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Human NK Cell Education by Inhibitory Receptors for MHC Class I

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Summary

Natural killer (NK) cells recognize the absence of self MHC class I as a way to discriminate normal cells from cells in distress. In humans, this "missing self" recognition is ensured by inhibitory receptors such as KIR, which dampen NK cell activation upon interaction with their MHC class I ligands. We show here that NK cells lacking inhibitory KIR for self MHC class I molecules are present in human peripheral blood. These cells harbor a mature NK cell phenotype but are hyporesponsive to various stimuli, including MHC class I-deficient target cells. This response is in contrast to NK cells that express a single inhibitory KIR specific for self MHC class I, which are functionally competent when exposed to the same stimuli. These results show the involvement of KIR-MHC class I interactions in the calibration of NK cell effector capacities, suggesting its role in the subsequent "missing self" recognition.

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Introduction

Major histocompatibility complex (MHC) class I molecules are critical elements of the vertebrate adaptive immune response, via their function as antigen-presenting molecules to T cells. MHC class I molecules are also recognized by several inhibitory receptors expressed on innate effectors, in particular NK cells (Hoglund et al., 1997; Parham, 2005; Raulet et al., 2001; Yokoyama and Plougastel, 2003). NK cells are large granular lymphocytes that are involved in innate immunity against viruses, bacteria, parasites, and other triggers of pathology, such as malignant transformation, all of which cause stress in affected cells (Moretta et al., 2002; Moretta and Moretta, 2004). NK cell responses are mediated by two major effector functions: direct cytolysis of target cells and production of chemokines and cytokines. Through the latter (e.g., interferon-γ [IFN-γ]), NK cells participate to the shaping of the adaptative T cell response (Martin-Fontecha et al., 2004). NK cell activity is governed by the dynamic balance between signals that either cooperate with or antagonize each other (Long, 1999; Vivier et al., 2004). The combinatorial engagement of activating and inhibitory cell-surface receptors determines whether NK cells will or will not kill target cells and/or produce cytokines during their effector phase of activation (Vivier et al., 2004).

NK cells express inhibitory receptors for MHC class I molecules, i.e., receptors for MHC class I molecules that are able to dampen NK cell effector function upon ligation. These inhibitory receptors include KIR (killer cell Ig-like receptors) in humans, Ly49 in the mouse, and CD94/NKG2A heterodimers in both species (Parham, 2005; Raulet et al., 2001). *KIR* and *Ly49* loci are extremely polymorphic, both in terms of gene numbers and alleles present (Parham, 2005; Trowsdale, 2001). The variegated expression of these genes adds to the complexity of NK cell MHC class I recognition (Parham, 2005; Raulet et al., 2001). In humans, KIR can harbor two (KIR2D) or three (KIR3D) extracellular C2-type Ig-like domains. KIR recognize groups of HLA class I molecules that are determined by the amino acids belonging to the C-terminal portion of the MHC class I α1 helix (Boyington and Sun, 2002). In particular, the recognition of inhibitory KIR2D depends to a large extent on the nature of the MHC class I amino acid present at position 80: KIR2DL1 recognizes the group of HLA-C molecules with a Lys^{80} residue (HLA-C2 specificity), whereas the KIR2DL2 and KIR2DL3 allelic forms recognize the group of HLA-C with an Asn^{80} residue (HLA-C1 specificity). Regarding inhibitory KIR3D, KIR3DL1 interacts with Bw4-containing HLA-B alleles, and KIR3DL2 has been reported to recognize HLA-A3 and HLA-A11 allotypes (Parham, 2005). In addition, the CD94-NKG2A heterodimers recognize the nonclassical MHC class I molecule HLA-E in humans and Qa1 in the mouse (Natarajan et al., 2002).

Upon encounter with cells with decreased expression of MHC class I molecules, i.e., "missing self" (Ljunggren and Karre, 1990), NK cells are no longer subject to inhibitory signals initiated by the engagement of MHC class I-specific receptors, promoting NK cell cytotoxicity and cytokine production. Interaction between MHC class I and their inhibitory receptors is thus thought to play a major role in the mechanisms of self tolerance during NK cell effector phases. Along this line, it has been reported that all mature NK cells express at least one self MHC class I-specific inhibitory NK cell receptor (the "at least one rule"), ensuring NK cell tolerance to self (Valiante et al., 1997). As KIR, Ly49, and their cognate MHC class I ligands are encoded by distinct chromosomes, this hypothesis implies mechanisms that would positively select NK cells expressing a self MHC class I-specific inhibitory receptor and/or negatively select NK cells lacking expression of a self MHC class I-specific inhibitory receptor.

A number of experiments in the mouse have clearly established the role of MHC class I to render NK cells functional (Dorfman and Raulet, 1996; Olsson et al., 1995). In particular, there is a correlation between the presence of H-2D^d and the function of the NK cell subset expressing its inhibitory receptor Ly49A (Olsson et al., 1995). These issues have been recently revisited showing that NK cells lacking self MHC class I-specific inhibitory receptor are present in the mouse as a hyporesponsive subset (Fernandez et al., 2005) and that engagement of inhibitory Ly49 molecules by self MHC class I molecule "licenses" NK cells to become functionally competent (Kim et al., 2005).

In humans, studies performed with peripheral blood NK cells from MHC class I-deficient individuals (patients with transporters associated with antigen processing [TAP] deficiency) have also shown an influence of MHC class I on the acquisition of NK cell function: NK cells from TAP-deficient patients exhibit an activation defect (Furukawa et al., 1999; Vitale et al., 2002; Zimmer et al., 1998). However, how MHC class I participate in the shaping of human NK cell function remains unknown (Yokoyama and Kim, 2006; Raulet and Vance, 2006). Here we address whether human NK cells are educated dependent upon the inhibitory MHC class I receptors they express. Our results show that the recognition of self MHC class I by inhibitory KIR is involved in the calibration of NK cell effector capacities during a developmental stage, allowing the subsequent recognition of the absence of self MHC class I during interaction with target cells.

Results

Characterization of Functional NK Cells in Human Peripheral Blood

The lack of reliable detection protocol for cytolytic versus noncytolytic subsets within a complex cell population hampered the precise characterization of "naturally occurring" killer NK cells in unmanipulated samples. In contrast to the standard Cr^{51} release assay that measures total target cell death, cytotoxic function can be assessed by flow cytometry via the cell-surface mobilization of CD107 (LAMP), a marker of intracytoplasmic cytolytic granules, as an indication of effector cell degranulation (Bryceson et al., 2005; Rubio et al., 2003; Wolint et al., 2004). The CD107 assay correlates with the Cr^{51} release assay: NK-resistant targets do not induce CD107 cell-surface mobilization, whereas antibody-coated cells (antibody-dependent cell cytotoxicity [ADCC]) and sensitive MHC class I$^-$ cells do (data not shown). This assay allowed us to identify "naturally occurring" killer NK cells in freshly isolated human peripheral blood samples and to compare this cytolytic potential with IFN-γ production in a multiparameter flow cytometric analysis. We first focused on NK cell stimulation by the erythroleukemic cell line K562, as a prototypical example of a MHC class I-deficient tumor cell target. Heterogeneity among three activated NK cell subsets, CD107$^+$IFN-γ$^-$, CD107$^+$IFN-γ$^+$, and CD107$^-$IFN-γ$^+$ NK cells, could be documented upon K562 stimulation (Figure 1A, left), indicating that only a subset of human peripheral blood NK cells (14.0% ± 1.0%, n = 9) acts as steady-state killer cells in this assay and that NK cell cytotoxic function and cytokine secretion can occur in the same cells but do not precisely correlate.

We next documented the phenotype of cytolytic and IFN-γ-producing NK cells by using a panel of monoclonal antibodies (mAb) reacting with NK cells. It has been proposed that the density of CD56 (neural cell adhesion

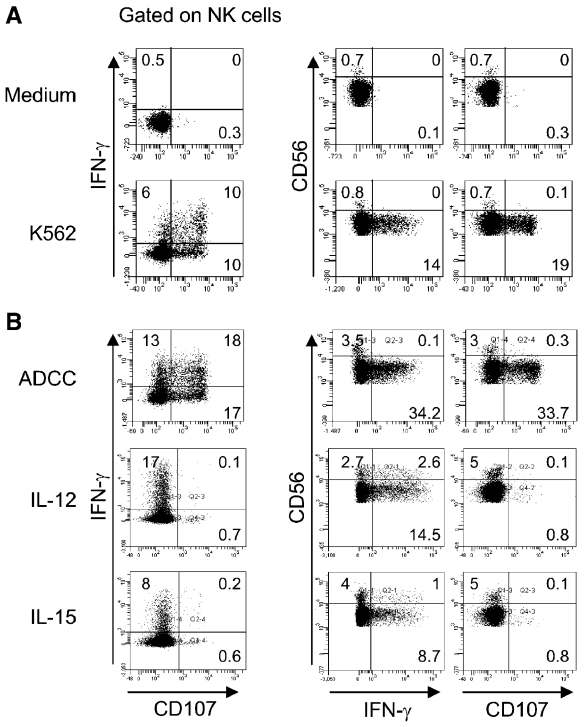

Figure 1. A Multiparameter Flow Cytometric Assay Reveals the Presence of NK Cell Subsets with Various Functional Capacities

(A) PBMC preparations were incubated in the presence or absence of K562 cells for 4 hr at an effector:target ratio of 5:1. Samples were analyzed by flow cytometry and gated on NK cells (CD3$^-$CD56$^+$ lymphocytes). Dot plots of one representative experiment of nine are represented. Numbers indicate the mean percentages of NK cells in each quadrant.

(B) PBMC preparations were incubated as in (A) but in the presence of antibody-coated target cells (P815), IL-12 (20 ng/mL), or IL-15 (10 ng/mL). Dot plots of one representative experiment of nine are represented. Numbers indicate the percentages of NK cells in each quadrant.

molecule, 140 kDa isoform) at the NK cell surface discriminates between two functionally distinct NK cell subsets: cytolytic CD56^{dim} NK cells and cytokine-producing CD56^{bright} NK cells (Cooper et al., 2001). In contrast, K562 stimulation induces IFN-γ production mainly by CD56^{dim} NK cells, whereas IFN-γ production by CD56^{bright} NK cells is marginal (Figure 1A, right). Similar results were observed for tumor necrosis factor-α secretion (data not shown). Stimulation of freshly isolated NK cells by ADCC by antibody-coated P815 mouse mastocytoma cells yielded comparable results as by K562 stimulation (Figure 1B). NK cell treatment with IL-12 or IL-15 for 4 hr primarily resulted in IFN-γ secretion, but not in CD107 cell-surface mobilization (Figure 1B). When NK cells were stimulated with these cytokines, a higher fraction of IFN-γ-producing NK cells was included in the CD56^{bright} NK cell fraction (Figure 1B), more consistent with previous distinction between CD56^{dim} and CD56^{bright} NK cells (Cooper et al., 2001). In conclusion, only a minor fraction of freshly isolated peripheral blood NK cells are reactive with MHC class I-deficient tumor cells (K562), antibody-coated target cells, IL-12, or IL-15 in a 4 hr assay, and the dichotomy between cytolytic CD56^{dim} NK cells and cytokine-producing CD56^{bright} NK cells does not apply when freshly isolated NK cells were stimulated with tumor cells.

NK Cells Lacking MHC Class I-Specific Cell-Surface Receptors Are Hyporesponsive

In an attempt to characterize the subset of freshly isolated peripheral NK cells that are reactive with K562, antibody-coated target cells, or cytokines, we analyzed the expression of a large panel of molecules on the surface of CD107$^+$ or IFN-γ^+ NK cells. In particular, the recent findings that mouse NK cell activity was correlated to the cell-surface expression of receptors for MHC class I prompted us to directly address this issue in humans (Fernandez et al., 2005; Kim et al., 2005). We distinguished two reciprocal NK cell subsets: KIR$^-$NKG2A$^-$ NK cells, which do not express KIR2DL1, KIR2DS1, KIR2DL2, KIR2DL3, KIR2DS2, KIR2DS4, KIR3DL1, or CD94-NKG2A, and KIR$^+$NKG2A$^+$ NK cells, which express at least one of these receptors. Upon encounter with K562, KIR$^+$NKG2A$^+$ NK cells contain on average twice as much CD107$^+$ and IFN-γ^+ cells than do KIR$^-$NKG2A$^-$ NK cells (Figure 2A). No modification of KIR or NKG2A NK cell-surface phenotype could be observed upon 4 hr incubation with target cells (data not shown). Thus, healthy individuals have a substantial fraction of circulating NK cells that lack the MHC class I-specific receptors KIR and NKG2A (13% ± 6% of peripheral blood NK cells, Figure 2B) and exhibit dampened cytolytic and cytokine production ability upon interaction with the MHC class I-deficient target cell K562. Because of the differential surface expression of KIR and NKG2A between CD56^{dim} and CD56^{bright} NK cells (Andre et al., 2000; Cooper et al., 2001), we further compared the functional properties of more restricted and similar NK cell subsets by gating out CD56^{bright} NK cells from our analysis. Consistent with data in Figure 2A, CD56^{dim}KIR$^-$NKG2A$^-$ NK cells are hyporesponsive to K562 for both CD107 and IFN-γ induction as compared to CD56^{dim}KIR$^+$NKG2A$^+$ NK cells (Figure 2C). The absence MHC class I molecules on the surface of K562 cells rules out the possibility that other inhibitory receptors for MHC class I molecules are involved in the weak effector function of CD56^{dim}KIR$^-$NKG2A$^-$ NK cells. Importantly, the latter were also poorly reactive to antibody-coated target cells (ADCC, Figure 2D). Besides MHC class I-specific receptors, a large variety of inhibitory cell-surface molecules has been described (Daeron and Vivier, 1999; Ravetch and Lanier, 2000), suggesting that the hyporesponsiveness of CD56^{dim}KIR$^-$NKG2A$^-$ NK cells might result from the engagement of unknown inhibitory receptors that sense ligands on both human K562 cells and mouse P815 cells. However, this hypothesis was ruled out by the demonstration that CD56^{dim}KIR$^-$NKG2A$^-$ NK cells are also less responsive than CD56^{dim}KIR$^+$NKG2A$^+$ NK cells when stimulated with plate bound CD16 mAb, a cell-free stimulus (Figure 2D). Similar conclusions were drawn when KIR$^-$NKG2A$^-$ and KIR$^+$NKG2A$^+$ NK cells were compared independently of their expression of CD56 (data not shown).

Mechanisms of KIR$^-$NKG2A$^-$ NK Cell Hyporesponsiveness

The hyporesponsiveness of KIR$^-$NKG2A$^-$ as compared to KIR$^+$NKG2A$^+$ NK cells prompted us to consider the possibility that KIR$^-$NKG2A$^-$ cells were not mature NK cells or even not bona fide NK cells. In addition to their CD3$^-$CD56$^+$ cell-surface phenotype, KIR$^-$NKG2A$^-$ NK cells were similar to mature KIR$^+$NKG2A$^+$ NK cells, as judged by their intracellular content in granzyme B and perforin (Figure 3A). PEN5 (CD162R), a specific marker of mature NK cells in peripheral blood (Andre et al., 2000), was also expressed on KIR$^-$NKG2A$^-$ and KIR$^+$NKG2A$^+$ NK cells at comparable amounts (Figure 3). The only three noticeable differences between the cell-surface phenotype of KIR$^-$NKG2A$^-$ and KIR$^+$NKG2A$^+$ NK cells reside in the reduced cell-surface expression of CD94 and CD226 (DNAM-1), as well as the reduced size of the CD56^{bright} subset within KIR$^-$NKG2A$^-$ NK cells. However, KIR$^-$NKG2A$^-$ were undistinguishable from KIR$^+$NKG2A$^+$ NK cells on the basis of the cell-surface expression of 16 other cell-surface receptors including the natural cytotoxicity receptors (NKp30, NKp46, NKp44), NKG2D, CD8α, CD2, CD85j (ILT-2, LIR-1), CD244 (2B4) CD11b, and CD11c (Figure 3).

Importantly, CD16 is expressed at similar surface levels between KIR$^-$NKG2A$^-$ and KIR$^+$NKG2A$^+$ NK cells, despite the dampened CD16-dependent cytolytic and cytokine production in KIR$^-$NKG2A$^-$ NK cells (i.e., ADCC or CD16 mAb-coated plates, Figure 2D). Of note, the IFN-γ production in response to PMA and ionomycin stimulation was comparable between KIR$^-$NKG2A$^-$ and KIR$^+$NKG2A$^+$ NK cells, over all PMA concentrations tested (Figure 4A and data not shown). We next analyzed the production of IFN-γ by these two reciprocal NK cell subsets in response to cytokines. Although KIR$^-$NKG2A$^-$ NK cells produce IFN-γ upon treatment with IL-12 and IL-18, their response was lower than that of KIR$^+$NKG2A$^+$ NK cells (Figure 4B). Short treatment with cytokines can also prime NK cell cytotoxicity. In particular, the MHC class I$^-$ B lymphoblastoid cells L721.221 are resistant to freshly isolated NK cells but sensitive to cytokine-activated NK cells. We therefore tested whether cytokine treatment arms both

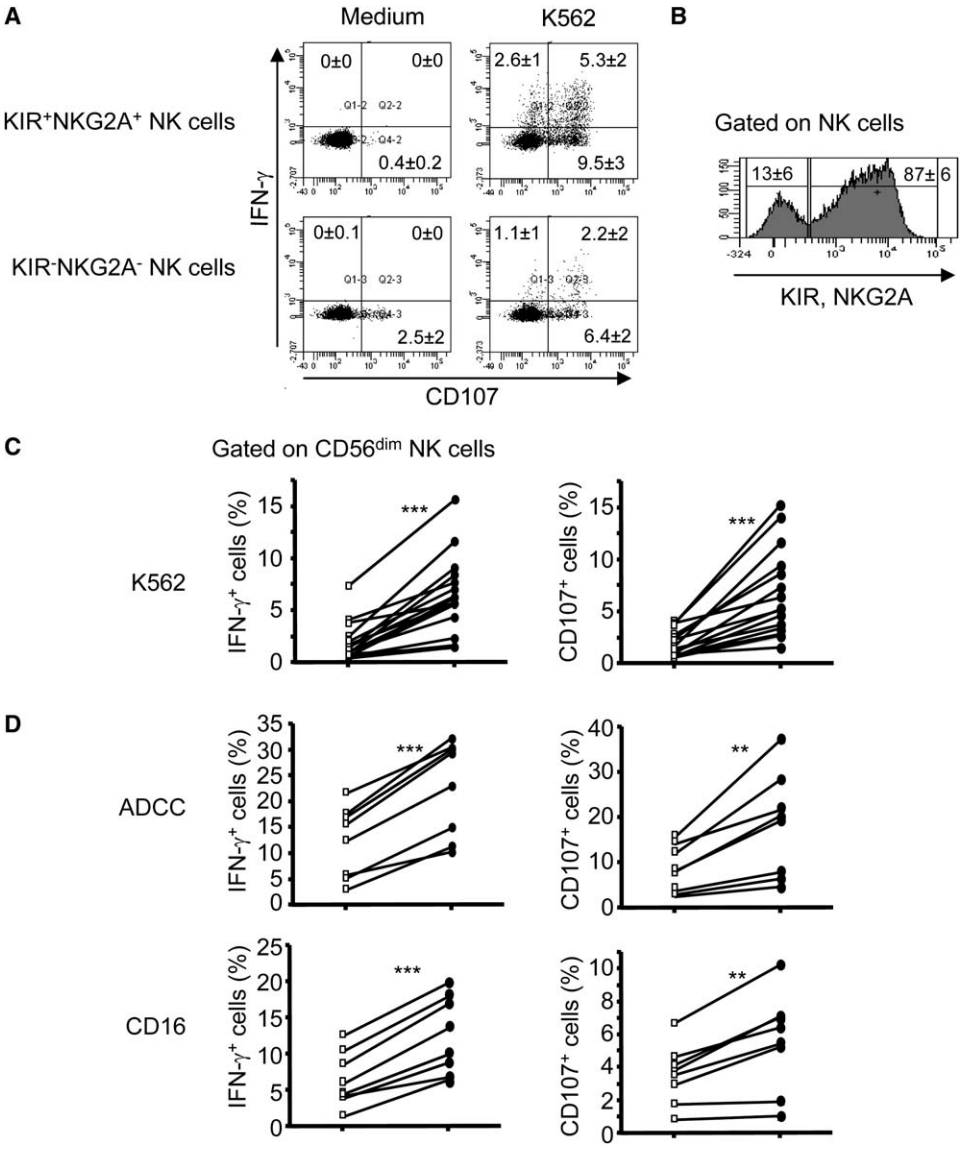

Figure 2. NK Cells Lacking Surface HLA Class I Receptors Are Hyporesponsive to Tumor Cells and CD16 mAb-Coated Plates

(A) Two reciprocal cell subsets were defined within peripheral blood NK cells: KIR$^-$NKG2A$^-$ NK cells that do not express KIR2DL1, KIR2DS1, KIR2DL2, KIR2DS2, KIR2DS4, KIR3DL1, or NKG2A, and KIR$^+$NKG2A$^+$ NK cells that express at least one of these receptors. PBMC preparations were incubated as in Figure 1A. Dot plots of one representative experiment of nine are represented. Numbers indicate the mean percentages ± SD of NK cells in each quadrant (n = 9).

(B) PBMC preparations were stained with CD3 and CD56 mAb to define NK cells. KIR and NKG2A expression was assessed with a mixture of KIR and NKG2A mAb as in (A). Histograms of one representative experiment of nine are represented. Numbers indicate the mean percentages ± SD of NK cells (n = 9) in corresponding areas.

(C) PBMC preparations were stained with CD3 and CD56 mAb to define NK cells. KIR and NKG2A expression was assessed with a mixture of KIR and NKG2A mAb as in (A). CD56^{bright} NK cells were gated out. PBMC preparations were incubated with K562 cells for 4 hr. Results indicate the percentage of reactive NK cells within each NK subset (n = 17). ***$p < 0.001$. Each dot and line represents the results from one individual. Open symbols correspond to KIR$^-$NKG2A$^-$ NK cells; closed symbols correspond to the NK cell subset expressing a least one of these MHC class I receptors (KIR$^+$NKG2A$^+$).

(D) As in (C), but PBMC preparations were incubated with antibody-coated P815 cells (ADCC) or plate bound CD16 mAb for 4 hr. Results indicate the percentage of reactive NK cells within each NK subset (n = 8); **$p < 0.01$; ***$p < 0.001$.

KIR$^-$NKG2A$^-$ and KIR$^+$NKG2A$^+$ NK cells and abolishes their functional differences. Upon 24 hr incubation in the presence of IL-15 or IL-12 and IL-18 followed by a 4 hr encounter with L721.221 cells, KIR$^-$NKG2A$^-$ NK cells were again less potent than KIR$^+$NKG2A$^+$ NK cells for both cytotoxicity and IFN-γ production (Figure 4C). CD56^{bright} NK cells were gated out from these analyses (Figures 4B and 4C) to more precisely compare KIR$^-$NKG2A$^-$ and KIR$^+$NKG2A$^+$ NK cells, but similar data were obtained when CD56^{bright} were included in the analysis (data not shown). Thus, the functional difference between KIR$^-$NKG2A$^-$ and KIR$^+$NKG2A$^+$ NK cells may affect a common signaling element downstream of various NK cell-activating pathways (including but

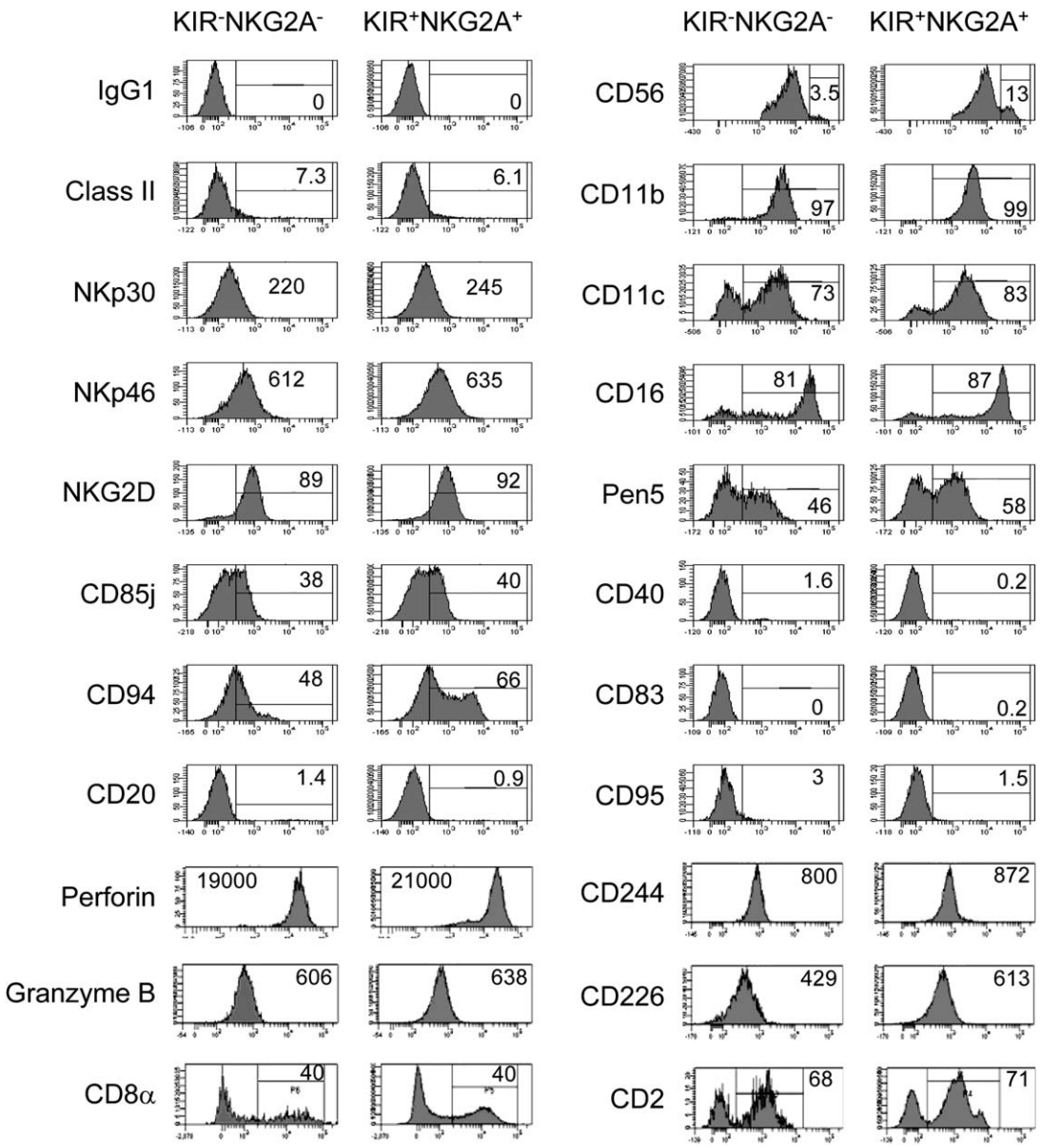

Figure 3. Phenotype of Hyporesponsive KIR$^-$NKG2A$^-$ NK Cells

PBMC preparations were stained as in Figure 2A.

Samples were analyzed for the cell-surface expression of indicated cell-surface molecules by flow cytometry. Numbers indicate the % of NK cells in corresponding areas, or the total mean fluorescence intensity for perforin, granzyme B, NKp30, NKp46, CD244, and CD226. Histograms of one representative experiment of three are represented.

not only ITAM-dependent pathways) and upstream of transcription factors' activation.

Impact of Inhibitory KIR and Cognate MHC Class I on NK Cell Function

As IL-15 and IL-12 + IL-18 were unable to abolish the low responsive state of KIR$^-$NKG2A$^-$ NK cells against MHC class I-deficient target cells, we tested other potential recovery signals. It is striking that freshly isolated KIR$^-$NKG2A$^-$ NK cells have features shared with freshly isolated NK cells from HLA class I-deficient individuals, including defects in K562 killing and ADCC (Furukawa et al., 1999; Zimmer et al., 1998). The lack of receptors (KIR and NKG2A) in one case and the lack of ligands (MHC class I molecules) in the other thus leads to similar human NK cell phenotypes. We therefore directly tested whether MHC class I influences the responsive capacity of NK cells bearing cognate or noncognate NK cell receptors and limited our analysis to KIR by gating out NKG2A$^+$ NK cells. The extreme polymorphism of both *HLA class I* and *KIR* loci in the human population (Parham, 2005) required us to design our study at the molecular level, taking into account the genotypes of our cohort of healthy donors at these loci. We selected individuals that express both a restricted subgroup of *HLA-C* alleles and the cognate inhibitory *KIR* gene. A first group of individuals was thus selected on the basis of the homozygous expression of the C2 group of *HLA-C*

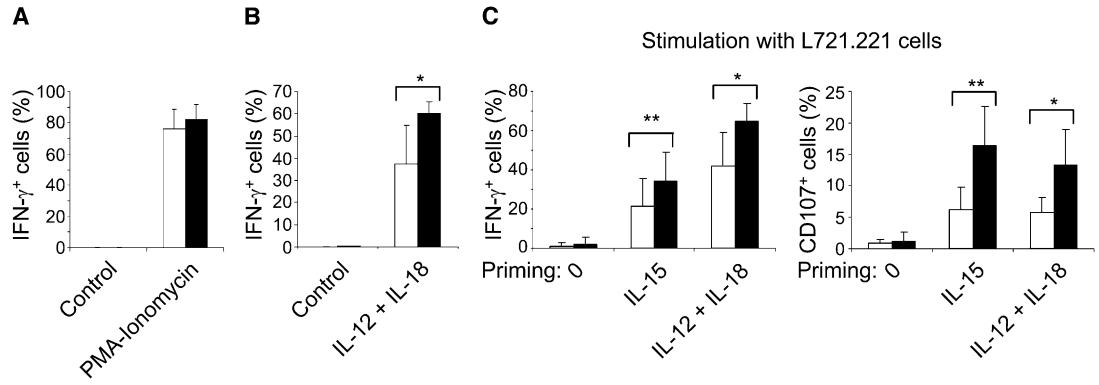

Figure 4. Characterization of Hyporesponsive KIR$^-$NKG2A$^-$ NK Cells

(A) PBMC preparations were incubated in the presence or absence of PMA (1 ng/mL) and ionomycin (0.5 μM) for 4 hr. Histograms indicate the mean percentages ± SD of reactive NK cells within each NK subset (n = 7).

(B) As in Figure 2C, but PBMC preparations were incubated for 24 hr with IL-12 (5 ng/mL) and IL-18 (20 ng/mL) in combination. Results indicate the mean percentages ± SD of reactive NK cells within each NK subset (n = 5); *p < 0.05.

(C) As in Figure 2C, but PBMC preparations were incubated for 24 hr with IL-15 (10 ng/mL) or IL-12 (5 ng/mL) and IL-18 (20 ng/mL) in combination. Cells were washed and incubated for an extra 4 hr with L721.221 cells. Histograms indicate the mean percentage ± SD of reactive cells within each NK subset (n = 5). *p < 0.05; **p < 0.01.

alleles (HLA-C2) and the presence of the *KIR2DL1* gene that encodes the inhibitory KIR recognizing HLA-C2 molecules (Figure 5A). In these individuals, we analyzed the reactivity of NK cells that express KIR2DL1 in the absence of other MHC class I receptors (KIR and NKG2A, referred as to KIR2DL1$^+$others$^-$ NK cells). For each sample, we compared these results to that obtained for the "reciprocal" subset of NK cells, i.e., NK cells that express a single KIR (product of the *KIR2DL2* gene) that does not interact with HLA-C2 (referred as to KIR2DL2$^+$others$^-$ NK cells). The effector function of KIR$^-$NKG2A$^-$ NK cells was also analyzed as a hyporesponsive control for each sample. KIR2DL1$^+$others$^-$ NK cells were fully competent for IFN-γ production and CD107 mobilization in response to trigger by K562, ADCC, and CD16 mAb-coated plates (Figures 5B and 5C). In marked contrast, both KIR2DL2$^+$others$^-$ and KIR$^-$NKG2A$^-$ NK cells were hyporesponsive to the very same stimuli in the same donor (Figures 5B and 5C). These results indicate that the expression of an inhibitory KIR at the NK cell surface is not sufficient to abolish the hyporesponsiveness detected in KIR$^-$NKG2A$^-$ NK cells and that the presence of a HLA class I ligand for the inhibitory KIR in the individual is necessary to remove NK cell hyporesponsiveness.

If this model was correct, one could predict that the KIR2DL1$^+$others$^-$ NK cell subset would be hyporesponsive in individuals in which KIR2DL1 has no cognate HLA-C2 ligand. We directly tested this hypothesis by analyzing a second cohort of individuals, who were selected for the homozygous expression of the C1 group of *HLA-C* alleles (*HLA-C1*) and the presence of the *KIR2DL2* gene that encodes for the inhibitory KIR recognizing HLA-C1 molecules (Figure 6A). In these individuals, KIR2DL1$^+$others$^-$ NK cells were hyporesponsive for all stimuli, the same way as KIR$^-$NKG2A$^-$ NK cells (Figures 6B and 6C). In contrast, KIR2DL2$^+$others$^-$ NK cells were competent in the same donors (Figures 6B and 6C). Most mAb recognizing KIR are crossreactive with inhibitory KIR-L and their activating KIR-S counterparts as a result of the extreme homology between these molecules (Stewart et al., 2005). All the above data were obtained with *KIR* genotyped individuals, in which we could unambiguously infer the KIR2DL1 NK cell-surface phenotype to inhibitory *KIR2DL1* alleles and the KIR2DL2 NK cell-surface phenotype to inhibitory *KIR2DL2* alleles. Similar data were obtained with individuals bearing activating *KIR2DS1* or *KIR2DS2* genes (data not shown) as well as with individuals that are deficient in all activating *KIR* genes but *KIR2DS4* (A haplotype donors) (see Figure S1 in the Supplemental Data available with this article online). These results suggest that activating KIR do not play a critical role in NK cell education, consistent with the absence of NK cell phenotype in KIR-S-deficient individuals (Parham, 2005), as well as in mice that are deficient for all activating Ly49-dependent signals (i.e., KARAP(DAP12)-deficient mice) (Bakker et al., 2000; Tomasello et al., 2000). As expected from a NK cell education process that depends upon the interaction between inhibitory KIR and self HLA, the competence of KIR2DL1$^+$others$^-$ NK cells in C2C2 individuals and of KIR2DL2$^+$others$^-$ NK cells in C1C1 individuals is stable with time. This was observed when NK cells were stimulated with antibody-coated targets and IFN-γ production was measured (Figure S2). Similar results were obtained with the use of CD107 mobilization as a read-out and K562 as well as CD16 mAb-coated plates as stimuli (data not shown).

Thus, peripheral blood NK cells expressing an inhibitory KIR-recognizing self HLA class I were functionally reactive to various MHC class I-deficient stimulation (i.e., target cells and mAb-coated plates). In contrast, NK cells expressing no inhibitory KIR or NKG2A (Figure 2) or expressing inhibitory KIR that do not interact with self MHC class I molecules (Figure 4) were hyporesponsive to the same stimuli. An important prediction derived from this model is that the KIR2DL1$^+$other$^-$ and the KIR2DL2$^+$other$^-$ NK cell subsets would be equally responsive when isolated from C1C2 heterozygous individuals. Stimulation of freshly isolated C1C2 PBMC with plate bound CD16 mAb indicates that this was indeed the case (Table 1).

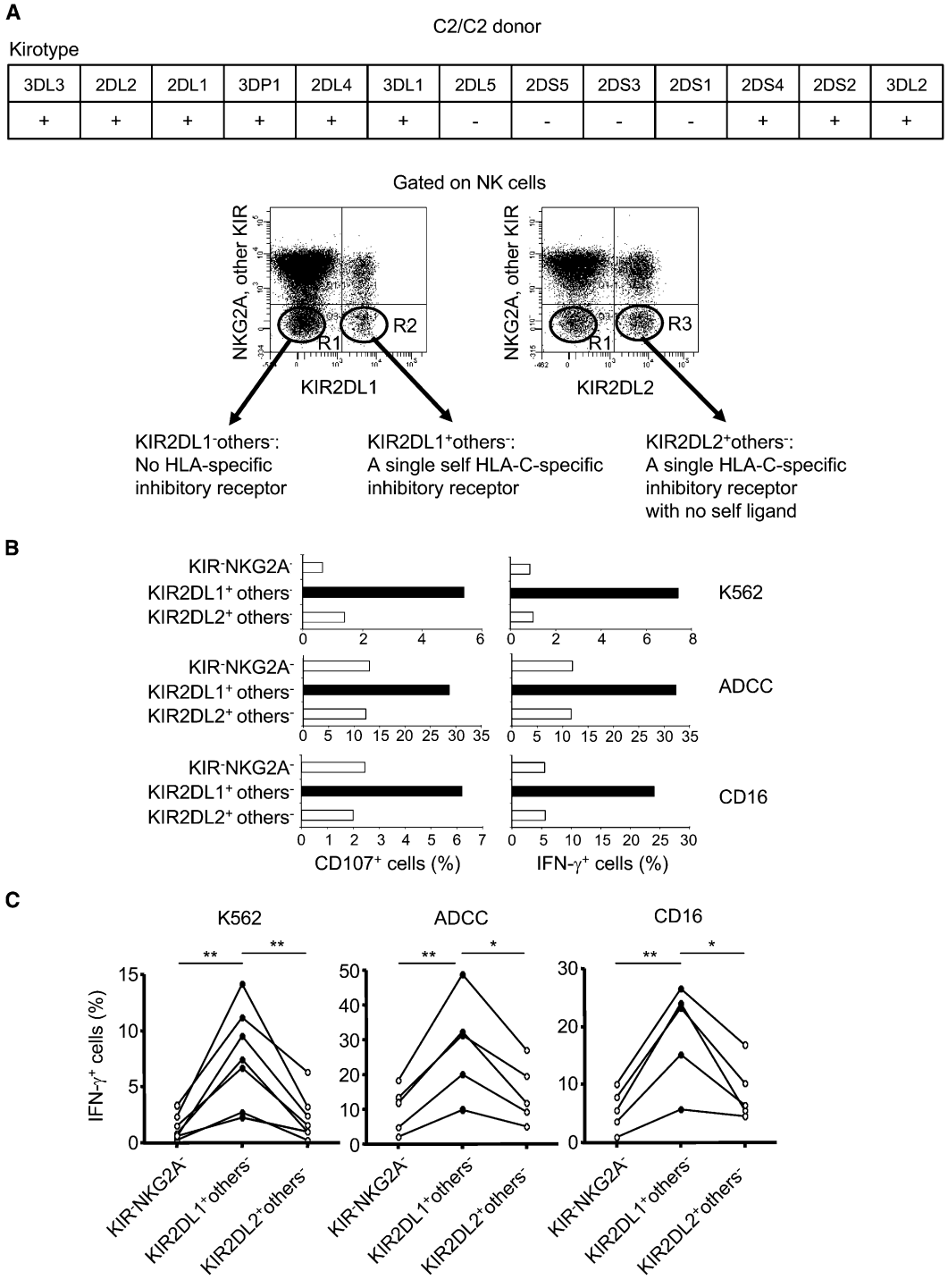

Figure 5. Evidence for a Role of Inhibitory KIR and HLA Class I in the Acquisition of NK Cell Function in C2C2 Individuals

(A) Top: The KIR genotype (kirotype) of a representative C2C2 KIR2DL1$^+$ donor. Bottom: Gating of the indicated NK cell subsets.

(B) PBMC preparations were incubated for 4 hr in the presence of indicated stimuli and analyzed by 6-color flow cytometry. The bars indicate the % of CD107$^+$ or IFN-γ^+ cells in the indicated NK cell subsets. The bars corresponding to the NK cell subset that expresses a KIR interacting with self HLA class I are in black. The bars corresponding to the NK cell subset that does not express NKG2A or a KIR interacting with self HLA class I are in white. A representative experiment of seven performed with six other C2C2 individuals is shown.

(C) As in (B) for a group of C2C2 donors (n = 7 for K562 stimulation; n = 5 for ADCC and CD16 stimulation), including three donors with no *2DS1* and *2DS2* genes. Each dot and line represents the results from one individual. Open symbols correspond to the NK cell subsets that do not express NKG2A or a KIR interacting with self HLA class I. Closed symbols correspond to the NK cell subset expressing a KIR interacting with self HLA class I.

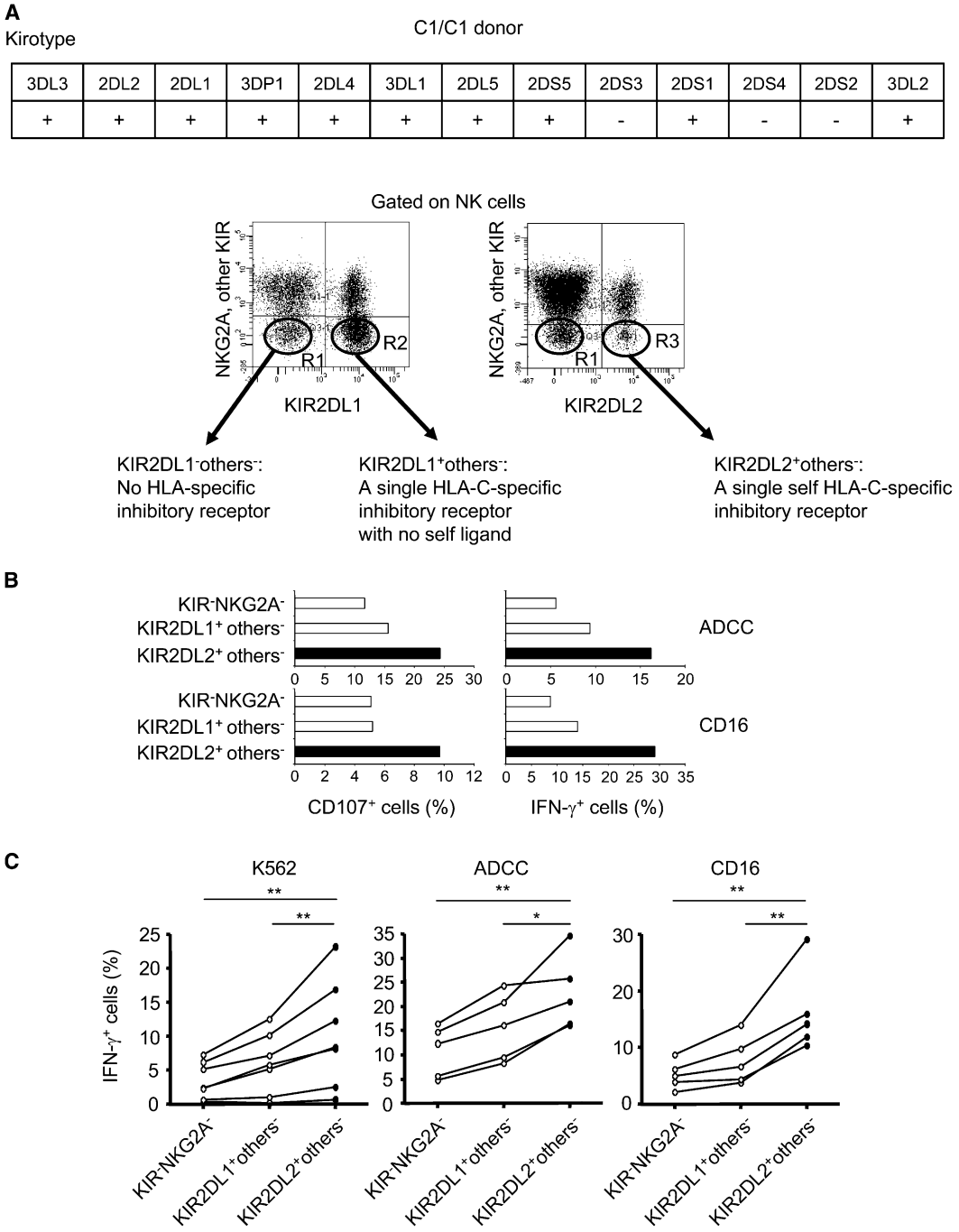

Figure 6. Evidence for a Role of Inhibitory KIR and HLA Class I in the Acquisition of NK Cell Function in C1C1 Individuals

(A) Top: The KIR genotype (kirotype) of a representative C1C1 KIR2DL2$^+$ donor. Bottom: Gating of the indicated NK cell subsets.

(B) PBMC preparations were incubated for 4 hr in the presence of indicated stimuli and analyzed by 6-color flow cytometry. The bars indicate the % of CD107$^+$ or IFN-γ^+ cells in the indicated NK cell subsets. The bars corresponding to the NK cell subset that expresses a KIR interacting with self HLA class I are in black. The bars corresponding to the NK cell subset that does not express NKG2A or a KIR interacting with self HLA class I are in white. A representative experiment of seven performed with six other C1C1 individuals is shown.

(C) As in Figure 5B for a group of C1C1 donors (n = 7 for K562 stimulation; n = 5 for ADCC and CD16 stimulation), including three donors with no *2DS1* and *2DS2* genes. Each dot and line represents the results from one individual. Open symbols correspond to the NK cell subsets that do not express NKG2A or a KIR interacting with self HLA class I. Closed symbols correspond to the NK cell subset expressing a KIR interacting with self HLA class I.

Discussion

These studies were originally set up to precisely define the subset of circulating NK cells that exert a naturally occurring cytotoxic function. It has been previously shown that human NK cells can be divided in two functionally distinct subsets: CD56^{dim} and CD56^{bright} NK cells. These NK cell populations differ in their trafficking

Table 1. Function of NK Cell Subsets in HLA-C1C2 Heterozygote Individuals

	% IFN-γ^+ Cells		
Donors	KIR$^-$NKG2A$^-$	KIR2DL1$^+$others$^-$	KIR2DL2$^+$others$^-$
#7401	9.5	22	19.4
#7400	8.7	15.5	14.9
#6682	4	8.4	9.7
#6434	24.1	34.5	32.1
#7430	19	32	32.5

PBMC preparations isolated from five representative C1C2 donors were stimulated for 4 hr with plate bound CD16 mAb. Cells were stained as indicated in Experimental Procedures. Results indicate the percentage of IFN-γ-producing cells within the KIR$^-$NKG2A$^-$, KIR2DL1$^+$others$^-$, and KIR2DL2$^+$others$^-$ NK cell subsets.

properties, the CD56^{bright} NK cells having a greater number in lymphoid organs than the CD56^{dim} NK cells, and the latter being the most prominent subset in blood (Ferlazzo and Munz, 2004). The CD56^{dim} NK cell population is also reported as being more prone to cytotoxicity and the CD56^{bright} subset being more prone to produce cytokines such as IFN-γ (Cooper et al., 2001). By using freshly isolated human peripheral blood mononuclear cell preparations and a multiparameter flow cytometric assay, we show that most, if not all, NK cells that are cytolytic and/or produce IFN-γ upon K562 or antibody-coated target stimulation belong to the CD56^{dim} subset, whereas NK cells that readily respond to IL-12 or IL-15 belong to the CD56^{bright} NK cell subset. It appears therefore more operational to define CD56^{dim} and CD56^{bright} NK cell subsets as "target cell responsive" and "cytokine responsive," respectively. Thus, the functional heterogeneity of human peripheral blood NK cells is more complex than a simple "cytolytic versus cytokine producing" dichotomy, underscoring the plasticity of NK cell responses to various stimuli.

In our search for correlates between NK cell phenotype and reactivity to tumor cells, we found that the cell-surface expression of MHC class I receptors was the most divergent difference between responsive and hyporesponsive NK cells. Indeed, the KIR$^+$NKG2A$^+$ NK cells contain most of CD107$^+$ and IFN-γ^+ NK cells upon stimulation with MHC class I$^-$ tumor targets, and also to some extent upon stimulation with cytokines. These results raise a number of issues related to NK cell education and tolerance to self. It has been indeed proposed that each NK cell clone expresses at least one self MHC class I-specific inhibitory receptor, ensuring NK cell tolerance to self (Valiante et al., 1997). However, we show here that a substantial subset of KIR$^-$NKG2A$^-$ NK cells is present in freshly isolated peripheral blood from normal individuals. Regardless of the imprecision in defining *KIR* genes, pseudogenes, and alleles, at least 13 *KIR* genes can be expressed as proteins in the human population (Parham, 2005). The paucity of available KIR mAb thus represents a limit in our study and prevents us from formally concluding that the NK cells that we define as KIR$^-$ do not express any inhibitory KIR (e.g., KIR3DL3, KIR2DL5). Despite this caveat, previous results with NK cell clones and supporting the "at least one rule" were obtained with a set of mAb comparable to that used in our study (Valiante et al., 1997). It is thus possible that KIR$^-$NKG2A$^-$ NK cells are counterselected by NK cloning procedures, suggesting a difference in the survival and/or proliferation requirements between KIR$^-$NKG2A$^-$ and KIR$^+$NKG2A$^+$ NK cells.

With KIR and HLA genotyping, our results strongly support that NK cells are not fully reactive against MHC class I-deficient stimuli unless their inhibitory receptors have recognized self MHC class I molecules prior to encounter with these stimuli. Taking into account the documented interaction between KIR and HLA, the similarity of reduced responsiveness seen on one hand in KIR$^-$NKG2A$^-$ NK cells as well as in KIR$^+$NKG2A$^-$ NK cells that do not interact with self MHC class I, and on the other hand in KIR$^+$ cells in MHC class I-deficient individuals (Furukawa et al., 1999; Vitale et al., 2002; Zimmer et al., 1998), is a strong argument to propose that NK cells must encounter self class I to become fully reactive. Similar data have been obtained in the mouse, where freshly isolated NK cells that lack the cell-surface expression of inhibitory receptors for self MHC class I were hyporesponsive to various stimulations, including NKRP1 or NKG2D mAb, and MHC class I-deficient targets (Fernandez et al., 2005; Kim et al., 2005). To explain these data, it has been proposed that the engagement of inhibitory receptors with self MHC class I provides a positive signal during NK cell maturation that would lead to the "licensing" of fully competent peripheral NK cells (Kim et al., 2005). This model implies opposite signaling capacities of inhibitory MHC class I receptors according to the stage of NK cell development: positive signaling during maturation and negative signaling during effector function. At present, we cannot formally rule out this model, which is compatible with our data. Importantly, NK cells that fail to engage MHC class I-specific inhibitory receptors (i.e., KIR$^-$NKG2A$^-$ NK cells and KIR$^+$NKG2A$^-$ NK cells that do not interact with self MHC class I) are bona fide NK cells with a number of features compatible with NK cell effector capacity, such as intact cell-surface phenotype, perforin and granzyme content, as well as potent reactivity to stimuli that bypass cell-surface receptors (PMA and ionomycin). Yet these cells are hyporesponsive to a number of stimuli, such as K562 cells, which are recognized by a combination of NK cell receptors including NKp30, L721.221 cells that are recognized by NKp46, 2B4, NTBA, and CD2 (A. Moretta, personal communication), immunoglobulin-coated mouse mastocytoma P815 cells (ADCC), as well as IL-12 and IL-18 to a lesser extent. One mechanism that could explain this broad hyporesponsiveness is that these cells express an inhibitory receptor, which is not specific for MHC class I. However, this possibility would be hard to reconcile with their hyporesponsiveness in our cell-free stimulation assay (CD16 mAb-coated plates), unless the inhibitory receptor would signal in absence of ligands or if the ligand would be expressed on NK cells themselves. Therefore, the simplest explanation to our findings rather is that the effect of KIR-MHC class I interaction on NK cell development operates at a common signaling element downstream of various NK cell-activating pathways (including but not only ITAM-dependent pathways) and upstream of transcription factors' activation. Consequently, the activating transduction circuits would be calibrated during NK cell maturation, and this calibration would integrate

inhibitory signals emanating from KIR ligation. NK cell maturation in the context of inhibitory signals would result in setting more efficient activating signaling circuits than in absence of inhibitory receptor ligation. In which anatomical sites and when such educating impact of KIR-MHC class I interaction takes place remains to be defined. This "calibration model" favors an earlier hypothesis based on the crosstalks between activating and inhibitory pathways during NK cell development (Fernandez et al., 2005). According to this report, NK cell maturation would be driven by some still-unknown receptors whose activating signals would lead to NK cell hyporesponsiveness unless the engagement of inhibitory receptors with self MHC class I attenuate these signals, allowing the export of fully competent NK cells (Fernandez et al., 2005; Raulet and Vance, 2006). Calibration is a constant theme in the selection of T and B lymphocytes (Fischer and Malissen, 1998), which could thus be extended to NK cells. It is also important to note that a very large fraction of NK cells that are KIR^+NKG2A^+ do not respond to classical NK cell stimulation (e.g., K562, ADCC, CD16 mAb-coated plates). We do not believe that this is purely because these cells have no self-specific MHC class I receptor, since only 5%–30% of NK cell expressing a self-specific MHC class I receptor are capable of functional reactivity with K562. This lack of response is also not due to limitations of the test and a plateau of detection, because PMA and ionomycin induce IFN-γ production by more than 75% of resting NK cells, and IL-2-activated NK cells contain up to 80% $CD107^+$ and IFN-γ^+ cells in the standard 4 hr K562 stimulation (data not shown). Other factors thus also contribute to NK cell reactivity in addition to MHC class I-driven education. Along this line, IL-12 could be one of these factors, as indicated by the fact that NK cells from *IL-12RB1*-deficient patients are hyporesponsive to in vitro stimulation by K562 (unpublished data).

What could be the biological relevance of the calibration of NK cell-activating pathways by the recognition of self MHC class I molecules via inhibitory receptors? NK cells from MHC class I-sufficient individuals and mice kill MHC class I-deficient targets, according to the "missing self" recognition. In contrast, NK cells from MHC class I-deficient individuals and mice are tolerant to self, as shown by the fact that they do not kill the very same MHC class I-deficient targets (Furukawa et al., 1999; Ljunggren and Karre, 1990; Raulet et al., 2001; Vitale et al., 2002; Zimmer et al., 1998). However, NK cells from MHC class I-deficient mice are fully protective against mouse cytomegalovirus infection (Tay et al., 1995). We thus propose that NK cell education on self MHC class I by inhibitory receptors does not result in a global anergy versus competence fate, but rather selectively allows NK cells to discriminate between cells expressing normal levels of MHC class I and cells with downregulated MHC class I expression, i.e., "missing self" recognition. Indeed, NK cell maturation in the context of inhibitory KIR or Ly49 ligation would increase the potential of activating signaling circuits. NK cell activation pathways would thus be fully activable upon subsequent encounter with MHC class $I^{dim/-}$ cells. In addition, it has been very recently reported that HLA class I ligands increase the frequencies of NK cells expressing cognate KIR (Yawata et al., 2006).

These data might be considered for the development of our understanding of autoimmunity and in the application of immune-based therapies. In particular, genetic epidemiologic studies have revealed a role for KIR and HLA in the susceptibility or resistance to a variety of pathologies, including autoimmune syndromes, cancer, and infectious diseases (Carrington and Martin, 2006). The current interpretation of these associations relies on the role of KIR-HLA interaction in the modulation of NK cell effector function during interaction with target cells. The present results suggest that the association between KIR, HLA, and human disease might also be the consequence of the role of KIR-HLA in NK cell education. Similarly, pioneering results have shown that donor versus recipient NK cell alloreactivity could eliminate leukemia relapse and graft rejection, while protecting patients against graft versus host disease (Ruggeri et al., 2002). During the first months post-hematopoietic transplant, donor NK cells develop in the recipient just as they would develop in the donor (Parham, 2005), becoming potentially alloreactive to the hosts. Our data introduce the possibility that these NK cells would rather be hyporesponsive, if they do not find proper educating MHC class I impact in the recipient. This scenario may be highly dependent upon the recipient's conditioning regimen as well as the dose of donor hematopoietic progenitors (i.e., the likelihood of interaction with donor or recipient MHC class I molecules), providing a rational basis for the variable outcome of these MHC-mismatched hematopoietic transplantation protocols (Parham, 2005).

Finally, it is most likely that other inhibitory receptors expressed by NK cells contribute to their education through MHC class I-dependent or -independent mechanisms. Education through other receptors would hide the apparent effect of KIR-MHC class I education, suggesting that the impact of this education is larger than we can directly demonstrate. In particular, this hypothesis would explain the residual activity in KIR^-NKG2A^- NK cells and in KIR^+NKG2A^- NK cells that do not interact with self MHC class I. Despite this possibility, it is remarkable that a role for the inhibitory KIR remains apparent, showing a dominant role for these molecules at the whole NK cell population level. More generally, a variety of other inhibitory receptors have been described on hematopoietic cells, several of them being expressed at early stages of cell differentiation (Daeron and Vivier, 1999; Long, 1999; Ravetch and Lanier, 2000). Thus, inhibitory receptors, other than MHC class I-specific receptors, might also educate a wider array of hematopoietic cells to discriminate between interacting cells that do, or do not, express self ligands.

Experimental Procedures

Cells and Patients

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation (Amersham Pharmacia Biotech) from whole-blood samples obtained from normal healthy volunteer donors, according to the local ethics committees on human experimentations (München, Marseille).

CD107 Mobilization Assay

Natural cytotoxicity was assessed with the MHC class I^- human erythroleukemic K562 target cells, and ADCC was assessed with

the P815 mouse mastocytoma cells coated with rabbit anti-mouse lymphocyte antibodies (Accurate Biochemicals). NK cells were tested for their cytolytic potential with the CD107 mobilization assay. In this test, FITC-CD107 mAb was added to the effector and target cell mixtures during a 4 hr incubation. $FITC^+$ cells were then scored by flow cytometry, revealing cells that have undergone granule-mediated cytotoxicity. Monensin was added during the assay to prevent the acidification of the endosomal compartment, which could alter the fluorescence of internalized CD107:FITC-CD107 mAb complexes (Rubio et al., 2003).

Cell Stimulation

For CD16 stimulation, $F(ab)'_2$ goat anti-mouse IgG (Immunotech) was coated to plastic wells for 2 hr in PBS at 37°C at 20 μg/mL. After washes, mouse CD16 mAb (Pharmingen) was incubated for 30 min at 4°C at 10 μg/mL in PBS. After washes, 5.10^6 PBMC were plated in each well in the presence of CD107 mAb and monensin as described in the next section.

mAb and Flow Cytometric Analysis

The following mouse mAb were used: APC-Cy7, APC-CD3 (SK7), APC-CD3 (UCHT1), APC-CD11c (B-Ly6), PE-Cy5-CD107a (H4A3), FITC-CD107a (H4A3), FITC-CD107b (H4B4), FITC-CD85j (GHI/75), FITC-CD20 (2H7), FITC-CD40 (5C3), and FITC-MHC class II (Tü39) (Becton Dickinson); and FITC-CD11b (BEAR1), FITC-CD16 (3G8), FITC-CD95 (UB2), FITC-CD83 (HB15a), PE-Cy7-CD56 (N901), PE-NKG2D (ON72), PE-NKp30 (Z25), PE-NKp46 (Bab281), PE-CD94 (HP-3B1), PE-NKG2A (Z199), PE-KIR2DL1/S1 (EB6), PE-KIR2DL2/L3/S2 (GL183), PE-KIR3DL1 (Z27), and PE-KIR2DS4 (FES172) (Beckman Coulter). Biotin-KIR and biotin-PEN5 (5H10) mAb were produced at Innate-Pharma and revealed with PerCP streptavidin (Becton Dickinson). NK cells were defined as the $CD3^-CD56^+$ cells within the lymphocyte size/granulometry gate. Two reciprocal NK cell subsets were distinguished: KIR^-NKG2A^- NK cells that do not express KIR2DL1, KIR2DS1 (CD158a, h), KIR2DL2, KIR2DS2 (CD158b, j), KIR2DS4 (CD158i), KIR3DL1 (CD158e1) or NKG2A (CD159a), and KIR^+NKG2A^+ NK cells that express at least one of these receptors. For $KIR2DL1^+KIR2DS1^-$ donors, $KIR2DL1^+others^-$ NK cells were defined as NK cells that stained positive with KIR2DL1, KIR2DS1 mAb and negative for a mixture of mAb reacting with KIR2DL2, KIR2DS2, KIR2DS4, KIR3DL1, and NKG2A. Reciprocally for $KIR2DL2^+KIR2DS2^-$ donors, $KIR2DL2^+others^-$ NK cells were defined as NK cells that stained positive with KIR2DL2, KIR2DS2 mAb and negative for a mixture of mAb reacting with KIR2DL1, KIR2DS1, KIR2DS4, KIR3DL1, and NKG2A.

For CD107 mobilization, INF-γ production, and perforin and granzyme B detection, PBMC were incubated 4 hr at 37°C in the presence of 5 μM monensin (Sigma), CD107 mAb, and various stimuli. Cells were then washed in PBS 2 mM EDTA and stained for extracellular markers. Thereafter, cells were fixed and permeabilized with IntraPrep reagent (Beckman Coulter). Intracellular IFN-γ, perforin, and granzyme B were detected with Alexa 647-IFN-γ (B27), PE-perforin (δG9), and FITC-granzyme B (Gb11) (Becton Dickinson). Four-, five-, and six-color fluorescent stainings were analyzed on a FACSCanto with the FACSDiva software (Becton Dickinson). Results are presented as mean ± SD of positive-stained cells, and statistical analysis has been performed with paired Student's t tests.

Genotyping

Genomic DNA was isolated from mononuclear cells via a standard salting-out method and quantified by spectrophotometry. KIR genotyping was performed by PCR-SSOP (Crum et al., 2000), by PCR-SSP with a PEL-FREEZ KIR Genotyping SSP Kit (Dynal Biotech, Brown Deer, WI), and/or with the KIR-SSO kit from One-Lamda. HLA-C typing was performed by PCR-SSOP (Williams et al., 2002) or with the HLA-C-SSO kit from One-Lamda. As *KIR2DL2* and *KIR2DL3* are alleles, they were both referred to as *KIR2DL2*. Similarly, *KIR3DL1* and *KIR2DS1* alleles were referred to as *KIR3DL1*.

Supplemental Data

Two Supplemental Figures can be found with this article online at http://www.immunity.com/cgi/content/full/25/2/331/DC1/.

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References

Andre, P., Spertini, O., Guia, S., Rihet, P., Dignat-George, F., Brailly, H., Sampol, J., Anderson, P.J., and Vivier, E. (2000). Modification of P-selectin glycoprotein ligand-1 with a natural killer cell-restricted sulfated lactosamine creates an alternate ligand for L-selectin. Proc. Natl. Acad. Sci. USA *97*, 3400–3405.

Bakker, A.B., Hoek, R.M., Cerwenka, A., Blom, B., Lucian, L., McNeil, T., Murray, R., Phillips, L.H., Sedgwick, J.D., and Lanier, L.L. (2000). DAP12-deficient mice fail to develop autoimmunity due to impaired antigen priming. Immunity *13*, 345–353.

Boyington, J.C., and Sun, P.D. (2002). A structural perspective on MHC class I recognition by killer cell immunoglobulin-like receptors. Mol. Immunol. *38*, 1007–1021.

Bryceson, Y.T., March, M.E., Barber, D.F., Ljunggren, H.G., and Long, E.O. (2005). Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells. J. Exp. Med. *202*, 1001–1012.

Carrington, M., and Martin, M.P. (2006). The impact of variation at the KIR gene cluster on human disease. Curr. Top. Microbiol. Immunol. *298*, 225–257.

Cooper, M.A., Fehniger, T.A., Turner, S.C., Chen, K.S., Ghaheri, B.A., Ghayur, T., Carson, W.E., and Caligiuri, M.A. (2001). Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood *97*, 3146–3151.

Crum, K.A., Logue, S.E., Curran, M.D., and Middleton, D. (2000). Development of a PCR-SSOP approach capable of defining the natural killer cell inhibitory receptor (KIR) gene sequence repertoires. Tissue Antigens *56*, 313–326.

Daeron, M., and Vivier, E. (1999). Biology of immunoreceptor tyrosine-based inhibition motif-bearing molecules. Curr. Top. Microbiol. Immunol. *244*, 1–12.

Dorfman, J.R., and Raulet, D.H. (1996). Major histocompatibility complex genes determine natural killer cell tolerance. Eur. J. Immunol. *26*, 151–155.

Ferlazzo, G., and Munz, C. (2004). NK cell compartments and their activation by dendritic cells. J. Immunol. *172*, 1333–1339.

Fernandez, N.C., Treiner, E., Vance, R.E., Jamieson, A.M., Lemieux, S., and Raulet, D.H. (2005). A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. Blood *105*, 4416–4423.

Fischer, A., and Malissen, B. (1998). Natural and engineered disorders of lymphocyte development. Science *280*, 237–243.

Furukawa, H., Yabe, T., Watanabe, K., Miyamoto, R., Miki, A., Akaza, T., Tadokoro, K., Tohma, S., Inoue, T., Yamamoto, K., and Juji, T. (1999). Tolerance of NK and LAK activity for HLA class I-deficient targets in a TAP1-deficient patient (bare lymphocyte syndrome type I). Hum. Immunol. *60*, 32–40.

Hoglund, P., Sundback, J., Olsson-Alheim, M.Y., Johansson, M., Salcedo, M., Ohlen, C., Ljunggren, H.G., Sentman, C.L., and Karre,

K. (1997). Host MHC class I gene control of NK-cell specificity in the mouse. Immunol. Rev. *155*, 11–28.

Kim, S., Poursine-Laurent, J., Truscott, S.M., Lybarger, L., Song, Y.J., Yang, L., French, A.R., Sunwoo, J.B., Lemieux, S., Hansen, T.H., and Yokoyama, W.M. (2005). Licensing of natural killer cells by host major histocompatibility complex class I molecules. Nature *436*, 709–713.

Ljunggren, H.G., and Karre, K. (1990). In search of the 'missing self': MHC molecules and NK cell recognition. Immunol. Today *11*, 237–244.

Long, E.O. (1999). Regulation of immune responses through inhibitory receptors. Annu. Rev. Immunol. *17*, 875–904.

Martin-Fontecha, A., Thomsen, L.L., Brett, S., Gerard, C., Lipp, M., Lanzavecchia, A., and Sallusto, F. (2004). Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. Nat. Immunol. *5*, 1260–1265.

Moretta, L., and Moretta, A. (2004). Unravelling natural killer cell function: triggering and inhibitory human NK receptors. EMBO J. *23*, 255–259.

Moretta, A., Bottino, C., Mingari, M.C., Biassoni, R., and Moretta, L. (2002). What is a natural killer cell? Nat. Immunol. *3*, 6–8.

Natarajan, K., Dimasi, N., Wang, J., Mariuzza, R.A., and Margulies, D.H. (2002). Structure and function of natural killer cell receptors: multiple molecular solutions to self, nonself discrimination. Annu. Rev. Immunol. *20*, 853–885.

Olsson, M.Y., Kärre, K., and Sentman, C.L. (1995). Altered phenotype and functon of natural killer cells expressing the major histocompatibility complex receptor Ly-49 in mice transgenic for its ligand. Proc. Natl. Acad. Sci. USA *92*, 1649–1653.

Parham, P. (2005). MHC class I molecules and KIRs in human history, health and survival. Nat. Rev. Immunol. *5*, 201–214.

Raulet, D.H., and Vance, R.E. (2006). Self-tolerance of natural killer cells. Nat. Rev. Immunol. *6*, 520–531.

Raulet, D.H., Vance, R.E., and McMahon, C.W. (2001). Regulation of the natural killer cell receptor repertoire. Annu. Rev. Immunol. *19*, 291–330.

Ravetch, J.V., and Lanier, L.L. (2000). Immune inhibitory receptors. Science *290*, 84–89.

Rubio, V., Stuge, T.B., Singh, N., Betts, M.R., Weber, J.S., Roederer, M., and Lee, P.P. (2003). Ex vivo identification, isolation and analysis of tumor-cytolytic T cells. Nat. Med. *9*, 1377–1382.

Ruggeri, L., Capanni, M., Urbani, E., Perruccio, K., Shlomchik, W.D., Tosti, A., Posati, S., Rogaia, D., Frassoni, F., Aversa, F., et al. (2002). Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science *295*, 2097–2100.

Stewart, C.A., Laugier-Anfossi, F., Vely, F., Saulquin, X., Riedmuller, J., Tisserant, A., Gauthier, L., Romagne, F., Ferracci, G., Arosa, F.A., et al. (2005). Recognition of peptide-MHC class I complexes by activating killer immunoglobulin-like receptors. Proc. Natl. Acad. Sci. USA *102*, 13224–13229.

Tay, C.H., Welsh, R.M., and Brutkiewicz, R.R. (1995). NK cell response to viral infections in beta 2-microglobulin-deficient mice. J. Immunol. *154*, 780–789.

Tomasello, E., Desmoulins, P.O., Chemin, K., Guia, S., Cremer, H., Ortaldo, J., Love, P., Kaiserlian, D., and Vivier, E. (2000). Combined natural killer cell and dendritic cell functional deficiency in KARAP/DAP12 loss-of-function mutant mice. Immunity *13*, 355–364.

Trowsdale, J. (2001). Genetic and functional relationships between MHC and NK receptor genes. Immunity *15*, 363–374.

Valiante, N.M., Uhrberg, M., Shilling, H.G., Lienert-Weidenbach, K., Arnett, K.L., D'Andrea, A., Phillips, J.H., Lanier, L.L., and Parham, P. (1997). Functionally and structurally distinct NK cell receptor repertories in the peripheral blood of two human donors. Immunity *7*, 739–751.

Vitale, M., Zimmer, J., Castriconi, R., Hanau, D., Donato, L., Bottino, C., Moretta, L., de la Salle, H., and Moretta, A. (2002). Analysis of natural killer cells in TAP2-deficient patients: expression of functional triggering receptors and evidence for the existence of inhibitory receptor(s) that prevent lysis of normal autologous cells. Blood *99*, 1723–1729.

Vivier, E., Nunes, J.A., and Vely, F. (2004). Natural killer cell signaling pathways. Science *306*, 1517–1519.

Williams, F., Meenagh, A., Patterson, C., and Middleton, D. (2002). Molecular diversity of the HLA-C gene identified in a caucasian population. Hum. Immunol. *63*, 602–613.

Wolint, P., Betts, M.R., Koup, R.A., and Oxenius, A. (2004). Immediate cytotoxicity but not degranulation distinguishes effector and memory subsets of CD8+ T cells. J. Exp. Med. *199*, 925–936.

Yawata, M., Yawata, N., Draghi, M., Little, A.M., Partheniou, F., and Parham, P. (2006). Roles for HLA and KIR polymorphisms in natural killer cell repertoire selection and modulation of effector function. J. Exp. Med. *203*, 633–645.

Yokoyama, W.M., and Kim, S. (2006). How do natural killer cells find self to achieve tolerance? Immunity *24*, 249–257.

Yokoyama, W.M., and Plougastel, B.F. (2003). Immune functions encoded by the natural killer gene complex. Nat. Rev. Immunol. *3*, 304–316.

Zimmer, J., Donato, L., Hanau, D., Cazenave, J.P., Tongio, M.M., Moretta, A., and Salle, H. (1998). Activity and phenotype of natural killer cells in peptide transporter (TAP)-deficient patients (type I bare lymphocyte syndrome). J. Exp. Med. *187*, 117–122.