Published in final edited form as:

Cancer Immunol Immunother. 2016 September ; 65(9): 1047–1059. doi:10.1007/s00262-016-1864-z.

NK cells maintain licensing status and are subject to KIR/KIR-ligand inhibition following *ex vivo* expansion

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Abstract

Infusion of allogeneic NK cells is a potential immunotherapy for both hematopoietic malignancies and solid tumors. Interactions between killer immunoglobulin-like receptors (KIR) on human NK cells and KIR-ligands on tumor cells influence the magnitude of NK function. To obtain sufficient numbers of NK cells for infusion, several approaches have been tested. One potent method uses cells from the K562 human erythroleukemia line that have been transfected to express activating 41BB ligand (41BBL) and membrane-bound interleukin15 (mbIL15); co-culture with these cells stimulates NK cell expansion and activation. The functional importance of KIRs on *ex vivo* expanded NK cells has not been studied in detail. We investigated how KIR/KIR-ligand interactions between expanded NK cells and target cells affect the function of expanded NK cells. After a 12-day co-culture with K562-mbIL15-41BBL cells, expanded NK cells maintained inhibition specificity and prior *in vivo* licensing status determined by KIR/KIR-ligand interactions. Addition of an anti-CD20 antibody (rituximab) induced NK-mediated antibody dependent cellular cytotoxicity (ADCC) and augmented killing of CD20+ target cells. However, partial inhibition induced by KIR/KIR-ligand interactions persisted. Finally, we found that extended co-cultures of NK cells with stimulatory cells transduced to express various KIR-ligand modified both the inhibitory and activating KIR repertoires of the expanded NK cell product. These studies demonstrate that the licensing interactions known to occur during NK ontogeny also influence NK cell function following NK expansion *ex vivo*. These findings can help donor selection and guide expansion of specific NK cell subsets for adoptive therapy of cancer.

Keywords

Ex vivo expansion; NK cells; Killer Immunoglobulin-like Receptors; HLA; ADCC

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Disclosures

The authors have no financial conflicts of interest.

Introduction

Ex vivo expanded and activated NK cells for adoptive transfer is an immunotherapeutic option for cancer, mainly due to the augmented cytotoxic capabilities of these expanded cells (1–5). Ongoing clinical efforts are investigating adoptive transfer of autologous and allogeneic NK cells following IL-2 stimulation or *ex vivo* expansion (6–9), with some early examples of potential clinical benefit (10, 11). Due to the limited number of NK cells in peripheral blood, *ex vivo* expansion can serve to generate a better source of NK cells. In addition to the potential for generating a several hundred-fold increase in NK cell numbers, *ex vivo* expanded NK cells demonstrate augmented expression of activating receptors, such as NKG2D and natural cytotoxicity receptors (NCRs), as well as increased tumor cytolytic activity as compared to non-expanded NK cells (1, 2).

The killer immunoglobulin-like receptors (KIRs) are an important family of receptors on human NK cells. There are fifteen KIR genes and two pseudogenes on human chromosome 19 (12). Individuals inherit widely different KIR genes, and the KIR haplotypes are highly diverse (13). Moreover, within a single individual, the protein products of the KIR genes are expressed clonally and somewhat randomly on NK cell surfaces, with some NK cells only expressing the product of a single KIR gene and other NK cells expressing the products of 2 or many more KIR genes (14).

Interactions between KIRs and KIR-ligands, together with other receptors on NK cells, regulate NK cell education and activation. For some KIRs, especially the inhibitory KIRs (iKIR), well-defined ligands are known; however the ligands for some inhibitory and many activating KIRs (aKIR) are still unidentified (12). Some of the known KIR ligands include those of the major histocompatibility complex (MHC) class I molecules, including HLA-C1 (C1), HLA-C2 (C2), and HLA-Bw4 (Bw4). Inhibitory KIR2DL1 recognizes HLA-C alleles with a lysine at amino acid position 80 (HLA-C2) and iKIRs 2DL2 and 2DL3 recognize HLA-C alleles with an asparagine at amino acid position 80 (HLA-C1) (15, 16). Several groups have shown that iKIRs 2DL2 and 2DL3 may also cross-recognize HLA-C2 alleles, but not to the same degree as they recognize HLA-C1 or that iKIR2DL1 interacts with HLA-C2 (17, 18). Inhibitory KIR3DL1 recognizes HLA-A and HLA-B alleles with a Bw4 motif within amino acid position 77 to 80 (19). Finally, aKIR2DS1 shares the same ligand (C2) as the iKIR2DL1, but aKIR2DS1 has a lower binding affinity to this ligand (20).

NK cells that express self-iKIRs (i.e. iKIRs that recognize the HLA expressed on normal tissues of the same individual) become “licensed” during their development (21). NK cells are unlicensed when they do not express any iKIR, or only express non-self iKIRs (i.e. iKIRs that do not recognize HLA expressed on normal tissues of the same individual). Once mature, licensed NK cells demonstrate augmented cytotoxic function (compared to unlicensed NK cells) against HLA-null tumor targets (or tumor cells that have down-regulated their HLA expression) (22). The differences between licensed and unlicensed NK cells have been intensively studied using freshly isolated NK cells; HLA-null tumor targets have been primarily used to differentiate the activation and cytotoxic capabilities of licensed vs. unlicensed NK cells (21, 23, 24). However, tumor cells in patients usually show some degree of HLA expression. Thus interactions between KIR repertoire on NK cells and KIR-

ligand repertoire on tumor cells, including licensing, play a considerable role in anti-tumor effects for immunotherapies that involve NK cells, both in the autologous and allogeneic settings (23, 25–29). However, the influence of interactions between iKIR expressed on NK cells and KIR-ligand on tumor cells, and the influence of prior *in vivo* licensing have not been well studied using *ex vivo* expanded NK cells. Whether *ex vivo* expansion and activation, combined with tumor antigen-specific mAb, could mitigate inhibition from KIR/KIR-ligand interactions or alter NK cell licensing status needs further investigation.

Several different pathways were reported to contribute to NK-mediated tumor lysis, including degranulation of perforin and granzyme containing particles, cytokine release, and induction of tumor apoptosis by Fas-L and TRAIL interactions (30). Among those mechanisms, degranulation and cytokine release have become important markers of NK cell activation and correlate with target cell lysis (29, 31). Here we use the NK cell degranulation marker, CD107a, to analyze the differences between the activation of licensed and unlicensed NK cells within the same donor, as well as between donors with various iKIR/KIR-ligand genotypes. Three different iKIR/KIR-ligand pairs (2DL1 with its ligand C2; 2DL2 and 2DL3 with their primary ligand C1; and 3DL1 with its ligand Bw4) have been the major focus for the licensing and inhibition interactions that involve iKIR (23, 28, 29). In this study we investigated the role of iKIR/KIR-ligand interactions on the cytotoxic and antibody-dependent cellular cytotoxicity (ADCC) function of *ex vivo* expanded NK cells, and the role of these interactions during the *ex vivo* expansion on modifying inhibitory and activating KIR repertoire of the expanded NK cells.

Materials and methods

Cell lines and media

The K562-mbIL15-41BBL cell line was developed at St. Jude Children's Research Hospital (32). The 721.221 EBV transformed B-cell line was a gift from Dr. Robert DeMars (University of Wisconsin-Madison) (33). Both cell lines are maintained in RPMI with 10% FBS, 2mM L-glutamine and penicillin/streptomycin.

Plasmid and transfection

HLA alleles were cloned into pcDNA3.0 (a gift from Dr. Deric Wheeler, University of Wisconsin-Madison) to generate HLA-C1 (C0801), HLA-C2 (C0401) and HLA-Bw4 (B4402) carrying plasmids. All three plasmids, as well as an empty vector pcDNA3.0, were separately transfected into the 721.221 cell line by nucleofection (Mirus and AMAXA). Transfected 721.221 cells were then selected with 1700mcg/mL Geneticin (Teknova), and stable expressing cells were sorted for HLA expression using FACS. Sorted cells were maintained in RPMI with 1700mcg/mL Geneticin. CD20 expression and HLA expression levels of transfected variants are shown in Supplemental Figure 1.

KIR and HLA genotyping

DNA from healthy donors (Table 1) was isolated from buccal swab or whole blood. KIR genes were genotyped using a SYBR green real time PCR method (34). KIR-ligand genes were genotyped by a sequence-specific primer PCR method (Olerup).

Ex vivo expansion of NK cells

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donor whole blood by ficoll density gradient centrifugation. K562-mbIL15-41BBL cells were irradiated with 100Gy and then mixed with 5 million PBMC at a 1:1 ratio in 10mL RPMI, with 100IU/mL IL2. Mixtures of PBMC and K562-mbIL15-41BBL cells were incubated at 37 degrees on a rocker at a constant speed in an incubator. Fresh RPMI with IL2 was added every 2–3 days to the culture. All cells in the expansion cultures were harvested between expansion days 12–15 for CD3 and CD56 staining to determine NK cell percentage followed by functional analyses in the degranulation assay.

In separate experiments, 721.221 HLA variants were irradiated with 100Gy and cultured together with fresh PBMC at a 1:1 ratio for expansion, under the same conditions as described above for expansion with K562-mbIL15-41BBL cells with the addition of 1ng/mL IL15. Expansion cultures were harvested between day 10 to 14 to determine inhibitory and activating KIR frequencies in the NK cells in the final product.

NK degranulation assay

Expanded NK cells were stimulated with 721.221 targets at a 1:1 ratio in 96-well round bottom plates at 37 degrees, in the presence of anti-CD107a antibody, with or without addition of rituximab. The lowest rituximab dose able to induce plateau-level CD107a expression of expanded NK cells stimulated with HLA-null 721.221 cells (E:T ratio=1:1) was selected (0.5ug/mL). After 1 hour of incubation with 721.221 targets, protein transport inhibitor (eBioscience) was added into the culture. After another 3 hours of incubation, cells were washed, stained with live/dead Aqua dye (Biolegend) and various mAbs, then analyzed on a Macsquant cytometer (Miltenyi) to determine the percentage of CD107+ NK cells in NK subpopulations defined by KIR staining (28, 29).

In order to compare the relative amount of CD107 expression between different NK donors, as well as NK expansions and stimulations performed on different days, data for all donors were normalized as follows. For each individual NK expansion culture, the percent of CD107+ NK cells from each NK subset being evaluated (i.e.: stimulation with HLA-C1, C2 or Bw4 expressing 721.221 variants, with or without addition of rituximab) was divided by the percent of CD107+ cells found in the iKIR-null NK cell subset stimulated by HLA-null 721.221 cells without rituximab treatment from the same experiment (the same expansion from the same donor).

Staining mAbs for flow cytometry

The following reagents were used for flow cytometry: PE/Cy7 anti-CD3 (clone HIT3a, Biolegend), APC anti-CD56 (clone HCD56, Biolegend), PE anti-CD107a (clone H4A3, eBioscience), FITC anti-2DL1 (clone REA 284, Miltenyi), PerCP anti-2DL2/DL3 (clone DX27, Miltenyi), BV421 anti-3DL1 (clone DX9, Biolegend), Aqua fixable viability dye (Biolegend), BV605 anti-CD3 (clone UCHT1, Biolegend), PE anti-2DL1/DS1 (clone 11PB6, Miltenyi).

Cytotoxicity assay

Target cells were labeled with ^{51}Cr for 1.5h, and mixed with expanded NK cells at various ratios (1:1, 2:1, 4:1) in 96-well plates for 4h at 37 degrees. Supernatants were harvested at the end of incubation and read by a gamma counter. Percent lysis of target cells was calculated as $100 \times (\text{Treatment-spontaneous}) / (\text{Maximum-spontaneous})$.

Statistical analyses

Statistical analyses were done with Prism5.0, using the Student t-test, one-way ANOVA or two-way ANOVA as indicated within the figure legends. Standard errors are indicated as error bars for each comparison in figures.

Results

Selective NK cell outgrowth following *ex vivo* culturing of PBMC with K562-mbIL15-41BBL cells

After 12 days of culturing PBMCs with irradiated K562-mbIL15-41BBL cells, NK cells were the major population identified to have expanded, with a purity between 70%–95% (Supplemental Figure 2A) and NK cell numbers increased by 150–250 fold (data not shown). This expansion and enrichment of NK cells in the final product enabled us to study rare NK cell subsets.

The distribution of KIR on fresh NK cells can be affected by donor KIR and KIR-ligand genotype but not by *ex vivo* expansion with K562-mbIL15-41BBL cells

We next evaluated whether distribution of specific KIRs on NK cells is affected by *ex vivo* expansion or donor HLA genotype. Among the 12 donors from which NK cells were expanded, the C1/C1 donors had a significantly higher frequency of 2DL2/3+ NK cells in their circulating PBMCs (Figure 1B, pre-Expansion); this higher frequency of 2DL2/3+ NK cells for these C1/C1 donors was still evident after the *ex vivo* expansion process using K562-mbIL15-41BBL cells (Figure 1B, post-Expansion). In addition, Bw4+ donors had a lower 3DL1 frequency than Bw4- donors, both pre- and post-expansion (Figure 1C). KIR2DL1 frequency was not affected by donor HLA-C genotype (Figure 1D), but 2DL2- donors had higher 2DL1 frequencies in post-expanded NK cells as compared to 2DL2+ donors (Figure 1E).

Inhibitory KIRs on *ex vivo* expanded NK cells retain inhibition specificity of their ligands

Following *ex vivo* activation and expansion, NK effector cells were stimulated with various HLA-expressing target cells. The iKIRs expressed on expanded NK cells inhibited NK cell activation, via interaction with the cognate KIR-ligand expressed on the 721.221 targets for each iKIR analyzed (Figure 2A and Supplemental Figure 3). Our results indicate that NK cell *ex vivo* expansion and activation (by K562-mbIL15-41BBL cells and IL2 for two weeks) does not prevent the iKIR on these NK cells from causing some level of NK cell inhibition when they encounter their cognate KIR-ligand. The amount of NK cell inhibition due to specific KIR-ligand recognition by iKIRs on *ex vivo* expanded NK cells was

relatively similar to the amount of inhibition on freshly harvested NK cells from PBMCs cultured overnight in IL2 (data evaluated for 6 donors, not shown).

Rituximab improves cytotoxicity of CD20+ targets by *ex vivo* expanded NK cells, and is still subject to iKIR/KIR-ligand inhibition

After NK cell expansion and activation, the expression of CD16 was similar to that measured on NK cells from freshly isolated PBMCs from the same donor (Supplemental Figure 2A). As expected, rituximab (an anti-CD20 monoclonal antibody) treatment resulted in increased percentage of degranulating NK cells (Supplemental Figure 2B). Despite the increase in degranulation, rituximab-induced ADCC is still subject to iKIR/KIR-ligand inhibitions (Figure 2B). This suggests that after a 12-day *ex vivo* expansion, where NK cells are in an activated state due to the continuous stimulation with both IL-2 and K562-mbIL15-41BBL, iKIR expressed on the expanded NK cells maintain their characteristics as on unstimulated NK cells in both natural cytotoxicity and ADCC settings.

Inhibition through iKIR/KIR-ligand interaction is not affected by the co-expression of non-ligated iKIRs

The data shown in Figure 2B indicate the co-expression of 1 or 2 non-ligated iKIR does not influence the activation of NK cells expressing a ligated iKIR. For example, when NK cells were stimulated with target cells that expressed HLA-C1 (the ligand for iKIR2DL2/3), the co-expression of 2DL1 and/or 3DL1 on NK cells with 2DL2/3 did not enhance the degranulation of expanded NK cells induced by the C1+ target cells (Figure 2B, 721-C1). In addition, other NK cell subsets that lacked 2DL2/3 expression (2DL1sp; 3DL1sp; 2DL1+3DL1+) all had significantly higher degranulation levels than 2DL2/3sp cells (Figure 2B, 721-C1). A similar response pattern, not influenced by non-ligated iKIRs, was demonstrated for HLA-C2 inhibition of NK cells with 2DL1, and for HLA-Bw4 inhibition of NK cells with 3DL1 (Figure 2B, 721-C2 and 721-Bw4).

***Ex vivo* expanded NK cells retain their licensing status determined by iKIR/KIR-ligand repertoire of the NK cell donor**

To compare licensed NK cells from unlicensed NK cells, we grouped 12 healthy donors (Table 1) according to their KIR-ligand (HLA-C1, C2 and Bw4) genotypes. The 4 C1/C1 donors (thus not carriers of C2) have licensed NK cells (NK subsets that express iKIR2DL2/3) that were more activated than their unlicensed NK cells (NK subsets that do not express iKIR2DL2/3), when stimulated with HLA-null 721.221 cells (Figure 3A, HLA-null 721.221, no rituximab). A similar licensing effect was also seen for the 5 donors that are Bw4+, with respect to its corresponding iKIR (3DL1) (Figure 3C, HLA-null 721, no rituximab). Rituximab treatment increased NK activation but the unlicensed NK cells still demonstrated less activity than the licensed NK cells from the same donors on HLA-null 721.221 cells (Figure 3A and 3C, HLA-null 721, +rituximab). These results are consistent with previous studies that utilized unstimulated NK cells (21), and demonstrate that the effect of NK licensing that occurred *in vivo* is retained after 12 days of *ex vivo* expansion with HLA-null cells (K562-mbIL15-41BBL).

In contrast, in C2/C2 donors, there was no difference in the amount of activation of the subset that should be licensed (2DL1sp NK cells) from the subset that should be unlicensed (2DL2/3sp NK cells) (Figure 3B, HLA-null 721). Previously published data demonstrate that there is some cross-reactive recognition of HLA-C2 by iKIR2DL2/3 (17, 18). Thus the 2DL2/3sp NK cells in Figure 4B might be considered “partially licensed”, thereby potentially accounting for their similar level of activation as the licensed 2DL1sp NK cells.

Inhibition due to iKIR/KIR-ligand interaction negates the augmented function of licensed vs. unlicensed NK cells following expansion

Inhibition resulting from iKIR and KIR-ligand interactions was reported to dominate over the licensing effect seen with unexpanded NK cells (23, 29). In this study, the same phenomenon is observed with expanded NK cells. When expanded NK cells from C1/C1 and C2/C2 donors were stimulated with C1 or C2-expressing 721.221 cells respectively, NK cells lacking self-iKIR (unlicensed) were more activated than NK cells expressing self-iKIR (licensed) due to KIR-ligand inhibition via self-iKIRs on licensed NK cells (Figure 3A HLA-C1 721, and Figure 3B HLA-C2 721). Similarly, the interaction between Bw4 and self-iKIR 3DL1 inhibited activation of licensed 3DL1sp cells down to the same level as their unlicensed iKIR-null NK cells (Figure 3C, HLA-Bw4 721). These results suggest that expanded NK cells that are unlicensed may play a more important role when the KIR-ligand that matches the NK donor (matched KIR-ligand) is expressed on target cells.

Altogether, the data in Figures 2 & 3 suggest that the difference between licensed and unlicensed NK cells detected in unstimulated NK cells (21, 23, 24) still applies for expanded and activated NK cells, even though they are cultured *ex vivo* with HLA-null cells (K562-mbIL15-41BBL) for 12–15 days. Whether a given target cell causes differential activation of licensed or unlicensed NK cells depends on what KIR-ligand the target cells express. In most combinations, the inhibitory effect caused by iKIRs recognizing their cognate ligand can override the augmented capabilities associated with prior licensing.

Specific KIR-ligand modifies the distribution of both inhibitory and activating KIRs on expanded NK cells

Besides K562-mbIL15-41BBL cells, many other cell lines can also serve as stimulatory cells for NK expansion, such as 721.221 cells (35). We expanded NK cells from a donor who expressed iKIR2DL1 and aKIR2DS1 using either HLA-null 721.221, or HLA-C2 expressing 721.221 stimulatory cells. Unlike the expansion results obtained when PBMC are cultured with K562-mbIL15-41BBL cells, 721.221 cell lines as stimulants resulted in a greater number of contaminating T cells in the expansion product (data not shown). To analyze NK subset frequencies, we gated out CD3+ cells. We found that the frequency of 2DL1+2DS1– NK cells following *ex vivo* expansion with 721.221 cells was significantly reduced when HLA-C2 was expressed (Figure 4B). Conversely, 2DL1–2DS1+ NK cell frequency was increased when NK cells were expanded with C2+ cells. The frequency of 2DL1+2DS1+ NK cells was reduced when expanded with C2+ cells (Figure 4B), which suggests that the inhibitory effect from 2DL1/C2 is stronger than the corresponding activating 2DS1/C2 interaction. The frequencies of 2DL2/3+ and 3DL1+ NK cells were decreased by expansion

with C1+ and Bw4+ 721.221 cells respectively (Figure 4B), but the decrease in 3DL1+ cells by Bw4+ stimulatory cells was not significant.

Discussion

In current clinical trials of expanded NK cell transfusion, the most common allogeneic NK resource is haploidentical NK cells. Even though iKIR/KIR-ligand mismatch between donor and recipient is preferred in this setting, several factors may influence NK function and patient outcome. Here we focused on the impact of each iKIR/KIR-ligand interaction pair on the degranulation level of *ex vivo* expanded NK cell subsets, tested against target cells with distinct KIR-ligand, alone or combined with CD20-specific mAb treatment to induce ADCC.

Ex vivo expanded NK cells have upregulated activating receptors, such as NKG2D and NKP44, and have superior tumor killing compared to freshly isolated NK cells (2). In our NK cell expansion model using K562-mbIL15-41BBL cells, we showed that expanded NK cells still maintain FcR expression, and their target cell lysis can be improved by including a target-cell antigen-specific mAb; in this case, rituximab. These data further support that adoptive transfer of expanded NK cells has the potential to be combined with tumor antigen-specific mAb to treat cancers via augmented ADCC (36).

Our data suggest that activation of *ex vivo* expanded NK cells is dependent upon the KIR-ligand expressed on the target cells, and whether the iKIR receptors on the NK cells recognize the KIR-ligand on the target cells (Figure 2B). However, the three 721.221 variants we used only represent one allele from each KIR-ligand group (C0801, C0401 and B4402 [threonine80]). Whether other alleles, especially Bw4-isoleucine80 alleles that show differential interaction with 3DL1 than threonine80 alleles (19, 37, 38), would have different impact on NK cell degranulation needs further investigation.

In this study, treatment with antigen-specific mAb to augment killing via ADCC did not overcome the influence of the iKIR/KIR-ligand interactions (Figure 2B). It suggests that iKIR/KIR-ligand mismatch between donor and recipient may still be beneficial when adoptive transfer of *ex vivo* expanded NK cells and mAb therapy are combined.

Besides iKIRs, there are other inhibitory receptors on NK cells that could be inhibited by HLA alleles, such as LIR1 and NKG2A (39). Even though the HLA alleles expressed on the 721.221 variants we made gave relatively specific inhibition to their cognate iKIR receptors (Supplemental Figure 3), there remains some low level of HLA-induced inhibition mediated by inhibitory receptors other than the iKIRs we evaluated on these cells (for example, the decreased CD107a of iKIR-null NK cells by HLA-C1 and C2, Supplemental Figure 3). LIR1/HLA and NKG2A/HLA-E interactions might account for some of this inhibition, but further validation by excluding the roles of LIR1 and NKG2A receptors on NK cells would be helpful.

Besides inhibitory function, iKIRs have also been shown to play a role in NK cell licensing (21, 22, 24). We hypothesized that during 12 days of *ex vivo* expansion with HLA-null cells, expanded NK cells might lose their original licensing status, because long-term stimulation

might overcome the tolerance of unlicensed cells. However, we found that NK cells preserve their original licensing status after *ex vivo* expansion with HLA-null cells (Figure 3, HLA-null 721). Consistent with data published on unexpanded NK cells (23, 29, 40), we found that inhibition induced by iKIR recognition of its cognate ligand normally dominated over the anticipated increase in effector function due to licensing effects (Figure 3). Interestingly, the impact from Bw4 inhibition on licensed iKIR3DL1sp cells seemed less potent at overriding the licensing difference compared to HLA-C inhibition for iKIR2DL1 or iKIR2DL2/3 (Figure 3).

Finally, we showed that donor HLA-C genotype has an influence on KIR2DL2/3+ NK cell frequency, and KIR2DL1 frequency is not affected by HLA-C, but might be influenced by 2DL2 genotype (Figure 1B). These results suggest that the presence of 2DL2 may interfere with 2DL1 expression. It is consistent with data published by Schonberg et al., demonstrating that HLA-C genotype affects 2DL2/3+ NK frequency overall, but affects 2DL1 frequency only in 2DL2- donors (41). For iKIR3DL1, our data are potentially discordant with data published by Yawata et al. showing that Japanese donors possessing two high-expressing 3DL1 allele copies have a higher frequency of iKIR3DL1+ NK cells in their PBMC if they also have Bw4 genes than those donors who do not have Bw4 genes (42). Our results showed the opposite effect of Bw4 on 3DL1 frequency (Figure 1B), however our healthy donors are of a different ethnicity (>80% Caucasian), and we did not differentiate high-expressing 3DL1 alleles from the rest. Our data also indicate that the expansion process does not significantly change this pattern (Figure 1B). Finally, our expansion results using KIR-ligand expressing 721.221 cells suggest that expression of KIR-ligand, HLA-C1 or Bw4, on the stimulatory cells selectively suppresses the expansion of NK cells with iKIR that recognize them (iKIR2DL2/3 and iKIR3DL1 respectively, Figure 4B). Interestingly, the presence of the KIR-ligand, HLA-C2, on the stimulatory cells selectively inhibits expansion of NK cells with iKIR2DL1 but augments expansion of NK cells with aKIR2DS1 (Figure 4B). A larger sample size will be important to confirm our observations, as there may also be an effect from allelic variation within KIRs and HLA that is overlooked with the small number of healthy donors we have evaluated.

Ex vivo expanded NK cells may differ from fresh PBMC derived NK cells in many ways, but they both express KIRs. Our data demonstrate that *ex vivo* expanded NK cells are still influenced by KIR/KIR-ligand interactions and by rules similar to the way KIR/KIR-ligand interactions regulate unstimulated NK cells. Overall, we have analyzed the effect of KIR/KIR-ligand on *ex vivo* expanded NK cell response in multiple ways, showing that: 1) there is a distinctive hierarchy of NK cell response depending on the iKIR expressed by expanded NK cells and KIR-ligand expressed by target cells; 2) NK cells expanded with HLA-null cells retain their response difference based on whether they were originally licensed or not, but the licensing effect is dominated by inhibition from iKIR recognition of KIR-ligand on target cells; 3) the presence of HLA-C vs. HLA-Bw4 on target cells inhibits activation of licensed NK cells to a slightly greater degree (when compared to activation seen for the donor's unlicensed cells); and 4) finally, KIR-ligand expressed on the stimulatory cells used for expansion can modify both inhibitory and activating KIR repertoire. Using *ex vivo* expanded NK cells in combination with immunotherapeutic mAbs to facilitate ADCC might have *in vivo* clinical significance, which could potentially be improved by considering the

influence of KIR/KIR-ligand interactions on ADCC function of these NK cells. This might be incorporated into future clinical testing via selection of optimal NK donors, as well as selection of optimal NK subpopulations (43) that consider the most beneficial interactions of KIR and KIR-ligand.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank all healthy donors participating in this study. Thanks also to Dr. Robert DeMars who provided 721.221 cells, and Dr. Deric Wheeler who shared the empty vector. This research was supported by a Hyundai Hope on Wheels Grant; Midwest Athletes Against Childhood Cancer; NIH Grants R01 CA166105 and R35 CA197078; and a grant from The Stand Up To Cancer Foundation and St. Baldrick's Foundation.

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Figure 1. Inhibitory KIR frequencies are affected by donor HLA genotype, but not by *ex vivo* expansion with K562-mbIL15-41BBL cells

PBMCs from healthy donors were expanded using K562-mbIL15-41BBL cells. **A**): Cells were pre-gated by lymphocyte, live cells, singlets and NK cells for KIR analyses. **B**): The percentages of 2DL2/3+ cells in pre- or post-expanded CD3-CD56+ cells from C1/C1 (n=4), C1/C2 (n=3) and C2/C2 (n=5) donors were compared to each other. Each line represents an individual healthy donor. Statistics between C1/C1 and C2/C2 donors are shown. There is no significant difference between C1/C1 and C1/C2 donors, or between C2/C2 and C1/C2

donors. **C**): The percentages of 3DL1+ cells in pre- or post-expanded CD3-CD56+ cells from Bw4+ donors (n=6) were compared to the percentages of 3DL1+ cells from Bw4- donors (n=4). **D**): The percentages of 2DL1+ cells in pre- or post-expanded CD3-CD56+ cells from C1/C1 (n=4), C1/C2 (n=3) and C2/C2 (n=5) donors were compared to each other. There is no significant difference between %2DL1+ cells from C1/C1, C1/C2 and C2/C2 donors, in either pre- or post-expanded NK cells. **E**): The percentages of 2DL1+ cells in pre- or post-expanded CD3-CD56+ cells from 2DL2-2DL3+ (n=6) donors were compared to that for 2DL2+2DL3+ (n=5) donors. There is no significant difference between %2DL1+ cells from 2DL2-2DL3+ and 2DL2+2DL3+ donors, in pre-expanded NK cells, but there is a significant difference in post-expanded NK cells. Two-way ANOVA was used to compare KIR frequencies in NK cells expanded from donors with different genotypes or expanded with various stimulatory cell lines. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

Figure 2. Inhibitory KIRs on expanded NK cells retain inhibition specificity

A): PBMCs from donor #18 were expanded with K562-mbIL15-41BBL cells. Expanded NK cells were stimulated with either HLA-null 721.221 cells or C1, C2, or Bw4-expressing 721.221 variants. CD107a+ cells were gated for 2DL1single positive (sp), 3DL1sp and 2DL2/3sp NK subsets by flow cytometry, and the percentage of CD107a+ cells was shown within the gate. **B):** Similar relationships as in Fig. 2A were found for expanded NK cells from 12 healthy donors using K562-mbIL15-41BBL cells, when stimulated with the 721.221-C1, 721.221-C2 and 721.221-Bw4 cell line respectively, in the absence or presence of rituximab (RT: 0.5ug/mL). Single positive (sp), double and triple-positive, as well as iKIR-null NK cell subsets were gated based on KIR2DL1, KIR2DL2/3 and KIR3DL1

expression by flow cytometry analysis. Two-way ANOVA analyses was used to compare normalized CD107a level between NK cells that only express cognate iKIR and NK cells that express different combinations of iKIRs. Statistics are only shown for the RT treatment group (black bars). (ns: not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

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Figure 3. Inhibition from iKIR/KIR-ligand interactions can override the licensing effect, and rituximab treatment does not overcome differences due to licensing or iKIR inhibition
A): Normalized CD107a levels of licensed NK cells (2DL2/3sp and 2DL1+2DL2/3+ NK cells, blue bars) expanded from C1/C1 donors (n=4) were compared to normalized CD107a levels of unlicensed NK cells (grey bars) expanded from the same donors, following stimulation with HLA-null, HLA-C1 or C2 expressing 721.221 cells, either with or without rituximab (RT) treatment (0.5ug/mL). **B):** Normalized CD107a levels of licensed NK cells (blue bars) expanded from C2/C2 donors (n=5) were compared to normalized CD107a levels

of unlicensed NK cells (grey bars) expanded from the same donors, following stimulation with HLA-null, HLA-C1 or C2 721.221 cells, either with or without RT treatment (0.5ug/mL). **C**): Normalized CD107a levels of licensed NK cells (3DL1sp, blue bars) expanded from Bw4+ donors (n=5) were compared to normalized CD107a levels of unlicensed NK cells (iKIR-null, grey bars) expanded from the same donors, following stimulations with HLA-null, HLA-C1, C2 or Bw4 721.221 cells, either with or without RT treatment (0.5ug/mL). In **A**, **B** and **C**, one-way ANOVA analyses (matching by donors) was used to compare CD107a levels between licensed and unlicensed NK cell subsets expanded from the same donor. (ns: not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

Figure 4. Inhibitory and activating KIR frequencies are affected by KIR-ligand expressed on stimulatory cell

PBMCs from healthy donors were expanded using 721.221 HLA-null or 721.221 HLA-C1/C2/Bw4 variants as stimulatory cells, at PBMC: stimulatory ratios of 1:1. **A**): KIR2DS1 and KIR2DL1 staining were distinguished by a mAb specific for 2DL1 and a mAb recognizing 2DL1 and 2DS1. **B**): The frequency of 2DL1+2DS1+, 2DL1+2DS1- and 2DL1-2DS1+ cells in NK cells expanded with HLA-null 721.221 cells was compared to the frequency of the same subset in NK cells expanded with HLA-C2 721.221 cells, regardless

of expression of other iKIRs on NK cells (n=4). The frequency of 2DL3+ cells and the frequency of 3DL1+ cells in NK cells expanded with HLA-null 721.221 cells were compared to the frequency of the same subset in NK cells expanded with HLA-C1 721.221 or HLA-Bw4 721.221 cells respectively, regardless of expression of other iKIRs on NK cells (n=4). Paired t test or two-way ANOVA (matched by donor) was used to compare KIR frequencies in NK cells expanded with various stimulatory cell lines. (ns: not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

Table 1

Healthy donor KIR and KIR-ligand genotypes

Sample ID	KIR 2DL1	KIR 2DL2	KIR 2DL3	KIR 3DL1	HLA-C*	HLA-Bw4
4	+	+	+	+	C1/C1	-
8	+	+	+	+	C1/C2	+
12	+	+	+	-	C1/C1	-
14	+	+	+	+	C2/C2	+
18	+	-	+	+	C2/C2	-
23	+	+	-	+	C1/C2	+
37	+	-	+	+	C1/C1	+
38	+	-	+	+	C1/C1	-
40	+	-	+	+	C1/C2	+
44	+	-	+	+	C2/C2	+
47	+	-	+	+	C2/C2	-
48	+	+	+	8	C2/C2	+

* Since HLA-C1 and HLA-C2 are the 2 main supersets of alleles for HLA-C, all individuals should be either C1/C1, C1/C2 or C2/C2