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Killer Ig-Like Receptor Ligand Mismatch Directs NK Cell Expansion In Vitro

Mingus J. J. Rose,*‡ Andrew G. Brooks,* Lisbeth A. Stewart, † Thi H. Nguyen, †‡ and Anthony P. Schwarer1†‡

NK cell alloreactivity is governed largely through failure to detect self-HLA class I ligands by the clonally distributed inhibitory killer Ig-like receptors (KIR) expressed on the NK cell surface. In this study, we investigated the extent to which HLA class I-KIR interactions influence human NK cell proliferation in the allogeneic setting. NK cells were cultured with feeder cells either matched or mismatched for inhibitory KIR ligands, the latter lacking one or more ligands present in the NK cell donor. In postculture cytotoxicity assays, the ability of polyclonal NK cells to kill KIR ligand-mismatched targets was enhanced by exposure to appropriately mismatched feeder cells in prior culture. This corresponded with an increased frequency of postculture donor NK cells expressing a given inhibitory KIR if the allogeneic feeder cells used in the culture lacked its ligand. Similar skewing of KIR distribution was seen in clonally expanded NK cells. Finally, a flow cytometry-based proliferation assay was used to show KIR-specific NK cell division in response to missing self. The findings demonstrate that KIR distribution among a population of alloresponding peripheral blood NK cells is shaped by the HLA class I environment. The Journal of Immunology, 2009, 183: 4502–4508.

Natural killer cells are lymphocytes of the innate immune system that are able to recognize and kill tumor, virus-infected, and allogeneic cells without prior activation (1, 2). Target selection in NK cells depends on the balance between competing activatory and inhibitory signals to the cell triggered by the binding of germline-encoded surface receptors to their ligands on the potential target (3). Normal autologous cells are spared from NK cell-mediated lysis due to recognition of self-HLA class I ligands by inhibitory receptors including CD94/NKG2A and inhibitory killer Ig-like receptor (KIR).2

HLA-specific NK cell inhibitory receptors recognize broad groups of HLA class I molecules rather than having single allelic restrictions. Potential targets are surveyed for overall HLA class I expression through the lectin-like receptor CD94/NKG2A and its recognition of the ubiquitously expressed nonclassical HLA-class I molecule HLA-E. The inhibitory KIR allow for more subtle immunosurveillance through recognition of epitopes shared by subgroups of HLA-A, HLA-B, and HLA-C allotypes. HLA-A3 and HLA-A11 are both recognized by the NK cell inhibitory receptor KIR3DL2. HLA-B allotypes containing the Bw4 public epitope are ligands for KIR3DL1. Finally, HLA-C molecules with a lysine residue at position 80 in their amino acid sequence, classed as group 2 HLA-C (HLA-C2) allotypes are recognized by KIR2DL1, whereas the reciprocal HLA-C molecules with an asparagine residue at position 80, classed as group 1 HLA-C (HLA-C1) allotypes are ligands for KIR2DL2 and KIR2DL3. Of these, the KIR ligands associated with the HLA-B and HLA-C loci in particular have been shown to be strong determinants of NK cell alloreactivity (4, 5).

Donor-vs-recipient NK cell alloreactivity in partially mismatched allogeneic hematopoietic stem cell transplantation (allo-HSCT) has been demonstrated to occur when HLA-B and/or HLA-C allele mismatch translates to a KIR ligand being present in the donor but absent in the recipient (KIR ligand mismatch) (6). The presence of such KIR ligand mismatches has been shown to correlate with NK cell-mediated graft-vs-leukemia, decreased relapse, improved engraftment, and improved overall survival in patients receiving haploidentical or partially mismatched unrelated HSCT for treatment of acute myeloid leukemia (7–9). Alternatively, others have contended that a KIR ligand mismatch is not essential in allo-HSCT for the beneficial NK cell alloreactivity to occur, but rather the pairing of KIR ligand absence in the recipient with the presence of the cognate inhibitory receptor in the donor’s KIR genotype is needed (the receptor/ligand model) (10, 11).

NK-based immunotherapy using mature NK cells from a mismatched donor has been demonstrated to be safe (12) and may be useful for pre-HSCT conditioning (13) as well as for treating post-HSCT relapse (12) and even treatment of some solid tumors (14). Although the KIR repertoire has been examined in the population of NK cells emerging from donor CD34+ cells after HSCT (15–17), less is known about how the KIR repertoire of mature donor NK cells would change following infusion into a mismatched recipient for immunotherapy.

In this study, we examined the proliferative response of mature peripheral NK cells to a KIR ligand-mismatched environment. The results demonstrate that NK cells in this context are inhibited from proliferating by the presence of KIR ligands on allogeneic cells. Accordingly, a KIR ligand- mismatched setting favors outgrowth of those NK cells expressing the cognate receptor for the mismatched ligand, and the KIR repertoire of the resulting NK
population is therefore skewed by the allogeneic HLA class I environment. To our knowledge, skewing of the repertoire of the NK population ex vivo has not been previously reported and may be important in transplantation or adoptive transfer of NK cells.

Materials and Methods

Donors

PBMCs were isolated by Ficoll-Paque density gradient centrifugation from whole blood donated by consenting healthy volunteers. Allele-level class I HLA typing and KIR genotyping were performed on peripheral blood samples from all donors by the Australian Red Cross Victorian Transplantation and Immunogenetics Service. Only those individuals with a homozygous group A KIR genotype (lacking activatory KIR2DS1/2/3 and KIR3DS1) were used as NK donors for cloning and bulk cultures as well as cytotoxicity experiments to negate the problem of dual specificity of mAb for both activatory and inhibitory forms of a given KIR. For CFSE assays, NK donors were selected with a KIR genotype that was either homozygous group A or lacking the gene for the activatory form of the given inhibitory KIR of interest. This study has been reviewed and approved by the Ethics Committee at the Alfred Hospital (Project 14/93).

Cell culture

Complete medium consisting of RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin was used for culture of the erythroleukemia cell line K562.

All NK cell cultures were performed using complete medium supplemented with PHA (Sigma-Aldrich) or recombinant human IL-2 (R&D Systems), PHA-activated lymphoblasts (PHA blasts) were generated from PBMCs, PBLs, or CD3+ cells by culture in complete medium supplemented for the first 2–3 days with 10 μg/ml PHA and 50 U/ml IL-2 thereafter.

NK cell purification and culture

PBLs were separated from PBMCs by depleting monocytes using 2- to 16-h culture in complete medium and subsequent retrieval of nonadherent cells. PBLs were next depleted of T cells by magnetic bead separation using anti-CD3 microbeads and LD separation columns (Miltenyi Biotec). CD3+ cell contamination was consistently <0.1% in the resulting lymphocyte population.

For expansion of NK clones, T cell-depleted PBLs were seeded at 5, 10, and 20 cells/well in 96-well tissue culture plates using complete medium supplemented with 2 μg/ml PHA for the first 24 h for NK activation and then culturing in 250 U/ml IL-2 for up to 4 wk. One × 10^6 irradiated (30 Gy) feeder cells per well were added at the initiation of the culture (day 1) in the form of PBMCs and again on culture day 5, this time in the form of PHA blasts of the same donor origin as the day 1 feeders.

For 14-day polyclonal NK cultures, T cell-depleted PBLs were seeded into T25 or T75 culture flasks at a concentration of 1 × 10^5 cells/well. CFSE-labeled cells were plated in triplicate at 5 × 10^4 cells/well onto 96-well U-bottom plates for coculture with irradiated (30 Gy) stimulator PHA blasts at a responder:stimulator ratio of 1:1 in complete medium supplemented with 250 U/ml IL-2. On day 6, triplicate wells were pooled and stained with propidium iodide and mAbs (SJ25C1)-PerCP and allophycocyanin; CD56 (NCAM16.2)-PE and allophycocyanin from R&D Systems. Corresponding isotype control mAbs (C1603/1)-PerCP and CD56 (NCAM16.2)-PE and allophycocyanin were included in all analyses. Data were acquired using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences) and analyzed using EXPO 32 ADC software (Beckman Coulter).

Cytotoxicity assay

NK cells were taken from culture and washed before use as effector cells in a standard 4-h 51Cr release assay for assessment of cytolytic activity. Briefly, target cells (K562 or PHA blasts) were labeled with 51Cr (PerkinElmer) for 1 h at 37°C then washed three times in PBS and resuspended in complete medium. Five thousand targets per well were added in triplicate wells to effector cells in 96-well V-bottom plates at E:T ratios indicated. After 4 h at 37°C, supernatants from each well were transferred to LUMAplates and 51Cr release was measured using a TopCount NXT (Canberra Packard; Meriden). The mean of triplicate wells was used to calculate the percentage of specific target cell lysis with the formula: (test well release – spontaneous release)/(maximum release – spontaneous release) × 100.

NK proliferation assay

T cell-depleted PBLs were labeled with 0.75 μM CFSE (Sigma-Aldrich) at a cell density of 1 × 10^6/ml in PBS. After 5 min at 37°C, cells were washed, first with PBS containing 1% FBS and then with 0.1% FBS, and resuspended in complete medium. CFSE-labeled cells were plated in triplicate at 5 × 10^4 cells/well onto 96-well U-bottom plates for 16-h culture in complete medium and subsequent retrieval of nonadherent cells. PBLs were next depleted of T cells by magnetic bead separation using anti-CD3 microbeads and LD separation columns (Miltenyi Biotec). CD3+ cell contamination was consistently <0.1% in the resulting lymphocyte population.

For expansion of NK clones, T cell-depleted PBLs were seeded at 5, 10, and 20 cells/well in 96-well tissue culture plates using complete medium supplemented with 2 μg/ml PHA for the first 24 h for NK activation and then culturing in 250 U/ml IL-2 for up to 4 wk. One × 10^6 irradiated (30 Gy) feeder cells per well were added at the initiation of the culture (day 1) in the form of PBMCs and again on culture day 5, this time in the form of PHA blasts of the same donor origin as the day 1 feeders.

For 14-day polyclonal NK cultures, T cell-depleted PBLs were seeded into T25 or T75 culture flasks at a concentration of 1 × 10^5/ml with medium supplements and irradiated feeder cells (NK:feeder ratio 1:1) were added as per clonal cultures.

Flow cytometry

Surface phenotype of cells was determined with the following mouse anti-human mAbs: CD3 (SK7)-PerCP and allophycocyanin and CD19 (SJ25C1)-PerCP and allophycocyanin; CD56 (NCAM16.2)-PE and allophycocyanin; CD158a (HP-3E4)-FITC and PE; KIR-NKAT2 (DX27)-FITC and PE; NK81 (DX9)-FITC and PE, all from BD Biosciences, as well as NKG2A (Z199)-PE from Immunotech and KIR2DL1 (143211)-allophycocyanin from R&D Systems. Corresponding isotype control mAbs were included in all analyses. Data were acquired using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences) and analyzed with EXPO 32 ADC software (Beckman Coulter).

Results

In vitro NK cell populations differ in alloreactivity depending on HLA class I environment of prior culture

A standard 4-h 51Cr release cytotoxicity assay was used to determine the functional impact of using HLA-disparate KIR ligand-mismatched feeder cells in bulk NK cultures. Polyclonal NK cells after a 14-day culture with autologous feeder cells exhibited minimal killing of allogeneic PHA blasts even though they had high functional capacity as ascertained by their killing of K562 cells. A representative example of three independent experiments is shown in Fig. 1A. However, NK cells taken from a parallel culture with allogeneic KIR ligand-mismatched feeders were able to lyse the allogeneic targets possessing the same KIR ligand repertoire as the feeder cells. A representative example of three independent experiments is shown in Fig. 1B. This corroborates early findings by Colonna et al. (19) who reported in 1993 that culture of NK cells from HLA-C1 or HLA-C2 homozygous donors with allogeneic stimulator cells homozygous for the opposite HLA-C subgroup led to the generation of NK cell lines specifically reactive against targets lacking whichever HLA-C subgroup was missing on the stimulator cells.

FIGURE 1. Cytotoxicity of cultured NK cells toward KIR ligand-mismatched targets is enhanced through use of appropriately mismatched allogeneic feeders. Allo specificity of postculture NK cells was measured using a standard 4-h 51Cr release assay. Effector cells were CD3+ PBLs cultured for 14 days in IL-2 with either autologous (A) or allogeneic KIR ligand-mismatched (B) feeder cells. NK cells exposed to KIR ligand-mismatched feeder cells during prior culture exhibited increased alloreactivity toward targets with a KIR ligand repertoire analogous to that of the feeder cells. Plots shown are representative of three independent experiments using NK cells from a Bw4 C1 C2 donor and allogeneic feeders/targets from a Bw4 C1 C2 donor (KIR ligand mismatched) or targets from a Bw4 C1 C2 donor (KIR ligand matched).
HLA-disparate allogeneic targets sharing the same KIR ligand repertoire (KIR ligand matched) as the effectors were spared from lysis (Fig. 1), showing that the NK cell alloreactivity was mediated by inhibitory KIR-HLA class I interactions. We therefore reasoned that the differences observed in alloreactivity after culture could reflect either enhanced function or increased frequency of the NK cell subset that specifically recognized missing self on the allogeneic feeder cells.

**Exposure to allogeneic KIR ligand-mismatched cells impacts on NK cell population KIR frequencies**

We next investigated the effect of culture with KIR ligand-mismatched feeder cells on the distribution of self-HLA class I receptors in a population of NK cells. Peripheral blood NK cells were grown as bulk cultures for 14 days in the presence of autologous or allogeneic feeders at a cell concentration of 1×10^6/ml and a ratio of 1:1. All NK donors had a KIR genotype lacking the stimulatory KIR2D5, KIR2D6, KIR2D7, KIR2D8, and KIR3D9 so that the observed culture outcomes could be attributed to inhibitory KIR in isolation from their stimulatory counterparts and also to ensure that positive staining with anti-KIR mAbs was definitive of inhibitory KIR expression.

When NK cells from a single donor were cultured in parallel with different feeder cells, no significant difference was seen in NK cell fold expansion between cultures (data not shown); however, the resulting polyclonal populations were seen to differ markedly in terms of their frequencies of KIR expression (Fig. 2). When feeder cells were mismatched with NK cells for a specific KIR ligand, then the frequency of the cognate KIR in the postculture NK population was higher than in autologous or allogeneic ligand-matched cultures.

This skewing was evident in all three donors tested when looking at the subpopulation of cells expressing the KIR of interest in the absence of the other KIR. The frequency of this subpopulation was examined because it allowed us to investigate subtle effects of a single KIR ligand by excluding the effect of inhibitory receptors for other HLA class I ligands present on the feeder cells.

**HLA class I genotype of feeder cells skews clonal expansion of NK cells**

Ruggeri et al. (6) have reported using a culture system for expanding peripheral blood NK clones using feeder cells obtained from a random pool of donors. We wanted to investigate whether this approach could be made more efficient by intentionally biasing the culture system to expand clones expressing a specific KIR of interest, thereby reducing the required cell numbers and materials. The same strategies for donor and feeder cell selection were applied as were previously used in bulk cultures. NK clones were expanded from T cell-depleted PBLs in contact with feeder cells from a chosen individual who lacked one KIR ligand that was present in the NK cell donor. Following Ruggeri et al. (6), cultures from plates with 20 or fewer growing wells were deemed likely to be clonal and harvested for analysis by flow cytometry. Occasionally, cultures exhibited mixed (positive and negative) staining for a given KIR and these polyclonal populations were excluded from analysis. Although there was considerable variation between donors in the overall numbers of clones expanded and the number of KIR represented in each postculture population, clones expressing the KIR predicted to be favored based on the feeder cell selection strategy (black bars) were consistently generated. Three of the four donors generated a greater number of clones when the NK cells expressed only the cognate receptor for HLA-C2, KIR2D1 (Fig. 3A, ■). Furthermore, aggregate data with KIR distribution among the pooled population of expanded NK clones from all donors showed that frequency of clones expressing KIR2D1L1, KIR2D3L3, or KIR3D1L1 was significantly higher when NK and feeder cells were mismatched for the cognate ligand (HLA-C2, HLA-C1, or HLA-Bw4, respectively). Clones expressing the KIR for which no ligand was present on the feeder cells are indicated by bold circles (Fig. 3B). The number of donors who are included in each group were three, three, and four for Bw4, C1, and C2 mismatch groups, respectively.

**Measure of ex vivo NK cell proliferative response to KIR ligand mismatch**

We hypothesized that the changed KIR frequencies observed in postculture NK cell populations were the result of clonal expansion rather than of receptor acquisition. The CFSE assay, described by Mannering et al., (18) to examine T cell proliferation, provided a means to test this hypothesis by giving a direct gauge of the proliferative response in a NK cell population to an allogeneic challenge. NK cells were isolated from healthy donors and labeled with CFSE then stimulated with allogeneic PHA blasts for 5 days in the presence of IL-2. PHA blasts were the chosen stimulator
cells due to their previous use in the cultures and also because they allowed exclusion of any remaining stimulators from final analysis by gating out any CD3+ events. Cell division as inferred by CFSE dilution was determined by flow cytometry for responder cells expressing the cognate receptor for HLA-C2, KIR2DL1, in the absence of other inhibitory KIR. Clones with this particular KIR repertoire (A) were consistently present in the population expanded from each of the donors. B, Pooled data showed that for each of the three experimental streams the distribution of KIR in the population of clones expanded was skewed toward clones expressing the KIR for which no ligand was present on the feeder cells (indicated by bold circles). Number of donors included in each group was three, three, and four for Bw4, C1, and C2 mismatch groups, respectively.

![FIGURE 3. KIR ligand-mismatched feeder cells bias outcome of NK clonal expansion in vitro. Peripheral blood NK cells from different donors were serially diluted and plated with irradiated allogeneic feeder cells for 3- to 4-wk clonal cultures. Growing clones were analyzed for surface expression of KIR by flow cytometry. Three experimental groups were set up, each representing a single KIR ligand mismatch, with NK and feeder cell donors selected to ensure presence of the ligand in the NK donors and absence of the ligand in the feeder cells. A, As a representative example of one of the three culture streams, NK cells from four different HLA-C2* donors were plated for clonal expansion with HLA-C* feeder cells. We predicted that this culture system would be biased toward NK clones that expressed the cognate receptor for HLA-C2, KIR2DL1, in the absence of other inhibitory KIR. Clones with this particular KIR repertoire (■) were consistently present in the population expanded from each of the donors. B, Pooled data showed that for each of the three experimental streams the distribution of KIR in the population of clones expanded was skewed toward clones expressing the KIR for which no ligand was present on the feeder cells (indicated by bold circles). Number of donors included in each group was three, three, and four for Bw4, C1, and C2 mismatch groups, respectively.](http://www.jimmunol.org/)

![FIGURE 4. KIR ligand mismatch directs ex vivo NK cell proliferation. Allogeneic stimulator cells mismatched with responder NK cells for a single KIR ligand were used to assess proliferative response in NK cells expressing the cognate KIR for the mismatched ligand. In the representative experiments shown, CD3+ PBLs were isolated from peripheral blood of a Bw4*C1* C2+ donor (A–F) or a Bw4*C1* C2- donor (G–I) and labeled with CFSE and then cultured with IL-2 for 5 days either alone (A, D, and G) or with irradiated allogeneic stimulator PHA blasts with the following KIR ligand repertoires: Bw4*C1* C2+ (B and E) and Bw4*C1* C2- (H) (KIR ligand match), Bw4*C1* C2- (C) (HLA-C2 mismatch), Bw4*C1* C2+ (F) (HLA-C1 mismatch), and Bw4*C1* C2- (I) (HLA-Bw4 mismatch). Cells were harvested on day 6 and stained with a panel of mAbs for analysis by flow cytometry. Plots shown are gated on PI− CD3− CD19− cells. NK proliferation in the KIR+ positive population was similar to the cells-alone background levels when allogeneic stimulator cells shared the same KIR ligand repertoire as the responders. However, ligand-mismatched stimulator cells elicited a strong proliferative response in those NK cells expressing cognate inhibitory KIR for the mismatched ligand.](http://www.jimmunol.org/)
changed functional capacity of the NK population toward certain allogeneic targets, as shown here in cytotoxicity assays. In contrast to Colonna et al. (19) who were able to demonstrate this effect in NK cells taken from HLA-C homozygous individuals only, we observed similar results in HLA-C heterozygous (C1+C2+) individuals. This difference is most likely to reflect the substantially different culture protocols used in the two studies. The earlier study tested NK cell lines generated from a starting NK population of 1 × 10^6 cells by stimulating with KIR ligand-mismatched PBMCs for 4 days, then expanding for 7 days with IL-2. The current study tested polyclonal NK cells generated from a starting population of several million peripheral NK cells broadly activated with PHA and expanded in the presence of mismatched stimulator cells for twice the duration in IL-2 as the previous study. The larger absolute numbers of NK cells at initiation of culture coupled with the longer period of cell expansion, including a restimulation with the mismatched feeder cells on day 5, may have all acted to increase the proportion of alloreactive cells from donors in this study above the matched feeder cells on day 5, may have all acted to increase the duration in IL-2 as the previous study. The larger absolute numbers of NK cells at initiation of culture coupled with the longer period of cell expansion, including a restimulation with the mismatched feeder cells on day 5, may have all acted to increase the proportion of alloreactive cells from donors in this study above the threshold for detection using the 51Cr release assay.

We used a strategy for biasing NK culture outcomes that involved deliberately mismatching NK and feeder cells for particular HLA-B or HLA-C subgroups corresponding to inhibitory KIR ligands so that feeder cells lacked a KIR ligand present in the NK cell donor. Alternatively, when cultures were performed with allogeneic feeders that were KIR ligand matched to the NK cells, KIR indicated (30). Bold, Positive response (CDI > 3); –, not tested. Each data point represents one of at least two experiments at a responder:stimulator ratio of 1:1.

<table>
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<th>Stimulator Cells</th>
<th>KIR2DL1 HLA-C2 positive</th>
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</table>

* In a 5-day coculture with allogeneic stimulator cells, CDI was calculated for responder NK cells that expressed the specific KIR indicated (top). Bold, Positive response (CDI > 3); –, not tested. Each data point represents one of at least two experiments at a responder:stimulator ratio of 1:1.

![Figure 5](image-url)  
**Figure 5.** NK cell subsets proliferate depending on lack of inhibitory ligands. NK cells from a HLA-C heterozygous (C1+c2-) individual were stimulated with HLA-C homozygous (C1’C2- or C1’C2+) PHA blasts and responses in the KIR2DL1+ and KIR2DL3+ populations were determined simultaneously. NK cell proliferative response to a HLA-C1 mismatch was seen in KIR2DL3+ but not in KIR2DL1+ cells in the responder population and vice versa for HLA-C2-mismatched stimulators.
A normal population of mature NK cells arising in the autologous context expresses a KIR repertoire which predominantly reflects the KIR genotype of the individual and is only very subtly influenced by HLA class I type (32–36). The same rule seems to hold for NK cells generated from transplanted donor CD34+ cells in allog-HSCT, so that the reconstituted peripheral NK cell population in the recipient posttransplantation tends to echo the KIR repertoire as seen in the donor (17, 33). However, the in vitro results of this study suggest that mature NK cells taken from donor peripheral blood and infused into a KIR ligand-mismatched recipient would quickly exhibit a population distribution of KIR very different from that of the donor, reflecting a predominance of cells with a KIR repertoire enabling response to the missing ligand.

The changes in KIR frequencies we observed in postculture NK populations were most probably due to clonal expansion rather than induction of receptor expression, as shown by the CFSE proliferation experiments. This finding was not surprising given that expression of KIR by mature NK cells, controlled by DNA methylation, is very stable both at the single-cell level and at the population level within a normal individual (37, 38). Although 50% KIR-negative NK cells are found in adult blood (27, 30), only 20% of these cells were able to be stimulated by IL-2 to up-regulate KIR (27). This 10% of the KIR-positive population would be likely to play only a minor role in the resultant skewing of the NK cell population. Furthermore, in contrast to IL-12, culture with IL-2 alone does not significantly affect the frequency of CD94/NKG2A-positive NK cells in a population (39).

Although the KIR ligand mismatches examined were seen to strongly influence NK cell expansion, they did not exert complete control over this proliferation as illustrated by the fact that in all cultures/experiments a significant proportion of the expanded population was KIR ligand-mismatched (28). This 10% of the KIR-positive population would be likely to play only a minor role in the resultant skewing of the NK cell population. Furthermore, in contrast to IL-12, culture with IL-2 alone does not significantly affect the frequency of CD94/NKG2A-positive NK cells in a population (39).

The use of PHA as a broad NK stimulator for the control over this proliferation as shown by the CFSE proliferation experiments. This finding was not surprising given that expression of KIR by mature NK cells, controlled by DNA methylation, is very stable both at the single-cell level and at the population level within a normal individual (37, 38). Although 50% KIR-negative NK cells are found in adult blood (27, 30), only 20% of these cells were able to be stimulated by IL-2 to up-regulate KIR (27). This 10% of the KIR-positive population would be likely to play only a minor role in the resultant skewing of the NK cell population. Furthermore, in contrast to IL-12, culture with IL-2 alone does not significantly affect the frequency of CD94/NKG2A-positive NK cells in a population (39).

Although the KIR ligand mismatches examined were seen to strongly influence NK cell expansion, they did not exert complete control over this proliferation as illustrated by the fact that in all cultures/experiments a significant proportion of the expanded populations did not express the inhibitory KIR relevant to the mismatched ligand. There are a number of possible explanations for these results. The use of PHA as a broad NK stimulator for the initiation of clonal and bulk cultures may have contributed to background NK proliferation, although this effect appeared minimal in pilot CFSE proliferation assays comparing PHA plus IL-2 with IL-2 alone as the assay medium (data not shown). Also, culture in IL-2 alone activates NK cells and has been shown to obscure subtle effects of receptor-ligand interaction seen in resting NK cells (40). Some proliferation may have been due to a bystander effect whereby NK cells responding to missing KIR ligand stimulate neighboring cells to respond through cytokine secretion and/or cell-cell contact and signaling through surface receptors. Finally, the influence of NK receptors, both stimulatory and inhibitory, other than those included in this study cannot be discounted.

Given the myriad of known NK cell receptors involved in immunosurveillance and the culture variables alluded to that may have obscured any emerging pattern, the mere fact that a significant effect could be seen looking at just three inhibitory receptor ligands serves to further underscore the established importance of these inhibitory KIR and their ligands in directing NK cell activity.

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Disclosures
The authors have no financial conflict of interest.

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Given the myriad of known NK cell receptors involved in immunosurveillance and the culture variables alluded to that may have obscured any emerging pattern, the mere fact that a significant effect could be seen looking at just three inhibitory receptor ligands serves to further underscore the established importance of these inhibitory KIR and their ligands in directing NK cell activity.

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Disclosures
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