# Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells

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**The relative contribution to cytotoxicity of each of the multiple NK cell activation receptors has been difficult to assess. Using *Drosophila* insect cells, which express ligands of human NK cell receptors, we show that target cell lysis by resting NK cells is controlled by different receptor signals for cytolytic granule polarization and degranulation. Intercellular adhesion molecule (ICAM)-1 on insect cells was sufficient to induce polarization of granules, but not degranulation, in resting NK cells. Conversely, engagement of the Fc receptor CD16 by rabbit IgG on insect cells induced degranulation without specific polarization. Lysis by resting NK cells occurred when polarization and degranulation were induced by the combined presence of ICAM-1 and IgG on insect cells. Engagement of receptor 2B4 by CD48 on insect cells induced weak polarization and no degranulation. However, coengagement of 2B4 and CD16 by their respective ligands resulted in granule polarization and cytotoxicity in the absence of leukocyte functional antigen-1–mediated adhesion to target cells. These data show that cytotoxicity by resting NK cells is controlled tightly by separate or cooperative signals from different receptors for granule polarization and degranulation.**

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Abbreviations used: ADCC, antibody–dependent cellular cytotoxicity; APC, allophycocyanin; ICAM, intercellular adhesion molecule; ITAM, immunoreceptor tyrosine-based activation motif; KIR, killer cell immunoglobulin-like receptor; LAMP, lysosomal-associated membrane glycoprotein; LFA-1, leukocyte functional antigen 1; SC, Schneider cell.

NK cells represent a subset of cytotoxic lymphocytes that is able to recognize and lyse tumor cells virus-infected cells, and immature dendritic cells without previous sensitization (1, 2). Besides expression of several receptors that mediate natural cytotoxicity, expression of CD16 (the low affinity receptor for IgG, FcγRIIIA) on a majority of NK cells renders them strong mediators of antibody-dependent cellular cytotoxicity (ADCC) against IgG-coated target cells (3, 4). NK cell function is regulated by a balance between activating and inhibiting receptor signals (5–7). Several types of inhibitory NK cell receptors recognize MHC class I molecules on target cells and prevent NK cell cytotoxicity toward normal cells. Down-regulation of MHC class I molecules on target cells may lead to NK cell–mediated lysis (8). Several structurally distinct activation receptors have been implicated in NK cell cytotoxicity, a complex process that involves adhesion, synapse formation, and granule polarization and exocytosis. Because of the multiplicity of receptor–ligand interactions between NK cells and target cells, it has been difficult to assign specific functions to individual receptors.

To overcome this complexity, we have developed a system based on the use of *Drosophila* insect cells as target cells (9, 10). Unlike mammalian cells, insect cells are not expected to express a multitude of ligands for adhesion and activation receptors of human NK cells. Therefore, they are better suited for investigations on the individual contribution of, and cross talk among, NK cell receptors. A notable advantage of such a reconstituted target cell system is that activation of normal, unmanipulated NK cells can be studied with physiologic ligands.

Here, we have focused on the contribution of three NK cell receptors to cytotoxicity by freshly isolated, resting human NK cells. Receptors CD16, leukocyte functional antigen (LFA)-1 (CD11a/CD18), and 2B4 (CD244) generally are considered as activation, adhesion,

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and costimulation receptors, respectively. CD16 is expressed on a large subset of peripheral blood NK cells, which also express intermediate levels of CD56 ($CD56^{dim}$). A phenotypically and functionally distinct $CD56^{bright}CD16^{-}$ subset of NK cells constitutes <10% of peripheral blood NK cells (11). $CD56^{dim}$ NK cells mediate ADCC through engagement of CD16. The CD16 receptor associates with signaling homodimers, or heterodimers of the FcR γ-chain and TCR ζ-chain, which carry cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs) (12). Signaling through ITAMs induces activation of Syk family kinases (13). Natural cytotoxicity receptors on NK cells, such as NKp30, NKp44, and NKp46, also associate with ITAM-containing chains (14). Except for two viral ligands of NKp46 and NKp30 (15, 16), the human ligands for the natural cytotoxicity receptors have not been identified. In this study, CD16 serves as a representative for ITAM-dependent signaling receptors.

LFA-1 is an integrin that is expressed by all leukocytes (17); it is a heterodimer of $\alpha_L$ (CD11a) and $\beta_2$ (CD18) polypeptides. This integrin participates in lymphocyte adhesion, arrest, and extravasation, and has a role in immunologic synapse formation (18). Patients who have leukocyte adhesion disorder, a syndrome that is caused by LFA-1 deficiency, suffer severe recurrent bacterial infections and impaired immunity (19). LFA-1 binds intercellular adhesion molecule (ICAM)-1 through ICAM-5 (17, 20). Adhesiveness through LFA-1 is regulated dynamically by changes in affinity and valency, which can be primed by cytokines or by target cell structures that engage activating receptors (17, 21). Although NK cells express other integrins, LFA-1 is believed to play a dominant role in target cell lysis (22). Studies of mouse $LFA\text{-}1^{-/-}$ NK cells showed that LFA-1 is necessary for efficient cytotoxicity by IL-2– or IL-12–activated NK cells (23, 24). Antibody blocking of LFA-1–ICAM interactions impairs ADCC and natural cytotoxicity by human NK cells (25, 26). Despite all of this information, it remains unclear if the major role of LFA-1 in NK cell cytotoxicity is to provide adhesion and formation of a tight interface with target cells, or to provide additional signals for delivery of the lethal hit. Recent studies suggested a signaling role of LFA-1 in perforin release by a subset of IL-2–pulsed primary NK cells (27), and in cytolytic granule polarization in long-term IL-2–cultured NK cells (10).

Receptor 2B4 generally is considered as a coactivator of NK cell cytotoxicity, because it enhances NK cell responses under limiting ITAM-mediated activation (28–30). 2B4 and its ligand, CD48, are members of the CD2 family of Ig-related proteins (31, 32). 2B4 is expressed by all NK cells, most γδ T cells, by a subset of αβ $CD8^+$ T cells (typically of the effector/memory phenotype), and by monocytes and basophils. NK cell activation through 2B4 is accompanied by phosphorylation of tyrosine-based motifs in the cytoplasmic tail and recruitment of signaling lymphocyte–activation molecule–associated protein and the Src-family kinase Fyn (33, 34). However, it is still unclear how 2B4 provides coactivation signals in the context of other receptor–ligand interactions and whether 2B4 is capable of triggering cytotoxicity independently of other receptor signals.

To dissect the respective contributions of activation receptor CD16, adhesion receptor LFA-1, and coactivation receptor 2B4 to target cell lysis by resting NK cells, different assays were used to measure cytolytic granule polarization and degranulation separately, rather than overall target cell lysis. One advantage of this approach over standard cytotoxicity assays was to shift the focus from the fate of insect target cells to the response of NK cells. NK cells lyse target cells through regulated exocytosis of cytolytic granules, as do other cytotoxic lymphocytes, which can result in perforin- or Fas ligand–dependent killing of target cells (35, 36). With *Drosophila* insect cells expressing ligands of human NK cell receptors, we now show that engagement of LFA-1 on resting NK cells signals for polarization of cytolytic granules, but does not induce degranulation or elicit cytotoxicity. CD16 signals for degranulation, and engagement of CD16 and LFA-1 leads to efficient target cell lysis. Coengagement of 2B4 and CD16 enhances the number of degranulating NK cells, and can induce cytotoxicity in the absence of LFA-1–ICAM-1 interaction.

## RESULTS

### Antibody-dependent cellular cytotoxicity by resting NK cells

Transfected *Drosophila* Schneider cell (SC)2 cells expressing ICAM-1, CD48, or ICAM-1 and CD48 (9) (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20051143/DC1) were used to study the requirements of target cell lysis by resting NK cells. SC2 cells were incubated with resting NK cells at different E/T ratios for 3 h at 37°C (Fig. 1 A). In contrast to IL-2–activated NK cells that lyse SC2 cells expressing ICAM-1 (10), freshly isolated, resting NK cells did not (Fig. 1 A). Resting NK cells did not lyse SC2, SC2-CD48, or SC2–ICAM-1–CD48 cells (Fig. 1 A). A rabbit antiserum was raised against *Drosophila* SC2 cells to test ADCC. SC2 cells preincubated with a $10^{-4}$ dilution of rabbit anti-SC2 serum were washed and incubated with resting NK cells at different E/T ratios for 3 h at 37°C. Efficient ADCC was observed with SC2–ICAM-1, SC2-CD48, and SC2–ICAM-1–CD48 cells, but not with SC2 cells (Fig. 1 A). Therefore, engagement of receptors for IgG, ICAM-1, and CD48 (CD16, LFA-1, and 2B4, respectively) in isolation was not sufficient to induce strong cytotoxicity by resting NK cells. However, coengagement of receptors for IgG with the receptor for ICAM-1 or the receptor for CD48 resulted in strong cytotoxicity by resting NK cells. These results show that resting NK cells can have strong cytotoxic activity, given the right signals, even in the absence of exogenous cytokines.

To test how adhesion of resting NK cells to SC2 cells may relate to cytotoxicity, conjugation assays were performed. As described previously (9), ∼20% of resting NK cells form conjugates with SC2–ICAM-1 cells within 20–30 min, ∼50% form conjugates with SC2–ICAM-1–CD48 cells, and <3% form conjugates with untransfected SC2 cells (Fig. 1 B). Coengagement of LFA-1 and CD16 increased

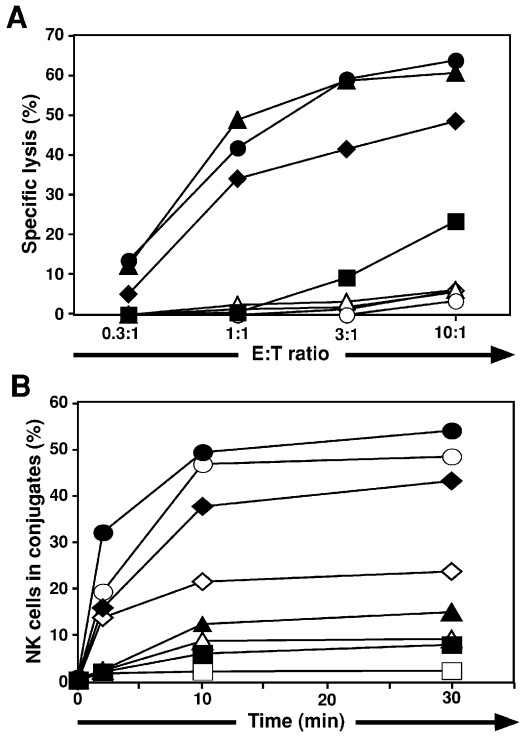


**Figure 1. ADCC by resting NK cells toward insect SC2 cells requires costimulation.** NK cells were mixed with SC2 (open squares), SC2–ICAM-1 (open diamonds), SC2–CD48 (open triangles), or SC2–ICAM-1–CD48 (open circles) cells or the above SC2 cells were preincubated with a rabbit serum raised against SC2 cells (filled symbols). (A) Cells were incubated for 3 h at 37°C. Specific lysis of SC2 cells was calculated from the percentage of propidium iodide–positive SC2 cells in duplicate samples, as determined by flow cytometry. (B) NK cells labeled with a green dye were mixed with SC2 cells labeled with a red dye, and incubated at 37°C for the indicated time. Cells were fixed and the fraction of NK cells in two-color conjugates was determined by flow cytometry. Points represent the average of duplicate samples. For cytotoxicity, the variation between duplicate samples was, on average, <10% of the calculated value of lysis. Representative experiments are shown.

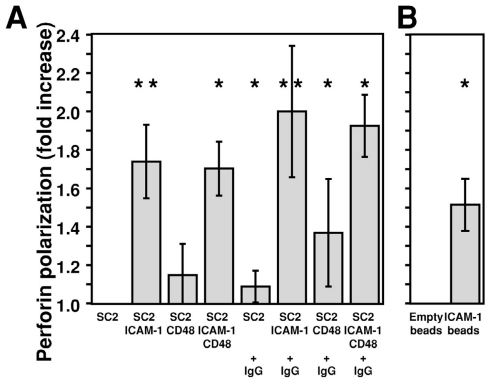


**Figure 2. ICAM-1 on insect cells induces granule polarization in resting NK cells.** (A) NK cells mixed with SC2 cells expressing indicated ligands were incubated for 20 min at 37°C, fixed, permeabilized, and stained with an antiperforin mAb and fluorochrome-conjugated secondary antibody. NK cells in conjugate with SC2 cells were scored for polarized perforin toward the target cell interface in six independent experiments (three for SC2–ICAM-1–CD48). (B) NK cells were mixed with protein A coupled beads coated with recombinant ICAM-1 Fc. Data are expressed as the fold increase of NK cells that display polarized perforin in three independent experiments. Error bars represent the SD. *$P < 0.05$ and **$P < 0.005$ relative to NK cells mixed with SC2 cells or empty beads, respectively.

conjugate formation to ~40% of resting NK cells (Fig. 1 B). However, coengagement of CD16 and 2B4 resulted in conjugate formation by only ~15% of resting NK cells. Engagement of CD16 or 2B4 alone resulted in <10% conjugate formation. Therefore, cytotoxicity did not correlate with conjugate formation. The results suggested that the contributions of LFA-1 and 2B4 to cytotoxicity were different.

### Granule polarization in NK cells induced by ICAM-1

The lack of cytotoxicity by resting NK cells mixed with SC2–ICAM-1 cells (Fig. 1 A) suggested that LFA-1 may not signal in resting NK cells, as it does in IL-2–activated NK cells, which lyse SC2–ICAM-1 cells (10). Cytolytic granule polarization assays were performed with resting NK cells to test whether engagement of LFA-1 by ICAM-1 alone was sufficient to induce polarization, as it is with IL-2–activated NK cells (10). SC2 cells were mixed with resting NK cells and incubated for 20 min at 37°C. Polarization of perforin-containing granules toward target cells was assessed by confocal microscopy, using reconstructed three-dimensional images. ICAM-1 expression on SC2 cells was sufficient to induce significant polarization of perforin-containing granules ($P < 0.001$; $n = 6$ independent experiments on NK cell preparations from separate donors; on average, 180 cell conjugates were scored for each transfectant with each donor) (Fig. 2 A). Data are displayed as fold-increase in polarization relative to ~30% (31 ± 4.7% [mean ± SD]; $n = 6$) random polarization observed with untransfected SC2 cells (Fig. 2 A). In contrast, rabbit IgG on SC2 cells did not induce much granule polarization. Expression of CD48 on SC2 cells induced little polarization and did not enhance the polarization observed with ICAM-1 alone (Fig. 2 A). However, the combination of CD48 and rabbit IgG on SC2 cells resulted in increased granule polarization relative to SC2-CD48 and SC2 cells coated with rabbit IgG alone ($P = 0.008$ and $P = 0.044$, respectively; Fig. 2 A). A contribution to the polarization toward SC2–ICAM-1 cells by receptors other than LFA-1 on NK cells, which may recognize ligands on insect cells, cannot be ruled out. Therefore, resting NK cells were mixed with beads coated with ICAM-1–Fc fusion protein for 20 min and evaluated for polarization of perforin-containing granules. To eliminate a potential contribution by CD16 on the NK cells, the ICAM-1–Fc fusion protein contained mutations in the human IgG1 Fc portion of the protein, which were designed to disrupt the interaction of the Fc region with CD16 (37). The value for the (random) contact of uncoated beads was similar to that observed with SC2 cells, and consistent with earlier data with IL-2–activated

NK cells (10). Polarization of perforin-containing granules was augmented significantly with ICAM-1–coated beads ($P = 0.023$; Fig. 2 B). Therefore, ICAM-1 is sufficient to induce granule polarization but not cytotoxicity in resting NK cells; this implies that LFA-1 delivers signals in resting NK cells, besides signals that are required for adhesion. The lack of granule polarization toward, and lack of lysis of, rabbit IgG–coated SC2 cells suggested that CD16 may not be able to engage its ligand and to signal in the absence of additional receptor–ligand interactions. To investigate this question further, additional assays for NK cell activation were used.

### Cell surface CD107a marks degranulation in resting NK cells

In cytotoxic lymphocytes, lytic granules are secretory lysosomes that contain a dense core, including various proteins that are involved in cytotoxic function (e.g., perforin, granzymes). The core is surrounded by a lipid bilayer that contains lysosomal-associated membrane glycoproteins (LAMPs) and Fas ligand (38, 39). Degranulation by cytotoxic T cells results in CD107a (LAMP-1) appearance at the cell surface and depletion of intracellular perforin (40, 41). We first examined whether CD107a colocalized with perforin, a key effector in NK cell cytotoxicity, by confocal microscopy in resting NK cells (42). Perforin colocalized with CD107a (Fig. 3 A), but $CD107a^+$ $perforin^-$ and $CD107a^-$ $perforin^+$ compartments also were observed in some cells. CD107a was not detected on the surface of resting NK cells by flow cytometry (Fig. 3 B). CD107a appeared at the surface of NK cells that were incubated with the sensitive target cell line K562 for 2 h at 37°C (Fig. 3 B), as reported recently (43). Likewise, cross-linking of CD16 with a mAb bound to $FcR^+$ P815 cells induced cell surface CD107a (Fig. 3 B). NK cells mixed with P815 cells or P815 cells incubated with isotype control mAb did not induce cell surface CD107a expression (Fig. 3 B). P815 cells incubated with a mAb to the activating NK cell receptor 2B4 induced a very small increase of NK cells with surface CD107a in some experiments (Fig. 3 B). The induction of CD107a surface expression correlated with target cell lysis by resting NK cells, which lysed K562 cells (Fig. 3 C, top),

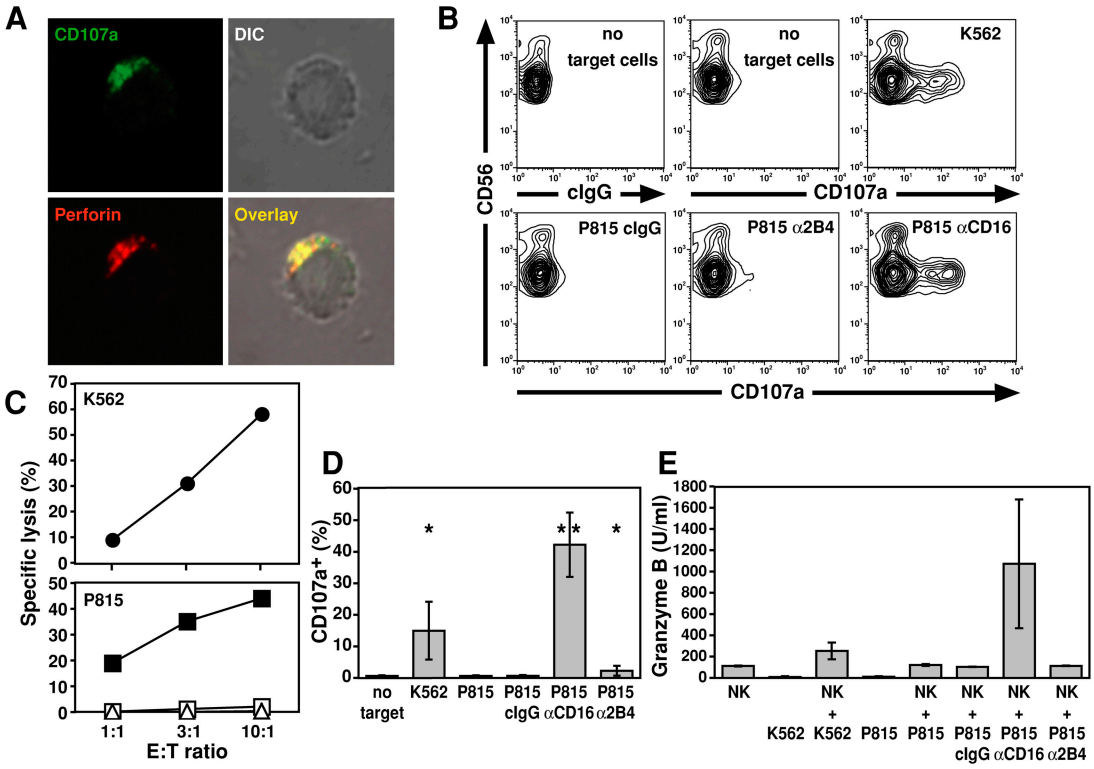


**Figure 3. Cell surface CD107a on resting NK cells correlates with granzyme B release and target cell lysis.** (A) NK cells were fixed, permeabilized, and stained with anti-CD107a and antiperforin mAbs. Left panels show confocal images of CD107a (green), perforin (red). Right panels show a differential interference contrast (DIC) image, and an overlay of all three images. (B–E) NK cells were mixed with K562 cells or with P815 cells preincubated with the indicated mAbs. (B and D) After 2 h at 37°C, the cells were stained with fluorochrome-conjugated anti-CD56 and anti-CD107a antibodies. NK cells were gated away from target cells on forward scatter/side scatter plots. (B) Profiles show CD56 versus CD107a or control antibody staining, as indicated. (C) In $^{51}Cr$ release assays, resting NK cells were incubated with K562 cells or with P815 cells incubated with isotype control (open squares), anti-CD16 (filled squares), or anti-2B4 (open triangles) mAbs. Specific lysis obtained in one representative experiment is shown. (D) The fraction of $CD107a^+$ NK cells is presented as the mean of six independent experiments. Bars indicate SD. *$P < 0.05$ and **$P < 0.005$ relative to NK cells incubated without target cells. (E) After 2 h, granzyme B release to supernatants was measured by ELISA. Values represent mean ± SD of three independent experiments.

and P815 cells incubated with anti-CD16 mAb (Fig. 3 C, bottom panel). Cytotoxicity and surface CD107a was not observed in the presence of isotype control or anti-2B4 mAb (Fig. 3 C, bottom).

$CD56^+CD107a^+$ NK cells were quantified in several independent experiments (Fig. 3 D). Sensitive target cells induced CD107a expression on only a fraction of NK cells. After incubation with K562 cells, 15 ± 9.1% (mean ± SD, $n = 7$ independent experiments) of the NK cells expressed surface CD107a (Fig. 3 D). Upon cross-linking by P815 cells that were incubated with anti-CD16 mAb, 42 ± 10% (mean ± SD, $n = 7$) of the NK cells expressed surface CD107a. NK cells alone, NK cells that were mixed with P815 cells, or P815 cells that were incubated with isotype control mAb did not induce cell surface CD107a (0.7 ± 0.20%, 0.7 ± 0.21%, and 0.7 ± 0.24%, respectively, mean ± SD; $n = 7$; Fig. 3 D). P815 cells that were incubated with a mAb to the activating NK cell receptor 2B4 induced a small increase of NK cells with surface CD107a (2.3 ± 1.6%, mean ± SD; $n = 7$; Fig. 3, B and C).

Degranulation reached a plateau at ~0.5–1 target cell per NK cell, and no further increase occurred up to 10 target cells per NK cell (unpublished data). Therefore, maximal degranulation was reached with E/Ts used in the degranulation experiments. Cell surface CD107a coincided with increased granzyme B release as quantified by ELISA on culture supernatants (Fig. 3 E). Although some granzyme B release was observed with NK cells alone, this basal release did not increase upon mixing with P815 cells or P815 cells that were incubated with isotype control or anti-2B4 mAbs (Fig. 3 E). Granzyme B release was augmented when NK cells were mixed with K562 cells or with P815 cells that were incubated with anti-CD16 mAbs (Fig. 3 E). In conclusion, cytotoxicity correlated well with the observed induction of cell surface CD107a and granzyme B release. Together, the results suggest that degranulation is necessary for resting NK cell–induced natural cytotoxicity and Fc receptor–dependent cytotoxicity, and that cell surface expression of CD107a is a marker of cytolytic granule exocytosis in NK cells.

Despite efficient target cell lysis, only a fraction of resting NK cells expressed surface CD107a after 2 h of incubation with sensitive K562 cells or P815 cells that were incubated with anti-CD16 mAbs. This could reflect (a) heterogenous expression of CD107a on resting NK cells, (b) that only a fraction of the NK cells degranulate and mediate cytotoxicity, or (c) that degranulation is underestimated because of a rapid CD107a reinternalization during the assay. First, uniformly high levels of CD107a were detected by intracellular staining of fixed and permeabilized resting NK cells with anti-CD107a mAbs, as compared with cells stained with an isotype control mAb (Fig. 4, A and B). Intracellular staining of perforin produced similar profiles; the $CD56^{dim}$ NK cells expressed slightly more perforin than did $CD56^{bright}$ NK cells (44 and unpublished data). Together, the data suggest that all resting NK cells are capable of expressing surface CD107a and mediating cytotoxicity if provided with signals for degranulation.

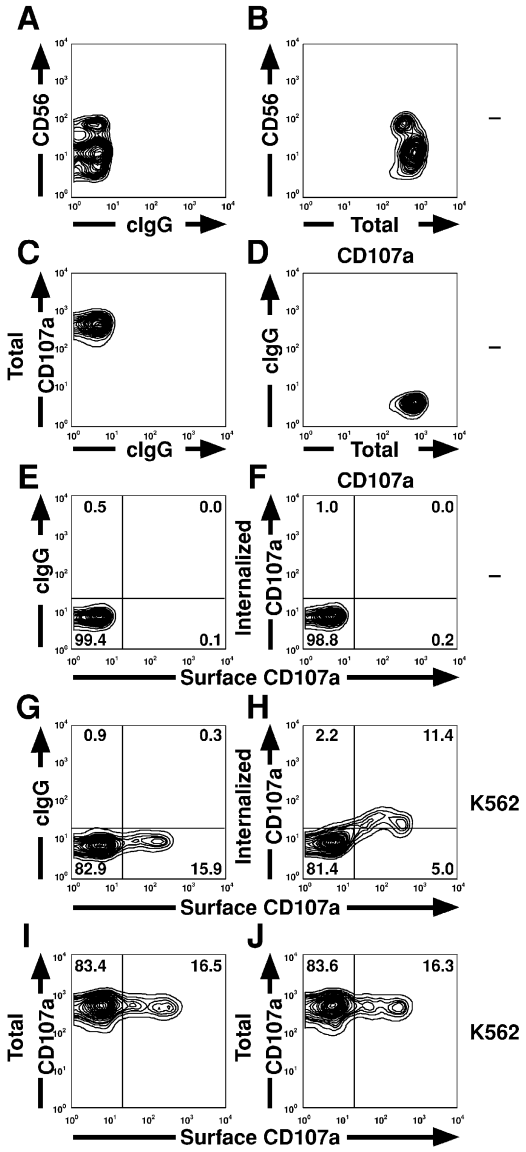


**Figure 4. CD107a at the surface of degranulated cells.** (A and B) Resting NK cells were stained with FITC-conjugated anti-CD56 mAb, fixed, permeabilized, and stained with isotype control (A) or anti-CD107a (B) mAbs. NK cells were gated by forward/side scatter gate. (C and D) Resting NK cells were stained with FITC-conjugated anti-CD56 mAb, fixed, permeabilized, and stained with biotinylated anti-CD107a mAb followed by streptavidin-APC (C) or streptavidin-PE (D). (E–J) Resting NK cells were incubated without (−) or with K562 cells, as indicated on the right. Resting NK cells were incubated for 2 h at 37°C. (F and H) Biotinylated anti-CD107a mAb was included during the incubation. Thereafter, cells were stained with FITC-conjugated anti-CD56 mAb and biotinylated anti-CD107a mAb followed by streptavidin-APC, fixed, permeabilized, and stained with streptavidin-PE (E–H) or biotinylated anti-CD107a mAb followed by streptavidin-PE (I and J). (J) NK cells were treated with cycloheximide. (C–J) Plots are gated on $CD56^{dim}$ NK cells. Numbers indicate the percentage of cells in each quadrant.

Second, to determine if detection of CD107a at the surface of a fraction of NK cells reflects the fact that few NK cells degranulate, or that CD107a internalization causes an

underestimate of degranulating NK cells, an assay was used to determine surface expressed and internalized CD107a in the same cells after the 2-h incubation. NK cells were incubated with biotinylated anti-CD107a during the assay, and were stained with streptavidin-allophycocyanin (APC) to reveal surface CD107a (x axis). This was followed by permeabilization and staining with streptavidin-PE to reveal internalized CD107a (y axis). Total intracellular CD107a produced comparable fluorescence intensities with streptavidin-APC or streptavidin-PE in $CD56^{dim}$ NK cells (Fig. 4, C and D). Resting NK cells alone did not express CD107a on the cell surface (Fig. 4, E and F). When incubated with anti-CD107a mAbs during the 2-h assay, no internalization of CD107a could be detected on resting NK cells alone (Fig. 4 F, top left quadrant). NK cells that were incubated with K562 cells displayed CD107a at the surface (Fig. 4, G and H), and most cells with surface CD107a had internalized some anti-CD107a mAbs (Fig. 4 H). In the course of the 2-h assay, very few cells appeared positive for internalized CD107a and negative for cell surface CD107a (Fig. 4 H, top left quadrant); this indicated that detectable levels of CD107a are present at the cell surface of cells that have degranulated, despite internalization. The percentage of NK cells with CD107a surface expression was not different between samples that had been stained with anti–CD107a mAbs after incubation with K562 cells, and those that were stained with mAbs during and after incubation with K562 cells.

Furthermore, only a fraction of the total lytic granule pool in resting NK cells is released during incubation with sensitive target cells, because the level of intracellular CD107a was not reduced detectably in those cells that had degranulated (Fig. 4 I). This was not due to rapid biogenesis of lytic granule components after activation, because preincubation of NK cells for 1 h with cycloheximide, an inhibitor of de novo protein synthesis, did not reduce intracellular CD107a levels in cells that displayed surface CD107a (Fig. 4 J). As observed in T cells (45), preincubation with cycloheximide did not inhibit NK cell degranulation (Fig. 4 J and not depicted). These results show that, although CD107a is internalized following degranulation, staining of cell surface CD107a after a 2-h incubation provides a good measure of NK cells that have undergone degranulation during the assay.

### Degranulation is induced by rabbit IgG but not ICAM on insect cells

IL-2–activated NK cells lyse *Drosophila* SC2 cells expressing ICAM-1 by an LFA-1–dependent mechanism (10). Furthermore, Fc fusion proteins of soluble ICAM-2 and -3, but not ICAM-1, induced perforin release in a subset of primary human NK cells that was pretreated with IL-2 (27). Therefore, insect SC2 cells expressing ICAM-1 or -2 were used to test for degranulation by resting NK cells. ICAM expression was higher on SC2–ICAM-1 cells than on SC2–ICAM-2 cells (Fig. S1). Resting NK cells formed conjugates with SC2–ICAM-1 and SC2–ICAM-2, but not with untransfected SC2 cells (9) (Fig. 5 A). However, when resting NK cells

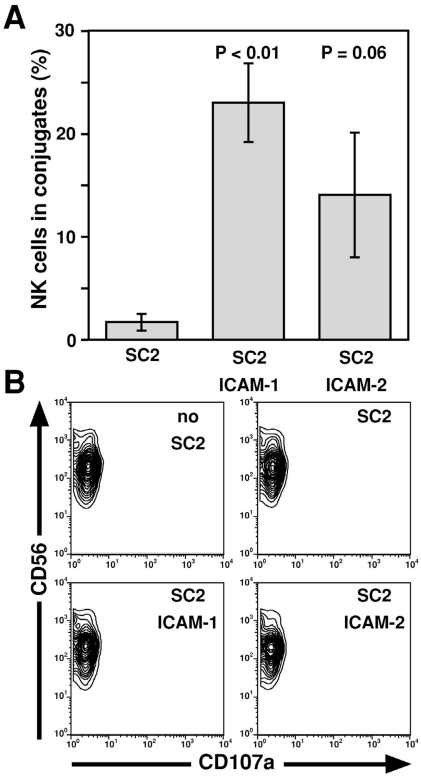


**Figure 5. ICAM on insect cells induce adhesion but not degranulation by resting NK cells.** (A) Resting NK cells labeled with a green dye were mixed with SC2 cells labeled with a red dye and incubated at 37°C for 10 min. Cells were fixed and the fraction of NK cells in two-color conjugates was determined by flow cytometry. Values represent the mean of three independent experiments. Bars represent the SD. (B) Resting NK cells were mixed with SC2–ICAM-1 or SC2–ICAM-2, and incubated at 37°C. After 2 h, cells were stained with fluorochrome-conjugated anti-CD56 and anti-CD107a antibodies. NK cells were gated on forward scatter/side scatter plots; the profiles show CD56 versus CD107a antibody staining. The profiles are representative of three or more independent experiments.

were incubated with SC2 or SC2–ICAM cells for 2 h at 37°C, no cell surface CD107a was induced (Fig. 5 B). This indicated that LFA-1 signaling is insufficient to induce degranulation by resting NK cells, and that signals for granule polarization and degranulation are uncoupled.

To evaluate if ligands of CD16 and 2B4 could induce degranulation, SC2 cells or SC2–CD48 cells, either preincubated with anti-SC2 rabbit serum or not, were incubated with resting NK cells for 2 h at 37°C (Fig. 6). Incubation of resting NK cells with IgG-coated SC2 cells was sufficient to induce surface expression of CD107a (Fig. 6, A and B). Surface CD107a was restricted to the $CD56^{dim}CD16^{+}$ NK cell subset (Fig. 6 A). These results showed that resting NK cells can be activated by rabbit IgG on SC2 cells, even in the absence of adhesion provided by LFA-1. SC2–CD48 cells did not induce surface expression of CD107a on resting NK cells (Fig. 6, A and B). Specific down-modulation of 2B4 surface expression after incubation of resting NK cells with SC2–CD48 cells (30–45% reduction in mean fluorescence inten-

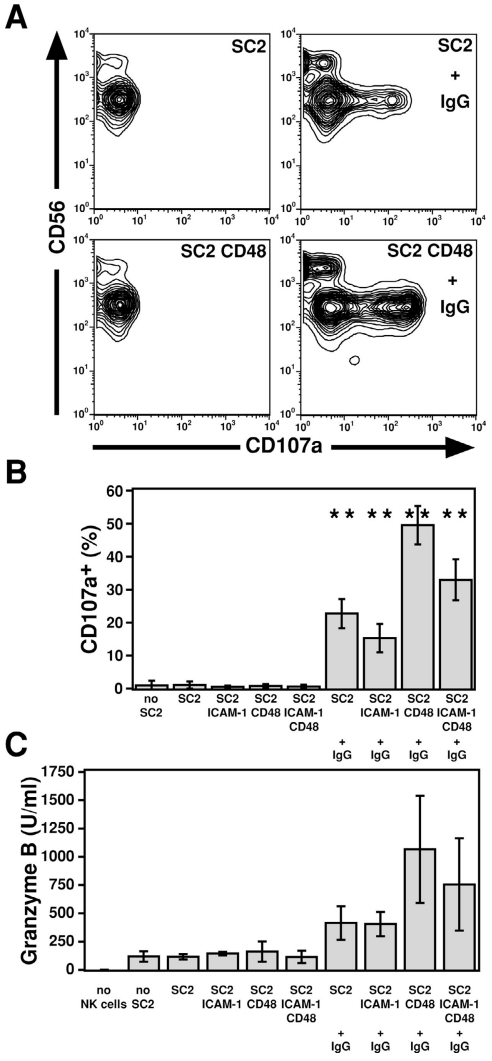


**Figure 6. CD16 engagement induces degranulation in resting NK cells.** (A–C) Resting NK cells were mixed with SC2, SC2–ICAM-1, SC2–CD48, or SC2–ICAM-1–CD48. Where indicated, SC2 cells were preincubated with a rabbit anti-SC2 serum (+IgG). Cells were incubated at 37°C for 2 h. (A, B) Thereafter, cells were stained with fluorochrome-conjugated anti-CD107a and anti-CD56 mAbs and analyzed by flow cytometry. (A) NK cells were gated on forward scatter/side scatter plots and profiles show CD56 versus CD107a mAb staining. (B) The percentage of $CD56^{dim}$ $CD107a^+$ NK cells is presented as the mean of six independent experiments. Bars indicate SD. ** $P < 0.005$ relative to NK cells incubated alone. (C) Granzyme B released in supernatants was measured by ELISA. Values represent mean ± SD of three independent experiments.

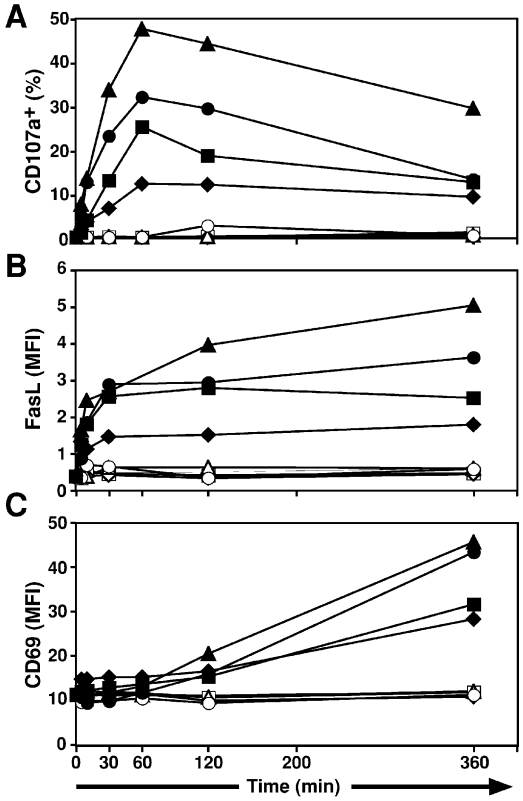


**Figure 7. Rapid coinduction of CD107a and Fas ligand at the surface of resting NK cells.** NK cells alone (line), or mixed with SC2 (open squares), SC2–ICAM-1 (open diamonds), SC2–CD48 (open triangles) or SC2–ICAM-1–CD48 (open circles) were incubated for 5, 10, 30, 60, and 120 min at 37°C. For Fc receptor stimulation, SC2 cells also were preincubated with a rabbit anti-SC2 serum (filled symbols). Cells were stained with fluorochrome-conjugated anti-CD56 and anti-CD107a mAbs (A), biotinylated anti-FasL mAb followed by fluorochrome-conjugated streptavidin (B), or fluorochrome-conjugated anti-CD69 mAb (C). The percentage of $CD56^{dim}CD107a^+$ NK cells (A), and the mean fluorescence intensity (MFI) of $CD56^{dim}$ NK cells for Fas ligand (B) and CD69 (C) is shown. Values represent the average of duplicate samples. A representative experiment is shown.

sity after anti-2B4 fluorescence–conjugated antibody relative to NK cells alone or NK cells mixed with SC2 cells; unpublished data) indicated that 2B4 does interact with CD48 on insect cells. SC2 cells expressing ICAM-1 and CD48 did not induce surface CD107a (Fig. 6 B), which indicated that a 2B4 signal, combined with LFA-1–dependent adhesion, is not sufficient to induce cytolytic granule exocytosis. CD16-triggered surface expression of CD107a clearly was augmented by coengagement of 2B4 with SC2–CD48 cells, but not by coengagement of LFA-1 with SC2–ICAM-1 cells (Fig. 6, A and B). Therefore, signals delivered by CD16 and by 2B4 engagement cooperate to enhance degranulation.

Granzyme B release was measured after culturing resting NK cells with SC2 cells for 2 h. Incubation of resting NK cells with SC2, SC2–ICAM-1, SC2–CD48, and SC2–ICAM-1–CD48 cells did not induce granzyme B release over spontaneous release (Fig. 6 C). In contrast, incubation with IgG-coated SC2 cells enhanced release by threefold over spontaneous release (Fig. 6 C). SC2–CD48 cells augmented the IgG-induced granzyme B release, whereas SC2–ICAM-1 cells did not (Fig. 6 C). Therefore, granzyme B release data confirmed those obtained with the degranulation assay.

The kinetics of degranulation by resting NK cells was analyzed. Cell surface expression of CD107a on resting NK cells was detected within 5 min of incubation with IgG-coated SC2 cells, and reached a maximum at 60 min (Fig. 7

A). Whereas the amplitude of degranulation varied among NK cells that were incubated with SC2 cells presenting different ligands (Fig. 7), the overall kinetics of degranulation were similar in all cases (Fig. 7 A). The rapid induction of CD107a expression at the cell surface correlated with that of Fas ligand (Fig. 7 B). In contrast, surface up-regulation of the lymphocyte activation marker, CD69, was detectable only 2 h after cell mixing (Fig. 7 C). Furthermore, cycloheximide, an inhibitor of de novo protein synthesis, had no effect on cell surface CD107a and FasL expression (Fig. 2 J and not depicted), but did abrogate CD69 up-regulation (not depicted). Therefore, resting NK cells degranulate rapidly, independently of new protein synthesis.

The co-stimulation of CD16 provided by recognition of CD48 by 2B4 was quantitated further, using titrations of rabbit IgG. Half-maximal degranulation, expressed as the percentage of NK cells with cell surface CD107a, was reached at a $\sim 2 \times 10^{-4}$ dilution of rabbit anti-SC2 serum (Fig. 8). Only $\sim 10^{-5}$ rabbit anti-SC2 serum was required to induce the same degree of degranulation with SC2–CD48 cells (Fig. 8). In repeated experiments, a 15-fold ($15 \pm 7.9$, $n = 6$ independent experiments) lower IgG concentration was required to induce the same level of degranulation with SC2–CD48 cells, as compared with SC2 cells. In addition, the frequency of degranulating $CD56^{dim}$ NK cells was higher at saturating IgG on SC2–CD48 cells as compared with SC2 cells ($81\% \pm 8.1\%$ and $61\% \pm 12\%$, respectively; $P < 0.001$; $n = 8$). To test if the increased degranulation frequency and the reduced activation threshold involved engagement of 2B4, a blocking anti-2B4 mAb was used (Fig. 8). Whereas the anti-2B4 mAb had no effect on degranulation induced by SC2 cells, it caused a reduction in the degranulation induced by SC2–CD48 cells; this implied that 2B4 contributes to the enhanced degranulation.

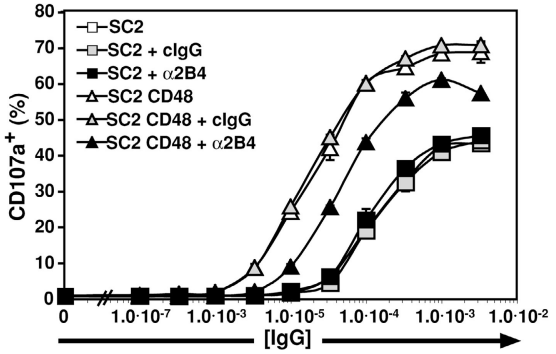


**Figure 8. 2B4–mediated signals lower the threshold for CD16-induced degranulation and increase the number of resting NK cells that degranulate.** NK cells were mixed with SC2 (open squares) or SC2-CD48 (open triangles) cells that had been preincubated with serial dilutions of a rabbit anti-SC2 serum. Cells were incubated at 37°C in the presence of no (empty), isotype control (gray shade), or anti-2B4 (filled) mAbs. After 2 h, cells were stained with fluorochrome conjugated anti-CD56 and anti-CD107a mAbs. The mean percentage of $CD56^{dim}CD107a^{+}$ NK cells of triplicate samples from one representative experiment is shown. Bars represent the SD.

## DISCUSSION

Cytolytic granule polarization and degranulation are two steps in NK cell–mediated cytotoxicity that are controlled separately by signals emanating from distinct receptors. Specifically, engagement of integrin LFA-1 by its ligand, ICAM-1, signals for polarization, whereas CD16 engagement by its ligand, IgG Fc, triggers degranulation. Neither polarization nor degranulation is sufficient for efficient target cell lysis; however, coengagement of CD16 and of LFA-1 results in strong cytotoxicity by resting NK cells. Furthermore, receptor 2B4 cooperates with CD16 to promote granule polarization and to enhance degranulation, such that cytotoxicity occurs in the absence of LFA-1–mediated adhesion. These conclusions were reached using normal, freshly isolated, resting NK cells in contact with insect cells expressing ligands of NK cell receptors, in the absence of antireceptor antibodies and cytokines. Variation was mainly quantitative, with the magnitude of responses differing between experiments and donors, whereas the relative responses induced by specific ligand combinations were remarkably consistent.

Engagement of LFA-1 by ICAM-1 is sufficient to induce cytolytic granule polarization in resting NK cells, as shown with purified ICAM-1–Fc fusion protein attached to beads. Granule polarization in NK cells is dependent on a signal controlled by the GTPase, Rac1 (46). The response of resting NK cells to autonomous signals from LFA-1 is a property that is not shared by T cells. Although a contribution by LFA-1 to granule polarization in T cells was described, polarization requires coengagement of the TCR (47, 48). Furthermore, LFA-1–dependent adhesion of T cells requires inside-out signals that can be delivered by the TCR, chemokine receptors, or other receptors (17, 21). In contrast, and in support of a unique signaling capacity of LFA-1 in NK cells, binding of resting NK cells to ICAM-1 is signal dependent, but is independent of inside-out signals from other receptors (9).

Granule polarization that was induced by LFA-1 binding to ICAM-1 and ICAM-2 was observed in IL-2–activated NK cells (10). Unlike resting NK cells, long-term IL-2–expanded NK cells kill insect cells expressing ICAM-1 (10). Perforin release that was induced by soluble Fc fusion proteins of ICAM-2 and -3, but not ICAM-1, was reported in a $CD8^{+}$ subset of human NK cells (27). Because the Fc portion was derived from human IgG1, it is not possible to exclude coengagement of CD16, which may explain the observed degranulation. We did not find evidence for degranulation induced by LFA-1 in freshly isolated, resting NK cells. Lysis of *Drosophila* SC2–ICAM-1 cells by IL-2–activated NK cells could be explained by the constitutive release of granzyme B, which is detectable in IL-2–activated NK cells (unpublished data). Combined with a polarization signal from LFA-1, this basal granzyme release may be sufficient to kill target cells.

In contrast to LFA-1, CD16 engagement, by its ligand, on insect cells is sufficient to induce rapid degranulation, as shown with a sensitive degranulation assay based on the dis-

play of the lysosomal protein CD107a (LAMP-1) at the cell surface. The signal from LFA-1 for polarization and the signal from CD16 for degranulation are separate, because LFA-1 provided no enhancement of degranulation, and CD16 provided only a small increase in polarization. The degranulation that was induced by CD16 engagement suggests that other ITAM-associated receptors, such as the natural cytotoxicity receptors NKp46, NKp44, and NKp30, also may be sufficient to induce degranulation. Because the human ligands for these receptors have not been identified, it is not possible to test the outcome of ligand engagement in transfected insect cells.

Cooperative signals from 2B4 and CD16 also can induce cytotoxicity by resting NK cells. Engagement of receptor 2B4 by CD48 on SC2 insect cells triggered no degranulation and little polarization. The lack of response is not due to a failure of 2B4 to engage its ligand on insect cells, because down-modulation of 2B4 was observed readily after a 2-h incubation of resting NK cells with SC2–CD48 cells. 2B4 is a strong coactivation receptor, because it greatly enhanced the degranulation that was induced by CD16, and provided good granule polarization when coengaged with CD16. A coactivation role of 2B4 in the NK-mediated lysis that was induced by ITAM-associated receptors was reported (30). The insect cell system used here goes further by showing a direct cooperation of 2B4 and CD16 signals in the absence of LFA-1–mediated adhesion. Because coengagement of CD16 and 2B4 by rabbit IgG and CD48 on SC2 cells resulted in weak conjugate formation, lysis of SC2 target cells by resting NK cells does not correlate with the strength of adhesion. Although adhesion through LFA-1 generally is required for efficient target cell lysis by NK cells (22, 24), our results show that LFA-1–independent cytotoxicity by NK cells can occur. However, the restricted expression of CD48 to hematopoietic cells imposes limits to such 2B4-dependent cytotoxicity. Conversely, ICAMs are expressed more widely (20, 49).

Only a fraction of resting NK cells undergoes degranulation, even when receiving maximal activation signals through CD16. For example, only ~15% of resting NK cells degranulated when incubated with K562 cells, even though efficient target cell lysis was observed. As shown recently, NK cells sorted for cell surface CD107a after incubation with K562 cells retained the ability to kill K562 targets, whereas the $CD107a^-$ subpopulation was not capable of lysis; this suggested that the $CD107a^-$ cells were incapable of degranulation (43). This could be explained by heterogeneity within the NK cell population in expression of activation receptors, or in activation thresholds for degranulation. Most NK cell activation receptors (e.g., NKp46, NKp30, DNAM-1, NKG2D, and 2B4) are expressed by most NK cells. Only ~60% of the resting $CD56^{dim}$ NK cell population underwent degranulation, even at a saturating dose of rabbit IgG on target cells, despite CD16 expression on all of these NK cells. Coactivation with 2B4 increased the frequency of degranulating NK cells to ~80%, which indicated that most cells are capable of degranulation if provided with strong signals. In addition, coengagement of 2B4 with CD16 lowered the threshold of activation, such that a 15-fold lower dose of rabbit IgG resulted in equivalent degranulation. Hyporesponsive NK cells may correspond to a recently described subset of mouse NK cells that lacks expression of inhibitory receptors for self, which nevertheless achieve tolerance through down-modulation of activation pathways (50).

Granzyme B release and cell surface appearance of Fas ligand correlated with the degranulation that was measured by surface staining of CD107a. Therefore, expression of surface Fas ligand seems to respond to the same signals as does degranulation, as previously reported with T cells (39). Supporting this notion, Fas ligand colocalizes with perforin and granzyme in an NK cell line (39).

A useful feature of the insect cell system used here is the possibility of studying NK cell activation in the absence or presence of inhibition that is mediated by MHC class I–specific receptors. The LFA-1–dependent cytotoxicity of IL-2–activated NK cell clones is inhibited by coengagement of LFA-1 with an inhibitory killer cell immunoglobulin-like receptor (KIR), upon binding to their respective ligands on insect cells, ICAM-1, and peptide-loaded HLA-C (10). Therefore, LFA-1–mediated signals are inhibited by KIR. Engagement of LFA-1 on NK cells by ICAM-1 on insect cells resulted in activation of the guanine exchange factor, Vav1, independently of actin polymerization (51). Vav proteins are central mediators of cytoskeletal reorganization through the activation of GTPases, Rac1 and Cdc42. Inhibition by KIR may be mediated by dephosphorylation of Vav1, which occurs independently of actin polymerization, as suggested by previous results (52). Therefore, signaling by LFA-1 and by inhibitory KIR are early events during NK–target cell interactions. Polarization of cytolytic granules at an early stage of conjugate formation may be a way to poise NK cells to kill by degranulation as soon as other activation receptors are engaged.

## MATERIALS AND METHODS

**Cells.** Human NK cell populations were isolated from peripheral blood by negative selection using an NK isolation kit (Miltenyi Biotec). Resting NK cells were resuspended in IMDM (Invitrogen) supplemented with 10% human serum (Valley Biomedical Inc.) and used within 1–4 d of isolation. These cells were 95–99% $CD3^-CD56^+$ as determined by flow cytometry. The human erythroleukemia cell line K562, and the mouse mastocytoma cell line P815 (both from American Type Culture Collection) were maintained in complete medium (RPMI 1640 supplemented with 2 mM L-glutamine and 10% FBS, all from Invitrogen). The transfection and maintenance of the *Drosophila* SC2 cell line has been described (9). Analysis by flow cytometry of human ligand expression on SC2 cells is discussed in supplemental Materials and methods (available at http://www.jem.org/cgi/content/full/jem.20051143/DC1).

**Antibodies.** mAbs used for staining of NK cells were anti-CD3 (UCHT1), anti-CD56 (NCAM 16.2), anti-CD69 (L78), anti-CD107a (H4A3), and anti-Fas ligand (CD178, NOK-1) (all from Becton Dickinson). For redirected antibody-dependent cellular cytotoxicity and blocking experiments, purified mouse IgG1 isotype control (MOPC-21, Sigma-Aldrich), anti-CD16 (3G8, Becton Dickinson), and anti-2B4 (CD244, C1.7, Beckman Coulter) mAbs were used. A rabbit serum was raised against

SC2 cells by repeated immunizations with SC2 cells and was used for stimulation of Fc receptors on NK cells. For granule polarization assays, cells were stained with an anti-perforin mAb (δG9, Pierce Chemical Co.) and revealed with an Alexa 568-conjugated goat anti–mouse IgG2b secondary antibody (Invitrogen).

**Cytotoxicity assays.** NK cell cytotoxicity toward SC2 cells was determined as described (10), with the following modification: SC2 cells were labeled with 4.0 μg/ml Cell Tracker Green (Invitrogen) for 30 min at room temperature. To induce ADCC, SC2 cells were preincubated with rabbit anti-SC2 serum diluted 1:10,000. Cytotoxicity assays with $^{51}$Cr-labeled K562 and P815 target cells were performed as described (53).

**Adhesion assay.** Conjugate formation between NK cells and SC2 cells was determined as described (9), with minor modifications. NK cells were labeled for 30 min with 1 μg/ml Cell Tracker Green CMFDA (Invitrogen). Likewise, SC2 cells were labeled with 4.0 μg/ml Cell Tracker Orange CMRA (Invitrogen). In some experiments, SC2 cells were subsequently added rabbit anti-SC2 serum to a final dilution of 1:10,000 and incubated for an additional 30 min at room temperature. The cells were washed three times. $10^5$ NK cells were mixed with $4 \times 10^5$ SC2 cells in 200 μl ice-cold HBSS (Biosource International) 5% FBS, incubated for the indicated times in a water bath at 37°C, vortexed, and added to 500 μl 0.6% paraformaldehyde in PBS. Cells were analyzed on a flow cytometer, and the fraction of NK cells in two-color conjugates was determined.

**Cytolytic granule polarization assay.** Polarization of perforin to SC2 cells or protein A beads coated with ICAM-1 Fc was examined as described (10). Full z-stacks of 40× fields were acquired. In subsequent analysis, conjugates between resting NK cells and insect cells were identified in the differential interference contrast image. Three-dimensional reconstructions of the images were used with the confocal z-series to determine whether the perforin-containing granules had polarized toward the target cells. The percent of NK cells in conjugates showing perforin polarized to the SC2 cells was determined for each individual transfectant. The percentages were normalized to the percentage polarization seen with untransfected SC2 cells.

**Mutation of human ICAM-1–Fc.** The sequences encoding the extracellular domains of human ICAM-1 were amplified by PCR and cloned into NheI, BamHI digested Cd5lneg1 (54), in frame with the CD5 leader peptide and the hinge, CH2, and CH3 regions of human IgG1. To exclude the possibility of CD16 binding to the recombinant ICAM-1–Fc, two mutations were introduced in the sequence corresponding to the Fc receptor-binding site on human IgG1. Leucine 235 and glycine 236 of the human IgG1 sequence near the junction of the hinge and CH2 domains of IgG1 (37) were mutated to glycine and leucine, respectively. The mutant was generated by QuikChange mutagenesis (Stratagene) with primers 5′-CCTCAGCACCTGAACTCGGGCTGGGACCGTCAGTCTTCC-3′ and 5′-GGAAGACTGACGGTCCCAGCCCGAGTTCAGGTGCTGAGG-3′.

**CD107a, Fas ligand, and CD69 staining.** For degranulation assays quantifying cell surface CD107a expression, $2 \times 10^5$ resting NK cells were washed twice in PBS and added to $10^6$ SC2 cells (or $4 \times 10^5$ K562 or P815 cells) in 200 μl complete medium. Cells were mixed by gentle pipetting, spun down for 3 min at 300 rpm, and incubated for 2 h at 37°C in 5% $CO_2$. Thereafter, the cells were spun down, stained with fluorochrome-conjugated anti-CD56 and anti-CD107a mAbs in PBS 2% FBS, and were added to 5 mM EDTA for 45 min on ice. The cells were washed, resuspended in PBS 2% FBS, and analyzed by flow cytometry (FACSCalibur, Becton Dickinson). In time-course experiments, cells were mixed as above; spun down at indicated times; and stained with fluorochrome-conjugated anti-CD56, anti-CD107a, and anti-CD69 mAbs. In assays staining for Fas ligand, the metalloprotease inhibitor KBR-8301 (a gift from K. Yoshino, Carna Bioscience, Kobe, Japan) was added at 10 μM during the assay, followed by consecutive staining with biotinylated anti-Fas ligand mAb and APC-conjugated streptavidin (Becton Dickinson). For intracellular staining, cells were stained with FITC-conjugated anti-CD56 and biotinylated anti-CD107a mAbs after the assay as described before, or added to biotinylated anti-CD107a mAbs during the 2-h incubation at 37°C as well. To determine cell surface expression of CD107a, cells were washed, resuspended in PBS 2% FBS, added to APC-conjugated streptavidin, and incubated for 45 min on ice. Thereafter, the cells were washed and fixed with 4% paraformaldehyde (Electron Microscopy Sciences) in PBS for 20 min at room temperature. The cells were washed and permeabilized in PBS 2% FBS and added to 0.5% saponin (Sigma-Aldrich) for 10 min on ice. To monitor endocytosed CD107a, cells were added to PE-conjugated streptavidin (GE Healthcare). Alternatively, to stain for total intracellular CD107a, streptavidin bound to anti-CD107a on the cell surface was blocked with 1 mM D-biotin (Invitrogen); the cells were washed and added to biotinylated anti-CD107a mAb, washed again, and finally incubated with PE-conjugated streptavidin. Cells were resuspended in PBS 2% FBS and analyzed by flow cytometry. Data were analyzed and plots were created with FlowJo software (Treestar Inc.). For blocking experiments with mAbs, NK cells were preincubated for 30 min with 10 μg/ml mAbs, mixed, and incubated with SC2 cells in medium with 10 μg/ml mAbs.

**Granzyme B ELISA.** For measurements of granzyme B release, $2 \times 10^5$ resting NK cells were washed twice in PBS and added to $10^6$ SC2 cells (or $4 \times 10^5$ K562 or P815 cells) in 200 μl complete medium. The cells were incubated for 2 h at 37°C in 5% $CO_2$. Thereafter, cells were spun down, and supernatants were aspirated and stored at −20°C. Granzyme B released into supernatants was quantified by ELISA (Sanquin).

**Statistics.** Statistical analysis was performed using paired Student's *t* test.

**Online supplemental material.** Fig. S1 depicts flow cytometric characterization of human ligand expression on transfected SC2 cells. Analysis of human ligand expression on SC2 cells can be found in supplemental Materials and methods. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20051143/DC1.

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