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Lytic Granule Polarization, Rather Than Degranulation, Is the Preferred Target of Inhibitory Receptors in NK Cells

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Natural cytotoxicity is achieved by polarized release of perforin and granzymes at the NK cell–target cell immunological synapse. Signals for granule polarization and degranulation can be uncoupled in NK cells, which raises the question of their respective sensitivity to inhibitory receptors. Expression of either HLA-C or HLA-E on the human cell line 721.221 blocked granule polarization, degranulation, and CD16-dependent MIP-1α secretion by NK cell clones that expressed inhibitory receptors of matching HLA specificity. To test inhibition of signals for polarization and degranulation separately, Drosophila S2 cells expressing ICAM-1 with either HLA-C or HLA-E were used. CD16-dependent degranulation and MIP-1α secretion were not fully inhibited, suggesting that other receptor–ligand interactions, which occur with 721.221 cells, contribute to inhibition. In contrast, HLA-C or HLA-E on S2 cells coexpressing ICAM-1 or ULBP1 were sufficient to block granule polarization induced by either LFA-1 or NKG2D, even during concomitant CD16-dependent degranulation. Similarly, expression of a ligand for NKR-P1A on S2 cells inhibited granule polarization but not CD16-induced degranulation. Therefore, granule polarization, rather than degranulation, is the preferred target of inhibitory receptors in NK cells. The Journal of Immunology, 2010, 185: 4698–4704.

Materials and Methods

Cells and Abs

Human NK cells were isolated and cloned as described (12) and screened for expression of CD155a, CD155b, and CD159a by flow cytometry. The HLA class I-negative cell line 721.221 (13), referred to as 221 cells, and 221-Cw3, 221-Cw15, and 221-E (0.221-AEH, a gift of D. Geraghty, Fred Hutchinson Cancer Research Center, Seattle, WA) cells were cultured as described (14). Drosophila S2 cells were transfected with the insect expression vector pAc5.1 (Invitrogen, Carlsbad, CA) along with the vector pNeoFly as described (15). Full-length lectin-like transcript 1 (LLT1) cDNA in pac5.1 was a gift of Y. Bryceson (Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, Rockville, MD). HLA class I on S2 cells were loaded with specific peptides (GAVDPLLKL for HLA-Cw3, QYDDAVYKL for HLA-Cw4, and VMAPRTLIL for HLA-E). Abs used were CD155a (EB6), CD155b (GL183), and CD159a (Z199) (Immunotech, Beckman Coulter, Miami, FL) for screening of NK clones, rabbit anti-S2 cell serum for stimulation through CD16 (7), anti-perforin for detecting perforin polarization (7), mAb F4/326 (anti–HLA-C; a gift of S.Y. Yang, Histogenetics, New York, NY), anti–HLA-E mAbs MEM-E06 and MEM-E07 (Exbio Antibodies, Vestec, Czech Republic), anti-CD54 (BD Pharmingen, San Diego, CA), anti-ULBP1 (R&D Systems, Minneapolis, MN), and anti-flag Ab, M2 (Sigma Aldrich, St. Louis, MO), for detecting expression of flag-tagged LLT1. To engage CD16 on NK cells, S2 cells were incubated with rabbit anti-S2 cell serum at a 1 × 10−5 dilution (unless indicated otherwise), and 221 cells were incubated with anti–HLA-DR IgG2a mAb L243 at 1 μg/ml.

Cytotoxicity, granule polarization, ELISPOT, and ELISA

NK cell cytotoxicity toward 221 cells was determined by a europium assay (2). Polarization of perforin-containing granules was determined as de-
scribed (7). Conjugates with polarized perforin were scored visually from three-dimensional confocal z-stacks. Perforin-containing granules were considered polarized when most of the fluorescence was concentrated in the lower quadrant of the NK cell (i.e., the quadrant that is closest to the target cell). The distinction between polarized and unpolarized granules was obvious in most cases. ELISPOT assays were set up to count the number of NK cells that secreted granzyme B (GrzB) above a set threshold level, as described (16). Effector and target cell suspensions were applied in triplicate at a 1:1 ratio and incubated at 37˚C for 3 h. Spots were enumerated with an ELISPOT plate reader (CTL Immunospot, Shaker Heights, OH). The number of ELISPOTs obtained with NK cells alone was subtracted from the experimental values. Cells (2 × 10^5 NK and target cells; 1:1) were incubated overnight at 37˚C. MIP-1α released was determined by ELISA (R&D Systems).

**Results**

**Inhibitory receptors for HLA-C and HLA-E block granule polarization and granzyme release induced by 221,221 cells**

A large panel of NK cell clones was assembled from peripheral blood of several individuals. To test for inhibition through KIR or CD94-NKG2A receptors, the HLA class I-negative human cell line 721,221 (referred to as 221 cells) transfected with HLA-Cw3 (221-Cw3), HLA-Cw15 (221-Cw15), or HLA-E (221-E) was used (Fig. 1A). CD158a is a ligand for the CD158b inhibitory receptors KIR2DL2 and KIR2DL3. HLA-Cw15 is a ligand for the CD158a inhibitory receptor KIR2DL1. HLA-E is a ligand for the inhibitory receptor CD94-NKG2A. The inhibitory function of receptors on NK cell clones was first established in cytotoxicity assays with transfected 221 cells (Fig. 1B). This was necessary because most Abs do not distinguish between inhibitory KIR2DL1 (CD158a) and activating KIR2DS1 (CD158b) or between inhibitory KIR2DL2/3 (CD158b) and activating KIR2DS2 (CD158b). Also, HLA-E binds to both inhibitory CD94-NKG2A and activating CD94-NKG2C receptors. A typical lysis assay with a clone coexpressing CD158b and NKG2A, consistent with GL183 and Z199 mAb staining (not shown). Every NK clone used in this study underwent such functional screening for expression of inhibitory receptors.

To test whether inhibitory receptors can block granule polarization, conjugates of NK cell clones with 221-Cw3 and 221-E cells were fixed, stained for intracellular perforin, and analyzed by three-dimensional imaging of confocal z-stacks. Perforin-containing granules in a CD158a-b⁺NKG2A⁺ NK cell clone polarized toward 221 target cells but not when mixed with either 221-Cw3 or 221-E resistant cells (Fig. 1C, 1D). As control, polarization was scored for NK cells in contact with the insect cell line S2 (Fig. 1D). In this and in many other experiments, granules appeared to be polarized toward S2 cells in 20–30% of NK cell contacts. As granules are often polarized to one side of NK cells, independently of any particular stimulus, one would expect random contacts with S2 cells to give the appearance of polarization in a fair proportion of NK cells. Nevertheless, the distinction between active granule polarization (>60% of conjugates) and inhibition of granule polarization (apparent polarization in 20–30% of conjugates, which is equivalent to the S2 cell control) was always clear. Inhibition of granule polarization was also observed with other NK clones and with the cell line NK92 transfected with inhibitory receptor KIR2DL1 (data not shown). Therefore, we conclude that inhibitory signals delivered by KIR and by CD94-NKG2A block the movement of lytic granules toward the NK cell immunological synapse.

To determine whether inhibition of target cell lysis was also achieved by a block in the fusion of lytic granules with the plasma membrane, a degranulation assay was developed. The release of GrzB by NK clones was measured by an ELISPOT assay. A large panel of NK cell clones expressing either CD158a, CD158b, or both were tested for their ability to release GrzB during mixing with either 221 cells, 221-Cw3 cells, or 221-Cw15 cells (Fig. 2A). The data are presented as the number of ELISPOTs relative to the number of spots obtained with 221 cells (i.e., in the absence of inhibition), and each data point represents a different NK clone. Degranulation was clearly inhibited whenever a specific KIR–HLA-C receptor–ligand combination was present, although the extent of inhibition varied among clones that shared a KIR phenotype. In addition, partial but significant inhibition was observed with CD158a-b⁺NKG2A⁺ clones mixed with 221-Cw15 cells (p = 0.006), consistent with the reported cross-reactivity of KIR2DL2 with group 2 HLA-C allotypes, such as HLA-Cw15 (17, 18). This cross-reactivity is also consistent with the more complete inhibition of double-positive CD158a-b⁺ clones by 221-Cw15 cells compared with inhibition of CD158a-b⁺ clones (p = 0.02).

The ability of NKG2A to inhibit GrzB release was tested using 221-E target cells (Fig. 2B). Inhibition was observed with every clone expressing NKG2A. Individual variability in the extent of inhibition was again observed among different clones. NK cell clones that do not express CD94-NKG2A are uncommon. Only four such clones were tested. As expected, no inhibition with 221-E target cells was observed (Fig. 2B). Therefore, engagement of inhibitory receptors by HLA class I on 221 cells resulted in inhibition of both granule polarization and GrzB release.

![Figure 1](http://www.jimmunol.org/)
GrzB and MIP-1α release induced by CD16 is not blocked by inhibitory receptor ligands on insect cells

It is not known how the many receptor–ligand interactions that occur during NK-221 cell contact contribute to the inhibition mediated by HLA class I-specific inhibitory receptors. To address this question, we used the *Drosophila* cell line S2 transfected with ligands of human NK cell receptors, including HLA class I ligands of inhibitory receptors. Stimulation through CD16 was achieved using a rabbit antiserum to S2 cells. Furthermore, S2 cells can be used to separate signals for polarization, through LFA-1, from signals for degranulation, through CD16 (7). Addition of increasing amounts of rabbit antiserum to S2 cells resulted in a small increase of GrzB release, relative to the level of GrzB released spontaneously by IL-2–activated NK cells (Fig. 3A). However, GrzB release was strongly increased when rabbit antiserum was added to S2 cells expressing ICAM-1 (S2–ICAM-1) (Fig. 3B). Half-maximal GrzB release occurred at an ~1 × 10^{-5} dilution of anti-S2 rabbit antiserum (Fig. 3A). Engagement of CD16 by rabbit IgG on S2 cells resulted also in the release of MIP-1α (Fig. 3B). Unlike GrzB release, MIP-1α secretion did not show a pronounced dependence on ICAM-1. MIP-1α release assays were preferred over IFN-γ and TNF-α secretion because IL-2–activated NK cells release a high basal level of IFN-γ and TNF-α.

To test for inhibition of GrzB and MIP-1α release, S2 cells were cotransfected with ICAM-1, β2–microglobulin, and either HLA-Cw3 (S2–ICAM-1+Cw3 cells) or HLA-E (S2–ICAM-1+E cells) (Fig. 4A). These cells were either loaded or not loaded with peptides that bound specifically to either HLA-Cw3 or HLA-E to test for inhibition of GrzB and MIP-1α release. As class I is transported “empty” to the surface of *Drosophila* cells, it cannot be recognized by inhibitory receptors unless loaded with exogenous peptide (10, 19). The inhibition induced by HLA class I on S2 cells was tested using the same cells, either before or after loading with specific peptides. With this approach, S2 cells provide a more rigorous test of HLA class I–dependent inhibition than that by use of independent transfected cells that do or do not express an HLA ligand for inhibitory receptors.

In a panel of 23 NK clones that expressed inhibitory CD158b, most of the clones showed no inhibition of GrzB release during mixing with S2–ICAM-1+Cw3 cells that had been loaded with an HLA-Cw3–specific peptide and incubated with rabbit antiserum at a dilution of 1 × 10^{-4} (Fig. 4B). Partial inhibition, which was beyond the SD, was observed with only three clones. More pronounced but still partial inhibition was observed with CD94-NKG2A+ NK cells during incubation with S2–ICAM-1+E cells loaded with an HLA-E–specific peptide (Fig. 4B). These results suggested that inhibition of NK cell clones by peptide-loaded HLA class I on insect cells was not as efficient as inhibition by HLA class I on human 221 cells. One possibility we considered was that stimulation of NK cells by IgG Fc on S2–ICAM-1 cells was stronger than stimulation by 221 cells and may thus have overridden inhibitory signals. To compare directly the relative strengths of signals, GrzB release was measured with bulk cultures of NK cells, from three unrelated donors, after mixing with 221 cells, S2–ICAM-1 cells, and S2–ICAM-1 cells coated with rabbit IgG. The number of GrzB ELISPOTs obtained was 675 ± 81 with 221, 117 ± 50 with S2–ICAM-1, and 553 ± 62 with S2–ICAM-1+IgG. Therefore, stimulation of GrzB release was not greater with S2–ICAM-1+IgG Fc than with 221 cells. We also tested whether peptide-loaded HLA-Cw3 on S2 cells would inhibit weaker CD16 activation signals by measuring inhibition over a broad range of anti-S2 cell serum concentrations. No inhibition of degranulation was observed at any of the dilutions tested (Fig. 4C).

As another readout for inhibition, CD16–induced secretion of MIP-1α was evaluated. MIP-1α release induced by rabbit IgG on S2–ICAM-1+Cw3 or S2–ICAM-1+E cells was not inhibited by...
peptide-loaded HLA-Cw3 or HLA-E, as shown with CD158a–b+NKG2A+ NK clones (Fig. 5A,5B). We conclude that CD16 signals for chemokine secretion are not inhibited by interaction of inhibitory receptors with peptide-loaded MHC class I molecules on insect cells.

The difference between inhibition by HLA class I on 221 and S2 cells may be due to the different activation signals used. Natural ligands on 221 cells activate natural cytotoxicity, whereas S2 cells coated with rabbit IgG activate NK cells via CD16. To test whether activation of NK cells specifically through CD16 was less sensitive to inhibition than activation through receptor–ligand interactions that promote natural cytotoxicity toward 221 cells, we took advantage of our observation that strong MIP-1α secretion was dependent on CD16 signals, as shown with IgG2a-coated 221 cells (Fig. 5C). Despite this CD16-dependent enhancement, MIP-1α secretion by a CD158a NK clone was completely blocked by HLA-Cw15 (Fig. 5C). Similar results were obtained with 221-Cw7 cells and a CD158b NK clone (Fig. 5D). Therefore, the reduced inhibition observed with Drosophila S2 cells compared with that of human 221 cells cannot be explained simply by activation through CD16.

Granule polarization toward ICAM-1–expressing insect cells is blocked by NK cell inhibitory receptors

Although GrzB release was not inhibited by HLA class I on S2 cells (Fig. 4), the resistance of S2–HLA-C cells from lysis by KIR+ NK clones (10) suggests that inhibition occurs at some other activation step. Indeed, analysis of NK cell clones bound to peptide-loaded S2–ICAM-1+Cw3 cells showed complete inhibition of granule polarization (Fig. 6A,6B). Likewise, polarization was completely inhibited in CD94-NKG2A+ NK cells mixed with peptide-loaded S2–ICAM-1+E cells (Fig. 6A, 6B). Inhibition of polarization was also observed with a CD158a+b–NKG2A+ clone incubated with...
S2–ICAM-1+Cw4 cells and with a CD158a–b+NKG2A+ clone and a CD158a–b–NKG2A+ clone incubated with S2–ICAM-1+E cells (data not shown). These results confirm that peptide-loaded HLA class I on Drosophila S2 cells is competent to induce functional inhibition of NK cells. We wished to test whether inhibition of polarization would still occur in the presence of strong CD16-mediated activation signals. We therefore tested whether granule polarization could still be inhibited while degranulation occurred by analyzing granule polarization in NK cells mixed with S2–ICAM-1+Cw3 or S2–ICAM-1+E cells that had been coated with rabbit IgG. Polarization was completely inhibited in a CD158a+b+NKG2A+ clone during incubation with IgG-coated S2–ICAM-1 cells expressing either HLA-Cw3 or HLA-E (Fig. 6B). These re-
sults demonstrate that granule polarization is preferentially in-
hibited over degranulation and that degranulation can still occur when polarization of the bulk of lytic granules is inhibited.

We then asked whether inhibitory receptors other than KIR and CD94-NKG2A would also block granule polarization. S2–ICAM-1+LLT1 cells were cotransfected with LLT1, a ligand for inhibitory receptor NKR-P1A (CD161) (20, 21). Because NKR-P1A is expressed on most NK cells, it was not necessary to isolate NK clones to test for inhibition. IL-2–activated NK cells were mixed with S2 cells expressing either ICAM-1 or ICAM-1 and ligands for inhibitory KIR and CD94-NKG2A, CD16-induced degranulation was not inhibited by ICAM-1 and LLT1 coexpression (Fig. 7B). We conclude that granule polarization is the preferred target for inhibition by different inhibitory receptors. Expression of ULBP1 on S2 cells is sufficient to induce granule polarization, which is blocked by inhibitory KIR

S2 cells expressing ULBP1, which is a ligand for activation receptor NKG2D, triggered polarization of perforin-containing granules in IL-2–activated NK cells (Fig. 8). We therefore tested whether NKG2D-dependent granule polarization was sensitive to inhibition, just as LFA-1–dependent polarization was. S2 cells coexpressing ULBP1 and HLA-Cw4 (S2–ULBP1+Cw4) were mixed with a CD158a+b–NKG2A+ clone. Addition of an HLA-Cw4–specific peptide completely inhibited polarization (Fig. 8A, 8B). As had been observed with S2–ICAM-1 cells, coengagement of CD16 by addition of antibody IgG to S2 cells did not overcome inhibition (Fig. 8B). Therefore, inhibitory receptors are not re-
sticted to inhibition of LFA-1 signals but can also prevent NKG2D-dependent signals for polarization.

We observed that ULBP1 expression on S2 cells was also a strong costimulator of CD16-dependent degranulation (Fig. 8C), similar to results obtained with S2–ICAM-1 cells (Fig. 3). This gave us an opportunity to test inhibition of degranulation induced by the combination of CD16 with NKG2D. A CD158a+b–NKG2A+ NK clone was mixed with S2–ULBP1+Cw4 cells coated with rabbit antiserum in the presence and absence of HLA-Cw4–specific peptide. No inhibition of degranulation was observed (Fig. 8C). These results confirm, using a different combination of activation signals, that granule polarization is the preferred target for inhibition by inhibitory receptors.
Discussion

We examined the sensitivity of granule polarization and degranulation by NK cells to inhibition by ITIM-containing receptors. Polarization of granules was always inhibited, irrespective of the inhibitory receptor tested or the cell on which the receptor ligand was expressed. In contrast, inhibition of degranulation, as measured by GrzB release, and of MIP-1α secretion was variable and de-
gregalation separately. To study HLA class I specificities on human 221 cells.

**FIGURE 8.** Polarization induced by NK2G2 signals is sensitive to in-
hibition by KIR2DL1 binding to peptide-loaded HLA-C on S2 cells. A. CD158a+b-NKG2A+ NK clone in contact with S2-ULBP1+Cw4 cells loaded with (+) or without (No) peptide, stained for perforin with an mAb

Factors that may contribute to the formation of a strong inhibitory
NK cell immunological synapse have not been defined yet. The complete inhibition of degranulation induced by HLA class I on 221 cells, but not on S2 cells, suggests that other receptor–ligand interactions between NK cells and 221 cells may contribute to inhibition. For example, inhibition of degranulation may require the additive effect of other ITIM-containing receptors, such as leukocyte-associated Ig-like receptor-1 and NKR-P1A. The human cell line 221 expresses LLT1 (Y. Bryceson, unpublished data), which is a ligand of NKR-P1A. We have found that expression of LLT1 on S2 cells is sufficient to block the granule polarization induced by ICAM-1. The *Drosophila* S2 cell system is well suited to determine the minimal requirements for inhibition, just as it has been to define requirements for activation of NK cells (7). It is worth noting that inhibitory KIR promotes clustering of activation

As shown with resting NK cells, CD16-induced degranulation can occur in the absence of detectable granule polarization (7). Likewise, we show here that GrzB release can persist during inhibition of granule polarization. Such unpolarized degranulation may involve fusion of lytic granules that are predocked at the plasma membrane. Even in the absence of stimulation, lytic granules in primary, resting NK cells can be observed at the plasma membrane by microscopy with live cells (25). The spontaneous release of granzymes by IL-2–activated NK cells may be

Inhibition of degranulation by HLA-C and HLA-E on 221 cells varied among NK clones from very good to incomplete. However, peptide-loaded HLA-C or HLA-E on S2 cells provided partial to very weak inhibition of degranulation. This difference in the inhibitory capacity of human 221 cells versus insect S2 cells was not due simply to the strength of activation signals. Inhibition of the degranulation triggered by 221 cells was not overcome by additional ADCC signals through CD16. Likewise, inhibition of the polarization induced by S2 cells was not overcome by CD16 signals. These data are consistent with the view that activation-inhibition signals are not simply in balance and that the outcome depends on an integration of signals, in which inhibitory signals dominate (1). It is possible that HLA class I expressed on mammalian cells provides stronger inhibition of NK cells than the inhibition conferred by peptide-loaded HLA-C and HLA-E on S2 cells. However, peptide-loaded HLA-C and HLA-E on S2 cells were sufficient to induce strong inhibition of granule polarization.

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which leads to synergistic NK cell activation through receptors NKG2D and 2B4. Inhibition of polarization in resting NK cells could not be investigated, as cloning of NK cells is required to isolate cells with defined inhibitory receptor expression.

The signals that control lytic granule polarization in NK cells are not well known, although specific components of the pathway have been identified (26–28). Several receptors in NK cells can contribute to cytolytic granule polarization and cytotoxicity (10, 29, 30). Using insect cells transfected with individual ligands of NK cell receptors, we have shown that engagement of LFA-1 by ICAM-1 alone is sufficient to induce granule polarization (7, 10). However, in mouse NK cells, ICAM-1 on beads induces actin cytoskeleton remodeling, and coligation with activation receptor NKG2D is required to induce granule polarization (30). We report here that expression of ULBP1 on S2 cells is sufficient to induce granule polarization in IL-2–activated human NK cells. Therefore, neither LFA-1 nor NKG2D is necessary, but each one is sufficient to induce polarization of granules in human NK cells, and in both cases polarization is sensitive to inhibition. It will be interesting to investigate how signaling by these two very different receptors converge to induce granule polarization.

The main conclusion is that inhibitory receptors are better equipped to stop granule polarization than to block GrzB and chemokine release. This was shown definitively by observing sustained GrzB and MIP-1α release by NK cells in which granule polarization was inhibited. The evidence obtained for inhibition of NK cells by HLA class I on Drosophila S2 cells is conclusive, because inhibition is dependent on addition of HLA class I-specific peptides. Persistent degranulation during inhibition of polarization in IL-2–activated NK cells suggests that it may occur in high inflammatory conditions. Prevention of NK cell cytotoxicity would be better achieved through inhibition of degranulation rather than polarization. However, the possibility of releasing the block in degranulation while maintaining inhibition of polarization endows NK cells with the potential to provide bystander killing while still refraining from direct attacks on MHC class I-positive cells.

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Disclosures

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