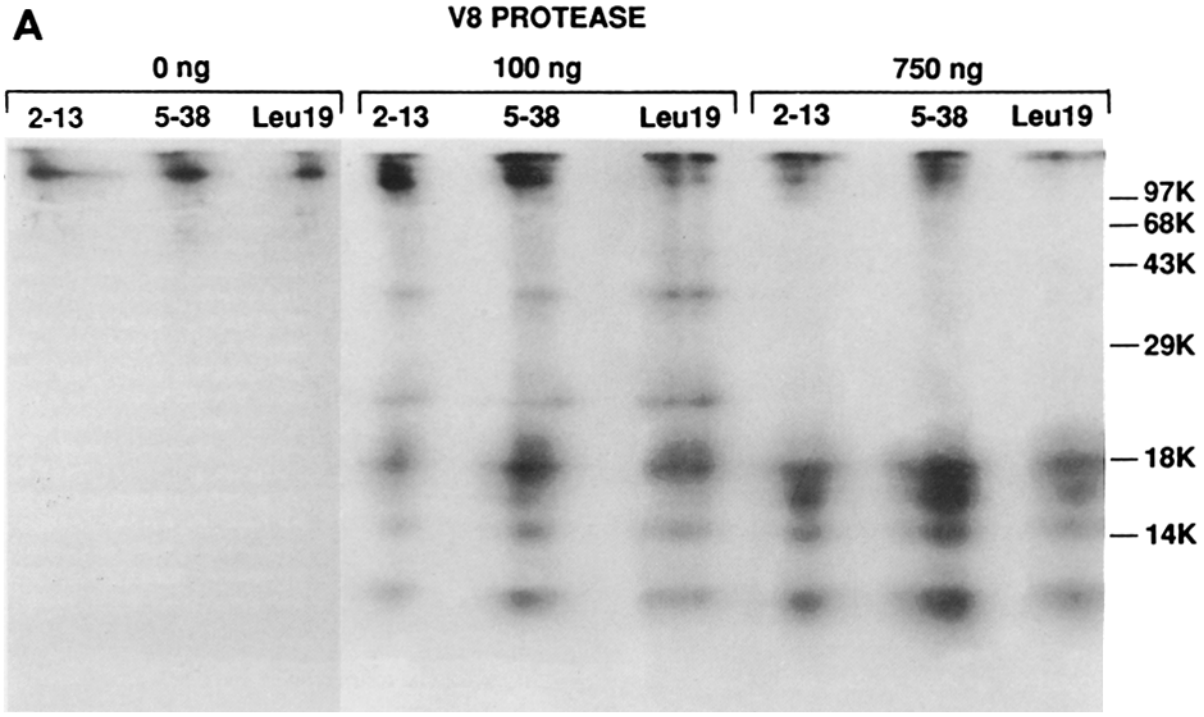


Figure 9. Sequential immunoprecipitation of 2–13, 5–38, and Leu 19 from KG1a5 cells. KG1a5 cells were labeled with ^{35}S-methionine and detergent solubilized. The lysates were substantially depleted of 2–13 antigen, 5–38 antigen, and Leu 19 antigen by immunoprecipitation with 2–13, 5–38, and Leu 19 mAbs, respectively. Each precleared lysate was once more immunoprecipitated with the same mAb and complete depletion of the relevant antigen was confirmed by SDS-PAGE. After depletion, the lysates were immunoprecipitated with the other mAbs. Immunoprecipitates were analyzed by 5% SDS-PAGE gel under reducing conditions.



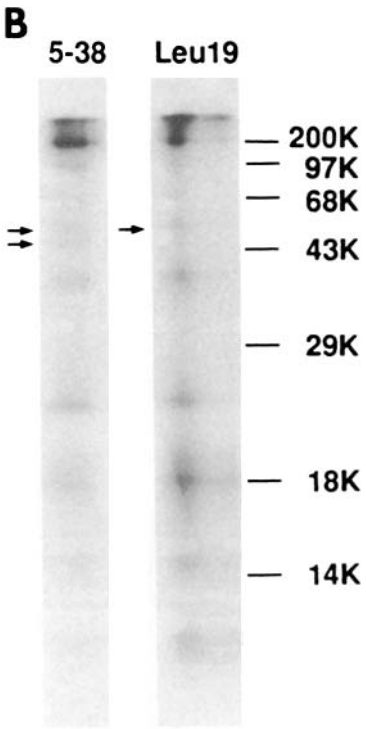


Figure 10. V8 protease digestion of 2–13, 5–38, and Leu 19 antigens from KG1a5 cells. ^{35}S-methionine labeled KG1a5 cells were detergent solubilized, precipitated with mAbs 2–13, 5–38, and Leu 19 and separated by SDS-PAGE as in Fig. 9. (*A*) The major bands were cut from the gels and digested with 0, 100, and 750 ng of *Staphylococcus aureus* V8 protease, and analyzed by SDS-PAGE (reducing conditions) using a 15% acrylamide gel. (*B*) 5–38 and Leu 19 proteins recovered as above were digested with 75 ng of V8 protease. (*Arrows*) indicate differences between these two digests.

resulted in nearly complete loss of the binding of 2–13, 5–38, and Leu 19 to NK cells while only slightly affecting the expression of Leu 7 (CD57), a glycolipid antigen (data not shown). These results confirm that the epitope recognized by 2–13 and 5–38 are amino acid rather than carbohydrate in nature.

Discussion

NK cells are defined as lymphocytes that lyse selected tumor cells or virally infected cells without prior sensitization or MHC gene restriction (1–3). Despite the fact that NK cells have a limited target cell range, no specific NK-associated surface receptor or target ligand has yet been defined, leading to speculation that such effectors lack a highly refined antigen recognition system. However, previous attempts to absorb NK activity with target cells suggested heterogeneity of both NK recognition and target structures (20, 21). In addition, NK cells have been shown to mediate hybrid resistance to parental bone marrow allografts (22, 23), a phenomenon that appears genetically restricted and directed at the products of noncodominant hematopoietic histocompatibility genes (24). Recently, we and others have provided evidence for alloantigen recognition by CD3$^-$ NK cells (8–10), and on the basis of these observations it would appear that at least some NK cells have the ability to distinguish target cells from one another.

In the present study, using an alloantigen-specific NK line as an immunogen, we generated two mAbs which inhibit alloantigen-specific cytotoxic activity of CD3$^-$ NK cells without affecting the ability of these cells to kill classical NK targets such as K562 cells. Immunofluorescence analysis of PBL, KG1 cells, and NK lines recognizing different targets revealed that the antigenic epitopes recognized by these two

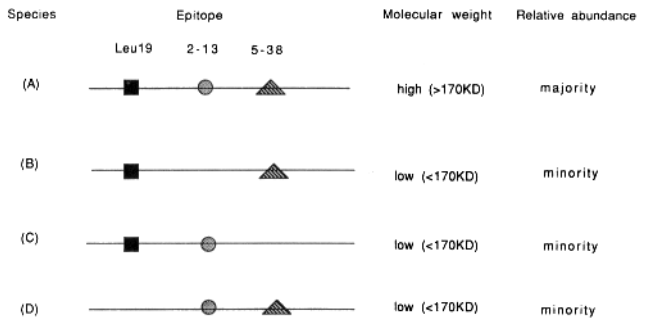


Figure 11. Proposed representation of CD56 isoforms.

mAbs are closely associated with, but not identical to the target molecule of Leu 19, a well-described anti-CD56 mAb. Thus, 2–13 and 5–38 block the staining of NK cells by fluorochrome-conjugated Leu 19 mAb; however, the staining patterns of Leu 19 on NK cells differs from that of 5–38, and Leu 19 had no effect on the lysis of allospecific target cells by NK lines, in contrast to 2–13 and 5–38. Sequential immunoprecipitation with these two mAbs and Leu 19, as well as analysis of the peptides resulting from V8 protease digestion revealed that each mAb recognizes overlapping and nonoverlapping isoforms of CD56, a molecule expressed selectively on lymphocytes with NK activity (5, 7). Fig. 11 summarizes in schematic form our interpretation of the current results. Since the isoforms of CD56 which lack one or more epitopes are always smaller than those which have the epitope (Fig. 9), and peptide mapping with V8 protease showed minor difference of these isoforms, we favor the view that different isoforms are generated by a combination of alternative splicing and posttranslational modifications rather than posttranslational modification alone. In addition, the epitopes recognized by 2–13 and 5–38 consist of amino acids (protein) rather than carbohydrate alone, since treatment of PBLs with neuraminidase or culture with tunicamycin did not reduce the binding of 2–13 and 5–38, whereas trypsin treatment of NK cells greatly reduced 2–13 and 5–38 antigen expression. Although it is not yet known how many isoforms of CD56 are present on NK cells, sequential immunoprecipitation confirmed the presence of at least four distinct isoforms. It is theoretically possible, and in our view likely, that more than four different isoforms exist on NK cells.

The finding of multiple CD56 isoforms on NK cells is consistent with the hypothesis that these isoforms constitute an allorecognition apparatus with limited heterogeneity. This view is also supported by the observation that although Leu 19, 2–13, and 5–38 mAbs stain most peripheral blood NK cells, the staining pattern for each allospecific NK line is distinct (Fig. 3 *A*). An alternative explanation for our findings is that CD56 plays an accessory role as a mediator of nonspecific adhesion between allospecific NK effectors and their targets. However, it is difficult to reconcile this view with the existence of multiple CD56 isoforms on NK cells, the variable expression of these isoforms on allospecific NK lines, and the failure of any of our anti-CD56 mAbs to affect the lysis by NK cells of "nonspecific" targets such as K562 erythroleukemia cells. Definitive evidence for either of these hypotheses will require studies of cells transfected with cDNA's for the relevant CD56 isoforms.

Since the hybridomas secreting 2–13 and 5–38 antibodies are efficiently lysed by peripheral NK cells, it is apparent that the CD56 isoforms play an important role not only in the interaction between NK effectors and their targets but also in the transduction of intracellular activation signals. Like CD56, the molecules CD2, CD16, and the recently described GL183 antigen have been shown to mediate signal transduction in NK cells (17, 25–27). The GL183 molecule is of particular interest since it was initially identified on some but not all NK clones (27); however, this molecule, like CD2 and CD16, is invariant in structure and, therefore, unlikely to be involved in alloantigen-specific interactions. The metabolic pathway affected by 2–13 and 5–38 mAbs (and, by inference, the natural ligands of CD56 on NK target cells) remain to be studied. Nonetheless, our results suggest that the rather confined expression of CD56 on NK cells and T cells with NK-like activity reflects a critical role for this molecule not only in the interaction of NK cells with their targets but also in the cytotoxic effector functions of NK cells.

Previous studies have suggested that CD56 is a single isoform of the neural adhesion molecule (N-CAM) (13), which is expressed on neural and muscle tissues as multiple isoforms and mediates homotypic adhesive interactions (28, 29). In chicken neural tissue, where N-CAM was initially identified (28, 30) numerous isoforms are generated from a single gene by differential RNA splicing and posttranslational modifications, including the addition of N-linked carbohydrates in polysialic acid (28, 30). Recently, N-CAM cDNA isolated from human muscle tissue has been cloned and sequenced (29). Human N-CAM has an immunoglobulin-like domain, suggesting that it may function as a receptor for ligands yet to be identified. In addition, Streuli et al. (31) have recently isolated cDNA encoding a cell surface molecule of undefined function, termed LAR, which has an extracellular region homologous to both the Ig-like and non–Ig-like domain of N-CAM. Thus, it is possible that 2–13 and/or 5–38 cross-react with N-CAM and N-CAM–like molecules such as LAR. In normal lymphoid tissues, CD56 is expressed predominantly on NK cells and a subset of T cells with NK-like activity (5, 7). Although this preferential distribution and the immunoglobulin-like structure of CD56 are consistent with an important role for this molecule in NK cell function, before the current studies, there has appeared virtually no evidence to support this view although CD56 is reported to be involved in cytotoxic activity and homotypic adhesion between NK cells and $CD56^+$ malignant neural cells (32). On the other hand, our results indicate that the CD56 isoforms recognized by 2–13 and 5–38 are directly involved in NK cell/target cell interactions, and since the LCL targets studied here lack any detectable expression of CD56, it is clear that the CD56 isoforms on NK cells mediate a heterotypic rather than homotypic interaction between NK cells and their targets. Although our limited peptide mapping analysis indicates that there are amino acid sequence differences between the CD56

isoforms on lymphoid cells, the relative contribution of these differences versus posttranslational modifications to the differential target cell recognition by distinct NK lines is unknown. Similarly, the nature of the natural ligand of these isoforms on NK targets remains to be elucidated.

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