Large Spectrum of HLA-C Recognition by Killer Ig–like Receptor (KIR)2DL2 and KIR2DL3 and Restricted C1 Specificity of KIR2DS2: Dominant Impact of KIR2DL2/KIR2DS2 on KIR2D NK Cell Repertoire Formation

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Large Spectrum of HLA-C Recognition by Killer Ig–like Receptor (KIR)2DL2 and KIR2DL3 and Restricted C1 Specificity of KIR2DS2: Dominant Impact of KIR2DL2/KIR2DS2 on KIR2D NK Cell Repertoire Formation

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The interactions of killer Ig–like receptor 2D (KIR2D) with HLA-C ligands contribute to functional NK cell education and regulate NK cell functions. Although simple alloreactive rules have been established for inhibitory KIR2DL, those governing activating KIR2DS function are still undefined, and those governing the formation of the KIR2D repertoire are still debated. In this study, we investigated the specificity of KIR2DL1/2/3 and KIR2DS1/2, dissected each KIR2D function, and assessed the impact of revisited specificities on the KIR2D NK cell repertoire formation from a large cohort of 159 KIR and HLA genotyped individuals. We report that KIR2DL2* and KIR2DL3* NK cells reacted similarly against HLA-C* target cells, irrespective of C1 or C2 allele expression. In contrast, KIR2DL1* NK cells specifically reacted against C2 alleles, suggesting a larger spectrum of HLA-C recognition by KIR2DL2 and KIR2DL3 than KIR2DL1. KIR2DS2* KIR2DL2* NK cell clones were C1-reactive irrespective of their HLA-C environment. However, when KIR2DS2 and KIR2DL2 were coexpressed, NK cell inhibition via KIR2DL2 overrode NK cell activation via KIR2DS2. In contrast, KIR2DL1 and KIR2DS2 had an additive enhancing effect on NK cell responses against C1C1 target cells. KIR2DL2/3/S2 NK cells predominated within the KIR repertoire in KIR2DL2/S2* individuals. In contrast, the KIR2DL1/S1 NK cell compartment is dominant in C2C2 KIR2DL2/S2* individuals. Moreover, our results suggest that together with KIR2DL2, activating KIR2DS1 and KIR2DS2 expression limits KIR2DL1 acquisition on NK cells. Altogether, our results suggest that the NK cell repertoire is remodeled by the activating and inhibitory KIR2D and their cognate ligands.

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Natural killer cells constitute the first line of defense against viral infections and tumor cells. These effectors of innate immunity discriminate self and nonself via inhibitory receptors that recognize HLA class I molecules in an allelic fashion. The absence or default of HLA class I expression on altered cells is a well-established characteristic of virally transformed and tumor cells, and leads to enhanced NK cell proli-feration, cytotoxicity, and cytokine production (1). These functional abilities confer to alloreactive NK cells a preponderant contribution to cell responses in allogeneic hematopoietic stem cell transplantation (HSCT) (2, 3). Similarly, a semiallogeneic context observed in pregnancy constitutes a new HLA environment that requires a complete modulation of self HLA molecules to avoid activation of NK cells via inhibitory receptors. Among inhibitory receptors, killer Ig–like receptors (KIRs) recognize mainly HLA-C molecules. The nonclassical HLA-E molecule presents a peptide from the signal peptide of HLA class I molecules and constitutes a second line of self-presentation. This molecule is recognized by the inhibitory heterodimer CD94/NKG2A, which is acquired before KIRs on NK cells during development (4). Finally, ILT-2 recognizes all HLA class I molecules by engaging the conserved β2-microglobulin and α3 domain of HLA class I molecules. KIRs are clonally expressed (5) on NK cells, leading to large combinations of KIR expression in different proportions of each KIR NK cell subset (6). During development, NK cells acquire a functional potential that is, in part, determined by the capacities of each NK cell to engage its inhibitory receptors with self HLA class I molecules (7). In addition, the number of KIR gene copies contributes to increased NK cell responsiveness (8). Different theories have been proposed to explain the formation of the KIR NK cell repertoire. Some groups have proposed a model following a sequential acquisition of KIR that is dependent on the HLA environment (9). In parallel, based on combinatorial analysis of KIR NK cells from haplotype AA individuals, Malmberg’s group (10) has proposed that the variegated
LIGAND SPECIFICITY OF ACTIVATING AND INHIBITORY KIR2D

Materials and Methods

Cells (PBMCs and cell lines)

PBMCs were isolated as previously described (15, 18). All blood donors were recruited at the Blood Transfusion Center (Etablissement Français du Sang, Nantes, France), and informed consent was given by all donors. HLA class I-deficient 271.221 lymphoblastoid cells, referred to as 221 cells, were used as non-HLA antigenic PBMCs and HLA-C*03:04 (C1) and HLA-C*04:01 (C2) transfected 221 cell lines used as feeders following the PBMC/EBV-B cells ratio at 10:1. KIR2DS2+ NK cells were cloned at 0.3 cell/well from the selected and amplified lines under limiting conditions in 96-well U-bottom microtiter plates with 50,000 irradiated autologous lymphocytes and 5000 irradiated HLA-C*03:04 (C1) and HLA-C*04:01 (C2) transfected 221 cell lines in a final volume of 100 μL. Growing colonies with a probability of monoclonality of 95% were kept for further analysis. NK cell lines and clones were cultured in RPMI 1640 medium (Life Technologies, Paisley, U.K.) containing glutamine (Life Technologies) and penicillin-streptomycin (Life Technologies) and supplemented with 10% human serum (Etablissement Français du Sang, Nantes, France) and 200 U/ml IL-2 (Chiron, Surens, France). Cells were maintained for 3 wk and expanded in their culture medium containing IL-2 without restimulation with feeders before functional analysis to decrease their spontaneous degranulation.

KIR2DL2 RT-PCR

Total RNA was purified from isolated KIR2DS2+ NK clones using NucleoSpin RNA XS (Macherey-Nagel). Qualitative KIR2DL2 RT-PCR was performed using a One-Step PrimeScript RT-PCR kit (TaKaRa, Japan) with KIR2DL2-specific primers from Thompson et al. (19).

Phenotypic analysis by flow cytometry

The NK cell–surface phenotype was determined by three- or four-color flow cytometry using the following mouse anti-human mAbs: anti–KIR2DL1-PE (Z199; Beckman Coulter), anti–KIR2DL2/2DS2-PE (GL183; Beckman Coulter Immunotech, Marseille, France), anti–CD3-PerCP (SK7), anti–CD56-allophycocyanin (B159; BD Biosciences), anti–CD107a-PE-Cy7 (H4A3; BD Biosciences), anti–CD16-FITC (3G8; BD Biosciences), anti–CD56-biotin (Z469/1; BD Biosciences), anti–KIR2DL3/2DS1/2-PE (F112), and anti–KIR2DL1/2/2DS1/2-FTC (1A6), generated and characterized in our laboratory and previously described (20). The KIR2DS2+ NK cell clone phenotype was determined using the following mouse anti-human mAbs: anti–KIR2DS4-FITC (143211; R&D Systems), anti–KIR2DL1/2DS1/2/2DS2-PE (GL183; Beckman Coulter Immunotech, Marseille, France), anti–CD3-PerCP (SK7), anti–CD56-allophycocyanin (B159; BD Biosciences), anti–KIR2DL1/2/2DS1/2-FTC (8C11), anti–KIR2DL3/2DS1/2-FTC (1F12), and anti–KIR2DL1/2/2DS1/2-FTC (1A6).

CD107a mobilization assay detected by flow cytometry

NK cells were preincubated with anti–CD107a-PE (HA43; BD Biosciences). NK cell degranulation was assessed after incubation for 5 h (negative
KIR2DL2+ and KIR2DL3+ NK cells from C1+ individuals showed with a lower frequency of positive cells (data not shown). Moreover, the frequency of CD107a+ KIR2DL2+ or CD107a+ KIR2DL3+ NK cells did not correlate with the presence or absence of particular C1 alleles; in contrast, some HLA class I molecules previously identified as C2 alleles were systematically excluded from functional studies. KIR2DL1 recognized HLA-C*04:01 (i.e., a “C2 allele”), but not HLA-C*03:04 (i.e., a “C1 allele”; Fig. 1A). However, KIR2DL2+ (Fig. 1C) and KIR2DL3+ NK cells showed a similar degranulation potential to their C1+ counterparts.

**Results**

**C1** activating KIR2DL2+ and KIR2DL3+ NK cells present a similar degranulation potential to their C1+ counterparts.

As a first step, we studied degranulation of KIR2DL NK subpopulations expressing either KIR2DL1, KIR2DL2, or KIR2DL3 using appropriate combinations of KIR-specific Abs targeting each of these receptors as previously described (20) (Fig. 1A). Thus, KIR2DL1+ KIR2DL2/3/S1/2+ NK cells were sorted using a combination of KIR2DL1/2/3/S2-specific 8C11 and KIR2DL2/3/S2-specific GL183 Abs from KIR2DS1+ genotyped individuals (Fig. 1A). KIR2DL2+ KIR2DL1/3/S1/2+ NK cells were sorted using a combination of a mix of KIR2DL3/S2-specific 1F12 with KIR2DL1-specific 143211 Abs and KIR2DL2/3/S2-specific GL183 from KIR2DS1+ genotyped individuals (Fig. 1A). KIR2DL3+ KIR2DL2/3/S1/2+ NK cells were sorted using a combination of KIR2DL2/3/S2-specific GL183 and KIR2DL1/S1-specific EB6 Abs from KIR2DL2/S2+ genotyped individuals (Fig. 1A). All sorted KIR2DL NK cells were expanded after in vitro stimulation. NK cells expressing inhibitory NKG2A receptors were systematically excluded from functional studies. KIR2DL1 recognized HLA-C*04:01 (i.e., a “C2 allele”), but not HLA-C*03:04 (i.e., a “C1 allele”; Fig. 1B). However, KIR2DL2+ (Fig. 1C) and KIR2DL3+ NK cells (Fig. 1D) similarly recognized both HLA-C*03:04 (C1) and HLA-C*04:01 (C2) molecules as shown by the CD107a upregulation assay. The same profile has been obtained for IFN-γ production with a lower frequency of positive cells (data not shown). Moreover, KIR2DL2+ and KIR2DL3+ NK cells from C1+ individuals showed a similar degranulation potential to NK cells from C1− individuals, as assessed against the HLA class I-deficient 221 cell line (Fig. 1C, 1D). This result was also observed for KIR2DL2+ and KIR2DL3+ NK cells amplified with 221 cells as feeders, eliminating a possible education of these NK cells by HLA-C molecules expressed by feeders (data not shown). In contrast, KIR2DL1+ NK cells showed efficient degranulation against the 221 cell line only in C2− individuals (Fig. 1B). Although variable from one individual to another, the frequency of CD107a+ KIR2DL2+ or CD107a+ KIR2DL3+ NK cells did not correlate with the presence or absence of particular C1 or C2 alleles. Altogether, these results suggest that the stringent C2 specificity of KIR2DL1 confers an NK education only in C2− individuals; in contrast, some HLA class I molecules previously identified as C2 ligands could be recognized by KIR2DL2 and KIR2DL3 receptors and could mediate, at least to some extent, education of KIR2DL2/3 NK cells. Thus, a preactivation of these KIR2DL NK cell subsets, notably by cytokine stimulation, seems sufficient to decrease their activation threshold.

**Broad HLA-C specificity of KIR2DL2+ and KIR2DL3+ NK cells but restricted C2 specificity of KIR2DL1+ NK cells**

To investigate the range of C2 specificity of KIR2DL2 and KIR2DL3, we evaluated the ability of KIR2DL NK cell subsets to recognize different C1 and C2 ligands, when compared with control KIR2DL1+ cells (Fig. 1E). All HLA-C transfected 221 cell lines similarly expressed HLA class I molecules except 221-C*15:02, which is less recognized by KIR2DL2 and KIR2DL3 NK cells (Fig. 1F). The C2 specificity of KIR2DL1 was confirmed toward four different HLA ligands: HLA-C*04:01, C*02:02, C*06:02, and C*15:01 with a hierarchy of recognition. Indeed, HLA-C*04:01 inhibited more efficiently KIR2DL1 NK cell degranulation than HLA-C*02:02 and HLA-C*15:01. HLA-C*06:02 was less but variably recognized by KIR2DL1 NK cells. Although the C1 allele HLA-C*03:04 was not recognized by KIR2DL1, another C1 allele HLA-C*08:02 (C1) partially inhibited KIR2DL1 NK cell degranulation. The same hierarchy of C2 allele-induced inhibition was also observed with KIR2DL2 and KIR2DL3 NK cells. Indeed, these cells, irrespective of their C1 molecule, were strongly inhibited by HLA-C*04:01, inhibited at an intermediate level by HLA-C*15:02, and less inhibited by HLA-C*02:02 and C*06:02. In contrast with KIR2DL1 cells, HLA-C*08:02 (C1) was less recognized than HLA-C*03:04 (C1) by KIR2DL2+ and KIR2DL3+ NK cell subsets. These results suggest broad HLA-C specificity of KIR2DL2 and KIR2DL3 cells, and a hierarchy of recognized HLA-C ligands within each C1 and C2 group.

**Stringent C2 allosreactivity of KIR2DS1+ NK cells**

In parallel with KIR2DL, we investigated C2 specificity of KIR2DS1 NK cells to validate our experimental approach before investigating HLA-C specificity of KIR2DS2 in this setting. We previously showed that different functional capabilities (degranulation, IFN-γ secretion, and proliferation) of KIR2DS1+ NK cell lines were triggered by 221-HLA-C*04:01+ (C2) cells, only among C2− individuals (15). In this study, we confirmed degranulation of sorted and in vitro expanded KIR2DS1+ KIR2DL2/3/S2+ NK2GA+ NK cells (Fig. 2A) by a broad panel of HLA-C1 transfected 221 cells (Fig. 2B), as illustrated for one representative individual (Fig. 2C). Interestingly, HLA-C*02:02 induced stronger CD107a expression than HLA-C*04:01 and HLA-C*06:02 by KIR2DS1+ NK cells. We cloned KIR2DS1+ KIR2D− NK cells and confirmed the absence of KIR2DS1+ NK cell allosreactivity against C2− target cells in C2− individuals (Fig. 2D). In contrast, C2− KIR2DS1+ NK cell clones showed strong allosresponsiveness against HLA-C*04:01- and HLA-C*02:02- (C2) target cells (Fig. 2D). The C2 specificity of KIR2DS1 was confirmed for four clones isolated from three C2− individuals. Our results suggest that KIR2DS1 shows stringent C2 specificity, like its inhibitory counterpart, KIR2DL1.

**C1 specificity of KIR2DS2**

To address the specificity of KIR2DS2 NK cells and reassess the rules governing activation of KIR2DS2+ NK cells, we used KIR2DS2+ KIR2DL1/3/S1− NK cell lines from KIR2DL3− genotyped individuals, obtained after sorting with a combination of the KIR2DL3/S2-specific 1F12 and KIR2DL1/S1-specific EB6 Abs (20), and subsequently in vitro expansion (Fig. 3A). Because the KIR2DS2+ NK cell population can coexpress the inhibitory KIR2DL2, we cloned KIR2DS2+ KIR2DL1/S1− NK cells and discriminated KIR2DS2+ clones from KIR2DS2− ones by KIR2DL2 RT-PCR analysis (Fig. 3B). Fig. 3C illustrates the degranulation of two KIR2DS2+ (KIR2DL2+ and KIR2DL2+) NK cell clones isolated from the same individual against 221, 221-C1 (HLA-C*03:04), and 221-C2 (HLA-C*04:01) cell lines. In accordance with their KIR2DL2 RNA profile, the degranulation of KIR2DS2+ KIR2DL2+ NK cell clones was strongly activated by the 221-C1 (HLA-C*03:04) cell line, but not by 221 or 221-C2 (HLA-C*04:01) cell lines. In contrast, degranulation of KIR2DS2+ KIR2DL2+ NK cell clones was strongly inhibited by both 221-C1 (HLA-C*03:04) and 221-C2 (HLA-C*04:01) cell lines. This observation confirms not only the broader specificity of KIR2DL2 NK cells (Fig. 1C), but also the
FIGURE 1. Comparable degranulation potential of C1− and C1+ activated KIR2DL2+ and KIR2DL3+ NK cells because of a large HLA-C specificity. (A) After depletion of CD3+ cells from PBMCs, KIR2DL1+ KIR2DL2/3/S1/2− NK cells (Nkp46+ 8C11+ GL183+) were cell sorted (7.2% of the population; day 0 [d0]) and amplified in vitro with irradiated allogeneic PBMCs and EBV-B cells as feeders. After 3 wk of stimulation (d20), the phenotype of these sorted and stimulated NK cells was assessed by flow cytometry and is illustrated for 1 representative KIR2DL1+ genotyped individual out of 11 studied individuals. Seventy-four percent of these amplified cells are KIR2DL1+ KIR2DL2/3/S1/2− NK cells (Nkp46+ 8C11+ GL183+) and 43% are NKG2A−. Following the same protocol, KIR2DL2+ KIR2DL1/3/S1/2− NK cells (Nkp46+ 1F12−′ 143211−′ GL183+) were cell sorted (11.9% of the population; d0) and amplified in vitro. After 3 wk of stimulation, the phenotype of these sorted and stimulated cells was assessed by flow cytometry and is illustrated for 1 representative KIR2DL2+ KIR2DL1/3/S1/2− genotyped individual out of 10 studied individuals. Sixty-eight percent of these amplified cells are KIR2DL2+ KIR2DL1/3/S1/2− NK cells (Nkp46+ 1F12−′ 143211−′ GL183+) and 34% are NKG2A−. Finally, KIR2DL3+ KIR2DL1/2/S1/2− NK cells (Nkp46+ GL183−′ EB6+) were cell sorted (21.9% of the population; d0) and amplified in vitro. The phenotype of these sorted and stimulated cells is illustrated for 1 representative KIR2DL3+ KIR2DL2− genotyped individual out of 15 studied individuals. Ninety-five percent of these amplified cells are KIR2DL3+ KIR2DL2/3/S1/2− NK cells (Nkp46− GL183−′ EB6−) and 70% are NKG2A−. Scatter plots displaying the CD107a+ KIR2DL+ NK cell frequency determined after 5-h degranulation assay at an E:T ratio of 1:1 in different stimulation conditions: medium, 221 cells, HLA-C*03:04 (C1) and HLA-C*04:01 (C2) transfected 221 cells (B) for selected and amplified KIR2DL1+ KIR2DL2/3/S1/2− NKG2A− NK cells from C2− (n = 7) and C2− (n = 4) individuals; (C) for selected and amplified KIR2DL2+ KIR2DL1/3/S1/2− NKG2A− NK cells from C1− (n = 4) and C1− (n = 6) individuals; and (D) for selected and amplified KIR2DL3+ KIR2DL2/3/S1/2− NKG2A− NK cells from C1− (n = 7) and C1− (n = 8) individuals. (E) Bars represent the mean of CD107a+ KIR2DL+ NK cell percentages (± SD) evaluated with a larger panel of C1 (HLA-C*03:01 and C*08:02) and C2 (HLA-C*04:01, C*02:02, C*06:02, and C*15:01) transfected 221 cells from previously studied individuals in (B)–(D). Educated NK cells (C1+ KIR2DL2+, C1+ KIR2DL3+, and C1+ KIR2DL3+). LIGAND SPECIFICITY OF ACTIVATING AND INHIBITORY KIR2D
dominant effect of NKR-induced inhibitory over activating signals. To determine the impact of the HLA-C environment on C1 reactivity of KIR2DS2+ NK cells, we evaluated the degranulation of 27 KIR2DS2+ KIR2DL2+ NK cell clones (Fig. 4A) and 14 KIR2DS2+ KIR2DL2+ NK cell clones (Fig. 4B) derived from 10 individuals (2 C1C1, 4 C1C2, and 4 C2C2) against 221, 221-C1 (HLA-C*03:04), and 221-C2 cells (HLA-C*04:01). Of note, we could not generate KIR2DS2+ KIR2DL2+ NK clones from C1C1 individuals. NK cell degranulation toward the 221 cell line varied from one KIR2DS2+ NK cell clone to another. Because this variability could be linked to expression of other inhibitory or activating NKRs, we assessed expression of unengaged inhibitory receptors, like KIR3DL1 and ILT2, and activating receptors (e.g., KIR2DS4, Nkp30, Nkp44, NKG2D, 2B4, and CD16). For some clones, KIR3DL1 and NCR expression could explain a high reactivity toward 221 target cells of some clones, such as the 1F9 clone from D9 individual. However, this heterogeneity was most probably linked to previous in vitro stimulation of NK cell clones. A CD107a mobilization assay showed increased degranulation of KIR2DS2+ NK cell clones with the 221-C1 (HLA-C*03:04) cell line in contrast with 221 and 221-C2 (HLA-C*04:01) counterparts, irrespective of the HLA-C background of individuals, even though few KIR2DS2+ KIR2DL1+/2DS1+ clones were obtained from C1C1 individuals. Overall, the degranulation patterns of KIR2DS2+ KIR2DL2+ clones were similar in C1+ (n = 11) versus C1- individuals (n = 8), because these clones were stimulated by the 221-C1 (HLA-C*03:04) cell line.

C2+ KIR2DL1+ cells are indicated in light gray and uneducated NK cells (C1- KIR2DL2+, C1- KIR2DL3+, and C2+ KIR2DL1+) in dark gray. To homogenize the data obtained from independent experiments, we present the data as the ratio of the degranulation obtained with HLA-C transfected 221 cells to the degranulation obtained with untransfected 221 cells.
кало EDTA Buffer with Sequence Specific Primer Size Marker (One Lambda). RNA was extracted from KIR2DS2+ KIR2DL1/3/S1 were cell sorted (10% of the population; d0) from KIR2DL3

and specific RT-PCR was then performed using KIR2DL2-specific PCR-SSP primers showing a specific RT-PCR product at 383 bp for some KIR2DS2+

clone in different culture conditions: medium, 221, HLA-C*03:04 (C1), and HLA-C*04:01 (C2) transfected 221 target cells at an ET ratio of 1:1.

representative KIR2DS2+ genotyped individual of 10 studied individuals. Seventy-four percent of these amplified cells are KIR2DS2+ KIR2DL1/3/2DS1

cells as feeders. After 3 wk of stimulation, the phenotype of these sorted and stimulated cells was assessed by flow cytometry and is illustrated for 1

figure 4B). The inhibitory C1-KIR2DL2 signaling bypassed the activating C1-KIR2DS2 signaling, highlighting the preponderant impact of KIR2DL2 on NK cell inhibition.

Additive effect of KIR2DL1 and KIR2DS2 against C1+ target cells

Taking into account that activation of KIR2DS2 in the presence of a C1 ligand should have an additive effect on the activation mediated by the lack of engagement of KIR2DL1, we then evaluated the impact of the coexpression of KIR2DS2 and KIR2DL1 on recognition of C1C1+ target cells. To this end, we sorted KIR2DL1+ KIR2DS2+ NK cells from KIR2DSL1 and KIR2DL3 negative genotyped C2+ individuals using anti-KIR2DL2/3DS2 (1F12)- and anti-KIR2DL1/1S1 (EB6)-specific Abs (Fig. 5A). The sorted KIR2DL1+ KIR2DS2+ cells were cloned and phenotyped by KIR2DL2 RT-PCR as previously described (Fig. 3B), to discriminate KIR2DL2+ NK cell clones. We observed a functional additive effect of KIR2DL1 and KIR2DS2 on the recognition of the 221-HLA-C*03:04 (C1) cell line (Fig. 5B, 5C). C2 ligands, like HLA-C*02:02 and HLA-

C*04:01, engaged KIR2DL1 receptors and inhibited KIR2DS2+ KIR2DL1+ NK cell degranulation.

The nature of KIR2D and HLA-C ligand affects the composition of the NK cell repertoire

To determine the functional impact of HLA-C–specific KIR2DL and KIR2DS receptor expression, we evaluated the phenotypic distribution of each KIR2D NK cell subset: KIR2DL1/S1+ KIR2DL2/3/ S2+, KIR2DL1/S1+ KIR2DL2/3/S2+, and KIR2DL1/S1+ KIR2DL2/3/ S2+ NK cell subsets within KIR2DL1/2/3/S1/2 NK cells, in a cohort of 159 KIR and HLA genotyped individuals (Supplemental Table I). Because almost all studied individuals were KIR2DL1+ genotyped (96%), this analysis was performed according to KIR2DL2, KIR2DL3, and KIR2DS2 genotype and HLA-C environment (Fig. 6A). In this cohort, all KIR2DL2+ individuals were KIR2DS2+ genotyped; thus, we assigned the individuals in one of three groups determined by the presence of KIR2DL3, KIR2DL2/ 3/S2, and KIR2DL2/3/S2 genes. All studied populations were similarly represented, regardless of the HLA-C (C1C1, C1C2, or C2C2) environment (data not shown). However, KIR2DL1/S1+ KIR2DL2/3/S2+ NK cell frequency was significantly decreased in KIR2DL2/S2+ individuals with a C2+ haplotype (Fig. 6A, Supplemental Table II). The double-stained population frequency was not significantly different in the studied groups. The KIR2DL2/3/ S2+ KIR2DL1/S1+ NK cell frequency was significantly higher in KIR2DL2/3/S2+ compared with KIR2DL3+ individuals (Fig. 6A, Supplemental Table II) with all HLA-C molecules, and in KIR2DL2/3/S2+ compared with KIR2DL3+ individuals with C1C1 and C2C2 molecules. Finally, we analyzed the distribution of each KIR2D NK cell subset, as defined by their mean frequencies in the pool of KIR2DL1/2/3/S1/2 NK cells (Fig. 6B), according to KIR2DL2, KIR2DL3, and KIR2DS2 genotypes and expression of either C1 or C2 molecules. The KIR2DL1/S1+ NK cell subset predominated in KIR2DL3+ individuals, and its mean frequency increased with the number of C2 alleles (mean frequencies are 36.1, 38.4, and 47.2%, respectively, in C1C1, C1C2, and C2C2 environments). In contrast, the presence of KIR2DL2 and KIR2DS2 correlated with increased mean frequencies of KIR2DL2/3/S2+ NK cells, particularly in KIR2DL2/S2+ individuals. Although KIR2DL2 and KIR2DL3 recognized a broad spectrum of HLA-C ligands, the higher affinity of KIR2DL2 than KIR2DL3 toward HLA-C ligands could account for decreased frequency of the KIR2DL1+ NK cell pool in the former individuals. The six individuals with a KIR2DL1+
genotype in our cohort were KIR2DL2/S2+, KIR2DL3−. We observed a significantly higher KIR2DL2/3/S2+ NK cell frequency (mean = 36.1 ± 5) in these individuals when compared with KIR2DL1+ KIR2DL2/S2+ 2DL3− individuals (mean = 20.3 ± 5, p = 0.005; Fig. 6C), possibly as a result of compensatory KIR2DL2/S2 expression in the absence of KIR2DL1 expression. Thus, KIR2DL1 substitution by KIR2DL2 is phenotypically marked in the KIR2D repertoire and possibly allows maintenance of self C2 recognition.

Significant impact of KIR2DS1 and KIR2DS2 expression on KIR2D NK cell repertoire formation

To determine the impact of KIR2DS1 gene on the KIR2DL1/S1 NK cell compartment, we assessed the frequency of KIR2DL1+ 2DS1−, KIR2DL1+ 2DS1−, and KIR2DL1− 2DS1+ NK cell subsets (Fig. 7A). Taking into account only the HLA-C environment, no difference in frequency was observed. However, a significantly decreased frequency of KIR2DL1+ 2DS1+ NK cells was observed particularly in B+ haplotypes regardless of the HLA-C molecules, linked directly to KIR2DS1 expression (Fig. 7A, Supplemental Table II). The KIR2DL1− KIR2DS1+ NK cell subset is significantly more represented in BB than AB haplotypes because of the increased number of KIR2DS1 allele copies. KIR2DS1 expression contributed to a significantly increased KIR2DL1/S1 NK cell pool, expressed with or without KIR2DL1 on NK cells (Fig. 7C, 7D). Using a 1F12/ GL183 Ab combination (20), we investigated the frequency of KIR2DL2+ 2DL3/S2− and KIR2DL3/S2+ NK cell subsets according to KIR2DL2, KIR2DL3, and KIR2DS2 genes and HLA-C environment (Fig. 7B, 7E). In accordance with the fact that KIR2DL2 and KIR2DL3 segregate as alleles (21), we observed that KIR2DL2*/ KIR2DL3/S2+ NK cell frequency is significantly higher in KIR2DL2/S2+ than KIR2DL2/3/S2+ genotyped individuals with a C2C haplotype (Fig. 7B). However, KIR2DL2+ KIR2DL3/S2− NK cell frequency was not twice in KIR2DL2/S2+ genotyped individuals that was observed in KIR2DL2/3/S2+ individuals with only one KIR2DL2 allele (Fig. 7E), which suggests either coexpression of KIR2DL2 with KIR2DS2, or decreased expression of KIR2DL2. Thus, the expression of the KIR2DS1 contributes to broaden significantly the pool of KIR2DL1/S1 NK cells even though it significantly limits the KIR2DL1+ 2DS1− cell subset frequency. In addition, C1 reactive KIR2DS2 expression seems to function to enlarge the KIR2DL2/3/S2 NK cell pool, and it is conceivable that its additive effect with KIR2DL1 may contribute to limit KIR2DL1 expression on NK cells.

In summary, our results suggest that together with KIR2DL2, activating KIR2DS1 and KIR2DS2 expression limits KIR2DL1 acquisition on NK cells.

Discussion

In this study, we revisited the HLA-C specificity of the main KIR2D and determined C1 reactivity of KIR2DS2 from selected KIR2DL− or KIR2DS− NK cell lines and clones, and investigated these specificities on KIR2D NK cell formation. Our results are in accordance with numerous studies (9, 12, 13, 17, 22–28), and notably
those obtained by Winter et al. (13) showing that KIR2DL2 and KIR2DL3 recognize C2 allotypes using NK92 infected with Vac-KIR2DL2 or -KIR2DL3. More recently, Moesta et al. (12) have shown that KIR2DL2 is a stronger receptor for HLA-C ligand than KIR2DL3. However, among C2 allotypes, we showed that HLA-C*04:01 ligand is better recognized than the other evaluated ligands (HLA-C*02:02, -C*06:02, and -C*15:01). These results are in line with a recent observation drawn from an analysis of

FIGURE 5. Additive effect of KIR2DL1 and KIR2DS2 against C1+ target cells. (A) After depletion of CD3+ cells from PBMCs, KIR2DS2+ KIR2DL1+ KIR2DL2/3/S1+ NK cells (NKp46+ EB6+ 1F12+) were cell sorted from C2+ KIR2DL3/S1+ genotyped individuals and amplified in vitro with irradiated allogeneic PBMCs and EBV-B cells as feeders. After 3 wk of stimulation, NK cells were cloned and only KIR2DL2+ NK cell clones verified by RT-PCR have been selected. The phenotype of one representative KIR2DS2+ KIR2DL1+ KIR2DL2/3/S1+ NK cell clone is shown. (B) CD107a mobilization assay was performed on four KIR2DS2+ KIR2DL1+ KIR2DL2/3/S1+ NK cell clones isolated from one C2+ individual. These results were confirmed for eight KIR2DS2+ KIR2DL1+ KIR2DL2/3/S1+ NK cell clones isolated from two different C2+ individuals. Bars indicate the mean of CD107+ NK cell percentages (± SD) in different culture conditions: medium, 221, HLA-C*03:04 (C1), and HLA-C*04:01 (C2) transfected 221 target cells at an E:T ratio of 10:1. (C) Representative density plots of KIR2DS2+ KIR2DL1+ KIR2DL2/3/S1+ (NKp46+ EB6+ 1F12+) NK cell degranulation observed in the different culture conditions.

FIGURE 6. The nature of KIR2D and HLA-C ligands directs the structure of the NK cell repertoire. (A) Representative density plot illustrating the different KIR2D NK cell subsets in the large cohort of HLA and KIR genotyped individuals (Supplemental Table I) grouped according to the presence of KIR2DL2/3 and 2DS2 genes (KIR2DL3+, KIR2DL2/3/S2+, and KIR2DL2/S2+) and HLA-C haplotype (C1C1, C1C2, and C2C2), KIR2DL1/S1+ KIR2DL2/3/S2+ (EB6+ GL183+) NK cell subset is indicated in white, KIR2DL1/S1+ KIR2DL2/3/S2+ NK cell subset is indicated in gray, and KIR2DL1/S1+ KIR2DL2/3/S2+ (EB6+ GL183+) NK cell subset is indicated in black. Box and whisker plot summarizing the frequency of each KIR2D NK cell subset out of all NK cells. (B) Pie charts depict the pattern of KIR2D composition in nine groups of individuals distributed following the presence of KIR2DL2/3 and 2DS2 genes (KIR2DL3+, KIR2DL2/3/S2+, and KIR2DL2/S2+) and HLA-C molecules (C1C1, C1C2, and C2C2). We summed KIR2DL1/S1+ KIR2DL2/3/S2+ (EB6+ GL183+) NK cell subset is indicated in white, KIR2DL1/S1+ KIR2DL2/3/S2+ NK cell subset is indicated in gray, and KIR2DL1/S1+ KIR2DL2/3/S2+ (EB6+ GL183+) NK cell subset is indicated in black. Box and whisker plot summarizing the frequency of each KIR2D NK cell subset out of all NK cells. (C) Scatter plots represent KIR2DL1+KIR2DS2+ NK cell frequency in KIR2DL1+ (n = 6) and KIR2DL1+ (n = 13) genotyped individuals. The mean frequency is indicated in each group by a black feature. Individuals have been identified following KIR2DS1 genotype and HLA-C molecules (C1C1, C1C2, and C2C2). Statistical significance (*p < 0.05, **p ≤ 0.001) between two groups was determined using the one-way ANOVA test (Supplemental Table II).
a large panel of single-HLA class I molecule beads based on Luminex, which showed a broader pattern of HLA-C recognition by KIR2DL2-Fc fusion protein, even using unloaded HLA class I molecules (12). Recently, we have shown that KIR2DL3+ NK cell lines equally recognized either C1C1 or C2C2 immature dendritic cells (29). The low affinity of the KIR2DL2/3 to HLA-C ligand is likely reinforced and stabilized within the immune synapse by other receptor–ligand interactions, thus allowing functional KIR-HLA interactions. Besides the wide spectrum of HLA-C recognition by KIR2DL2 and KIR2DL3, another important message of our study is the ability of activated KIR2DL2+ and KIR2DL3+ NK cells to recognize the “missing-self” irrespective of their HLA-C background. Our results are in agreement with those of Anfossi et al. (7), who suggested education of KIR2DL3+ NK cells mainly from a C1+ environment. Indeed, we observed that resting C12 KIR2DL3+ NK cells are less receptive to missing-self activation (data not shown); however, KIR2DL3 engagement with C2 ligands during NK development or cytokine stimulation could enhance the response to missing-self after a preactivation, resulting in a decrease of their activation threshold. This point is essential in particular pathological contexts, such as viral infection, tumor processes, and HSCT, where activation could increase the missing-self response of poorly educated cells such as C12 KIR2DL3+ NK cells or KIR2DL2+ NK cells.

In contrast with other approaches based on soluble KIR-Ig proteins, KIR2D transfected cells, or a vaccinia expression system, our results were drawn from NK cells purified from different individuals with a physiological KIR2D expression. The more sensitive cellular model probably allows assessment of low-avidity interactions between of KIR2D and HLA-C, which could explain discordant results with those obtained with binding assays using KIR2D-Ig fusion proteins (13). This is typically the case for soluble KIR2DS receptors, which did not bind to any of the HLA class I molecules expressed on a large panel of transfected cells (13). In our cellular model, we showed that KIR2DS2 recognizes only C1 ligands, in contrast with its inhibitory KIR2DL2 form, in

FIGURE 7. Significant impact of KIR2DS1 and KIR2DS2 expression on KIR2D NK cell repertoire formation. Representative density plot illustrating the different NK cell subsets expressing (A) KIR2DL1 or and KIR2DS1, studied in the large cohort of HLA and KIR genotyped individuals (Supplemental Table I) grouped according to the HLA-C molecules (C1C1, C1C2, and C2C2), KIR haplotype (AA, AB, and BB), and presence or absence of the KIR2DS1 gene. KIR2DL1* S1+ (EB6+ 143211+) NK cell subset is indicated in black, KIR2DL1* S1+ (EB6* 143211+) NK cell subset is indicated in gray, and KIR2DL1* S1+ (EB6* 143211+) NK cell subset is indicated in white. (B) Representative density plot illustrating the different NK cell subsets expressing KIR2DL2 and/or KIR2DL2/3/S2 studied in individuals grouped in function of the presence of KIR2DL2/3 and 2DS2 genes (KIR2DL3+, KIR2DL2/3/S2+, and KIR2DL2/S2+) and HLA-C molecules (C1C1, C1C2, and C2C2). KIR2DL2* 2DL2/3/S2 (1F12+ GL183+) NK cell subset is indicated in white; KIR2DL2+ 2DL2/3/S2* (1F12+ GL183+) NK cell subset is indicated in black. Box and whisker plot summarizing the frequency of each KIR2D NK cell subset out of all NK cells. Pie charts show the KIR2DL1 NK cell subset in nine groups of (C) KIR2DS1 genotyped individuals and (D) KIR2DS1+ genotyped individuals distributed following the presence of KIR2DL2/3 and 2DS2 genes (KIR2DL3+, KIR2DL2/3/S2+, and KIR2DL2/S2+) and HLA-C molecules (C1C1, C1C2, and C2C2). We summed KIR2DL1* S1+, KIR2DL1* S1+, and KIR2DL1* S1+ NK cell subsets, weighting them according to their frequency indicated in the pie chart. (E) Pie charts depict the pattern of KIR2D composition in the KIR2DL2/3/2DS2 NK cell pool in nine groups of individuals distributed following the presence of KIR2DL2/3 and 2DS2 genes (KIR2DL3+, KIR2DL2/3/S2+, and KIR2DL2/S2+) and HLA-C molecules (C1C1, C1C2, and C2C2). We summed KIR2DL2+ 2DL2/S2+ and KIR2DL2+ 2DL3/S2+ NK cell subsets, weighting them according to their frequency indicated in the pie chart. The size of the pie chart is proportional to the frequency of KIR NK cell pool and it is indicated (±SD) in the bottom left of each group in italics. The number of studied individuals in each group is indicated in the bottom right. Statistical significance (*p < 0.05, **p < 0.001) between two groups was determined using the one-way ANOVA test (Supplemental Table II).
all HLA-C environments. Failed C1/KIR2DS2 interactions using soluble KIR2DS2-Ig fusion proteins or KIR2DS2 tetramers (30) would suggest weak affinities. Thus, the few substitutions between the extracellular parts of both receptors could explain these apparent discrepancies. KIR2DS1 and KIR2DS2 show a lower affinity than their inhibitory counterparts with overlapping specificity, ensuring that inhibition signals override activation signals. However, in the absence of inhibitory KIR signals, the engagement of activating KIR2DS is sufficient to trigger NK cell responses.

The broader specificity of KIR2DL2 and KIR2DL3 toward HLA-C alleles also recognized by KIR2DL1 would explain the predominant expression of this compartment within the KIR2D NK cell repertoire. KIR2DL1 is predominantly represented in a C2C2 environment in the presence of KIR2DL3, but its representation is decreased in the presence of KIR2DL2 and KIR2DS2 genes. One possible explanation for this is that KIR2DL2 is a stronger competitor than KIR2DL3 for C2 ligands, explaining its predominant representation in the KIR2D NK cell pool in a C2C2 environment. Moreover, the high proportion of KIR2DS1+ NK cells in the KIR2DL1/s1 pool could contribute to the decreased frequency of KIR2DL1+ 2DS1+ NK cells. Thus, all activating and inhibitory KIR2D are functional, but the strength of HLA-C affinity is different, ensuring a hierarchy between these KIR2D, and explains, in part, the formation of the NK cell repertoire depending on the HLA-C environment. In this regard, we observed in KIR2DL1+ genotyped individuals that KIR2DL2/3/2DS2 expression was significantly increased compared with KIR2DL1+ genotyped counterparts, suggesting an adjustment of the KIR2D repertoire formation after the distribution of each pool. This observation may not be compatible with a sequential acquisition of KIR with KIR2DL1 expression after KIR2DL2 and KIR2DL3 as proposed by Uhrberg’s group (9). It is likely that the adjustment is more continuous and that the KIR2D expression can be readjusted according to other KIR2D expressed, as previously proposed (10).

The C2-expressing fetuses carried by group A KIR homozygous mothers constitute a risk factor for pre-eclampsia (31) and current miscarriage (32) during pregnancy. In contrast, fetal C1 mothers constitute a risk factor for pre-eclampsia (31) and re-expressed, as previously proposed (10).

The data on the association of maternal KIR3DL1 and paternal KIR3DL1+ Bw4 environment are compatible with a sequential acquisition of KIR with KIR2DL1 counterparts, suggesting an adjustment of the KIR2D repertoire formation. The impact of allelic KIR3DL1 and a Bw4 environment on KIR2D NK cell repertoire should be studied in a larger cohort. Altogether, our results suggest that the nature of the HLA-C-specific activating and inhibitory KIR2D expressed and the ligand environment directs the structure of NK cell repertoire.

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Disclosures

The authors have no financial conflicts of interest.


