Clinical & Translational Immunology

Australian and New Zealand Society for Immunology Inc.

Clinical & Translational Immunology 2020; e1151. doi: 10.1002/cti2.1151
www.wileyonlinelibrary.com/journal/cti

ORIGINAL ARTICLE

Identifying the optimal donor for natural killer cell adoptive therapy to treat paediatric B- and T-cell acute lymphoblastic leukaemia

Bree Foley[1], Clara Ta[1], Samantha Barnes[1], EmmadeJong[1], Michelle Nguyen[1,2], Laurence C Cheung[1,3], Anthony Buzzai[1], Teagan Wagner[1], Ben Wylie[1], Sonia Fernandez[2], Mark Cruickshank[2], Raelene Endersby[1], Ursula Kees[1] & Jason Waithman[1]

[1]Telethon Kids Institute, University of Western Australia, Nedlands, Australia
[2]School of Biomedical Sciences, University of Western Australia, Nedlands, Australia
[3]School of Pharmacy and Biomedical Sciences, Curtin University, Perth, Australia

Correspondence
Dr J Waithman, Telethon Kids Institute, The University of Western Australia, Northern Entrance, Perth Children's Hospital, 15 Hospital Avenue, Nedlands, PO Box 855, West Perth, WA 6872, Australia.
E-mail: jason.waithman@telethonkids.org.au

Received 7 January 2020;
Revised 10 June 2020;
Accepted 10 June 2020

doi: 10.1002/cti2.1151

Clinical & Translational Immunology
2020; **9**: e1151

Abstract

Objectives. Natural killer (NK) cells are an attractive source of cells for an 'off the shelf' cellular therapy because of their innate capacity to target malignant cells, and ability to be transferred between donors and patients. However, since not all NK cells are equally effective at targeting cancer, selecting the right donor for cellular therapy is critical for the success of the treatment. Recently, cellular therapies utilising NK cells from cytomegalovirus (CMV)-seropositive donors have been explored. However, whether these NK cells are the best source to treat paediatric acute lymphoblastic leukaemia (ALL) remains unclear. **Methods.** Using a panel of patient-derived paediatric B- and T-ALL, we assessed the ability of NK cells from 49 healthy donors to mount an effective functional response against these two major subtypes of ALL. **Results.** From this cohort, we have identified a pool of donors with superior activity against multiple ALL cells. While these donors were more likely to be CMV^{+}, we identified multiple CMV^{neg} donors within this group. Furthermore, NK cells from these donors recognised B- and T-ALL through different activating receptors. Dividing functional NK cells into 29 unique subsets, we observed that within each individual the same NK cell subsets dominated across all ALL cells. Intriguingly, this occurred despite the ALL cells in our panel expressing different combinations of NK cell ligands. Finally, we can demonstrate that cellular therapy products derived from these superior donors significantly delayed leukaemia progression in preclinical models of ALL. **Conclusions.** We have identified a pool of superior donors that are effective against a range of ALL cells, representing a potential pool of donors that can be used as an adoptive NK cell therapy to treat paediatric ALL.

Keywords: acute lymphoblastic leukaemia, cellular therapy, cytomegalovirus, natural killer cells

INTRODUCTION

Natural killer (NK) cells have the capacity to target transformed and malignant cells. Early studies utilising autologous NK cells as an immunotherapy showed limited clinical efficacy[1–3] which was attributed to a family of inhibitory receptors known as the killer immunoglobulin-like receptors (KIRs) that recognise allelic epitopes present on certain HLA-A, HLA-B and HLA-C molecules.[4,5] Inhibitory KIR prevents NK cell elimination of cancer cells upon recognition of self-HLA.[4] To overcome NK cell inhibition, subsequent studies explored the use of allogeneic NK cells, pairing donors and recipients with a mismatch between at least one KIR gene in the donor and HLA class I allele (KIR ligand), in the context of either haematopoietic stem cell transplantation (HSCT)[6] or adoptive transfer of NK cells directly.[7] Significant survival benefits following NK cell immunotherapy have been observed for acute myeloid leukaemia (AML) and paediatric patients with acute lymphoblastic leukaemia (ALL).[6,8–10] However, while these approaches work for some ALL patients, strategies to further enhance NK cell immunotherapies are warranted.

One strategy to improve adoptive NK cell therapy is to identify those NK cells most effective at eliminating cancer. We, and others, have identified that following cytomegalovirus (CMV) infection, unique subsets of NK cells expand that differ in their capacity to proliferate, survive, eliminate tumor cells, secrete cytokines, and mediate antibody-dependent responses.[11–17] Commonly termed adaptive NK cells, they typically express the activating receptor NKG2C, CD57 and an inhibitory KIR for self-HLA.[18] Furthermore, NKG2C$^+$ NK cells undergo epigenetic reprogramming of the *IFNG* locus following CMV infection, resulting in their ability to rapidly produce IFNγ once activated at a faster rate than naïve NK cells.[19] Clinically, both CMV reactivation[20] and expansion of NKG2C$^+$ NK cells[21,22] have been associated with reduced risk of leukaemic relapse following HSCT. Recently, the use of these adaptive NK cells as the source of cells for preclinical NK cell adoptive therapy has demonstrated promising results across a range of cancers including ALL[23,24] and phase I clinical trials are currently underway to use NKG2C$^+$CD57$^+$ adaptive NK cells from CMV-seropositive (CMV$^+$) donors to treat AML and solid tumors (NCT03081780; NCT03319459).

Although these findings are extremely encouraging, it is not clear whether NK cells from CMV$^+$ donors are the most effective NK cell subset against ALL. In this study, we examined the capacity of NK cells from 49 healthy donors to mount effective functional responses against a panel of patient-derived paediatric B- and T-ALL cells, which represent the major subtypes of childhood leukaemia. We identified a group of donors who exhibited superior responses against multiple ALL cells, representing a potential pool of donors that could be used to develop an adoptive NK cell therapy capable of treating a range of ALL subtypes. The pool of superior donors comprised CMV$^+$ and CMV^{neg} individuals, each of whom harboured discrete NK cell subsets that dominated against both B-and T-ALL despite variation in the HLA type expressed on the target ALL cells. Finally, adoptive transfer of NK cells from our superior donors was associated with improved survival in preclinical models of B- and T-ALL.

RESULTS

Highly effective NK cells against both B- and T-ALL identified in CMV$^+$ and CMV^{neg} donors

We sought to identify whether NK cells from CMV$^+$ donors have enhanced capacity to target the two major types of paediatric ALL: B- and T-ALL. We examined the capacity of resting NK cells from 49 healthy donors (CMV$^+$ $n = 32$, CMV^{neg} $n = 17$; Supplementary table 1) to degranulate (CD107a expression) or produce cytokines (TNFα) following co-culture with a panel of patient-derived leukaemic cells (Table 1). Overall, all ALL cells stimulated an effective response resulting in increased CD107a expression (Figure 1a) and TNFα production (Figure 1b). Between CMV$^+$ and CMV^{neg} donors, there was no significant difference in the proportion of cells exhibiting increased CD107a expression or TNFα production across the ALL cells. However, the range of responses between CMV$^+$ donors was greater than that of CMV^{neg} donors. As not all CMV$^+$ donors expand adaptive NK cells with enhanced effector function potential,[16] we were interested in whether an increased proportion of these cells correlated with enhanced function against ALL.

While the phenotype of adaptive NK cells can vary amongst individuals,[14,16] we focused our analysis on the broad adaptive NK cell subset, $NKG2C^{+}CD57^{+}$ NK cells and their ability to produce TNFα. Similar to previous reports,[16] we observe hat about 1/3 of our CMV^{+} donors have expanded $NKG2C^{+}CD57^{+}$ NK cells (Supplementary figure 1). Intriguingly, expansion of $NKG2C^{+}CD57^{+}$ NK cells correlated with enhanced TNFα production against all B-ALL cells (r-value > 0.5, $P < 0.05$), but only 1 T-ALL, MOLT-4 (Figure 1c). To further confirm this, we divided $CD56^{+}$ NK cells into 4 groups: $NKG2C^{+}CD57^{neg}$, $NKG2C^{+}CD57^{+}$, $NKG2C^{neg}CD57^{+}$ and $NKG2C^{neg}CD57^{neg}$ and assessed the ability of each of these subsets to produce TNFα against our panel of ALL cells (Figure 1d). Of the four subsets, $NKG2C^{+}CD57^{+}$ NK cells were the most effective against B-ALL, but this was not true for T-ALL. Collectively, these results demonstrate that although adaptive NK cells are effective against B-ALL, they may not be the most effective NK cells across all types of ALL.

Identification of superior donors against both B- and T-ALL

Next, we sought to determine whether donors with high responses against one ALL cell were responders against other ALL cells, given the degree of variation in responses observed in our initial screen. Unsupervised hierarchical clustering based on the percentage of $CD56^{+}$ NK cells expressing CD107a (Figure 2a) or producing TNFα (Figure 2b) revealed clustering of donors with similar responses against the 9 ALL cells. For CD107a, there was a cluster of 9 donors who all exhibited high responses against at least 6 of the different ALL cells (both B- and T-ALL). A similar cluster of 12 donors was observed for TNFα production, with moderate to high responses against the majority of ALL cells. These donors also had high responses against the class I-negative cell line, K562 (data not shown). Importantly, not all of these high-performing donors were CMV^{+}. In contrast, there was a small pool of donors who solely had strong activity against one leukaemic cell line, PER-145, yet this was only for CD107a as there was little TNFα produced by these donors. Furthermore, there were clusters of donors who had weak responses against most of the ALL cells accounting for about 1/4–1/3 of donors.

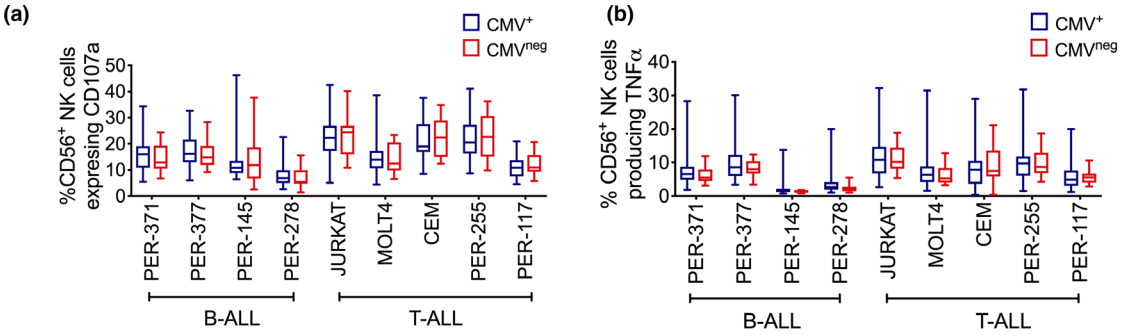
NK cells from superior donors differ in their recognition of B-ALL and T-ALL

NK cell recognition of paediatric ALL has previously been reported to occur through the activating receptors DNAM-1 and NKG2D.[9,25] We therefore examined the use of these receptors in the recognition of ALL by superior NK cells (a combination of donors identified in Figure 2; PB014, PB012, PB011, PB016, PB005, PB035, PB036 and PB041). Using gene-expression data, we observed similar levels of the NKG2D ligands: MICA, MICB, ULBP1 and ULBP2, and the DNAM-1 ligands: CD112 and CD155 across all cells in our panel (Figure 3a). Blocking NKG2D on our superior NK cells significantly decreased both CD107a expression and TNFα production against each T-ALL cell, whereas blocking DNAM-1 only had an effect against PER-117 (Figure 3b). While blocking NKG2D had some effect on B-ALL with significant decreases in CD107a and TNFα against PER-371 and PER-377, this was not as pronounced as T-ALL. Because of these discrepant results, we

Table 1. HLA class I typing of ALL cells

Cell line	ALL	HLA-A	HLA-B	HLA-C	KIR ligands[a]
PER-371	B-ALL	0301, 2902	3801, 5701	0602, 1203	C1 C2 Bw4
PER-377	B-ALL	0301	3501, 4501	0401, 0602	C2
PER-145	B-ALL	0101, 2402	3502, 3701	0402 ,0602	C2 Bw4
PER-278	B-ALL	0201, 3201	2705, 4402	0202, 0501	C2 Bw4
JURKAT	T-ALL	0301	07, 35	0401, 0702	C1 C2
MOLT-4	T-ALL	0101, 2501	1801, 5801	0602, 1203	C1 C2 Bw4
CEM	T-ALL	03, 31	08, 40	0304, 0701	C1
PER-255	T-ALL	0101, 3101	0801, 4001	0304, 0701	C1
PER-117	T-ALL	0101, 3001	0801, 1302	0602, 0701	C1 C2 Bw4

[a]KIR ligands determined by the presence of the C1 and C2 epitopes for HLA-C and the Bw4 epitope for HLA-A and HLA-B.



(c)

ALL Cell	PER-371	PER-377	PER-145	PER-278	JURKAT	MOLT4	CEM	PER-255	PER-117
r value	0.4884	0.5458	0.5619	0.6867	-0.0476	0.4691	0.04468	0.1198	0.1761
P value	0.0005	<0.0001	0.0001	<0.0001	0.751	0.0009	0.7655	0.4226	0.2363

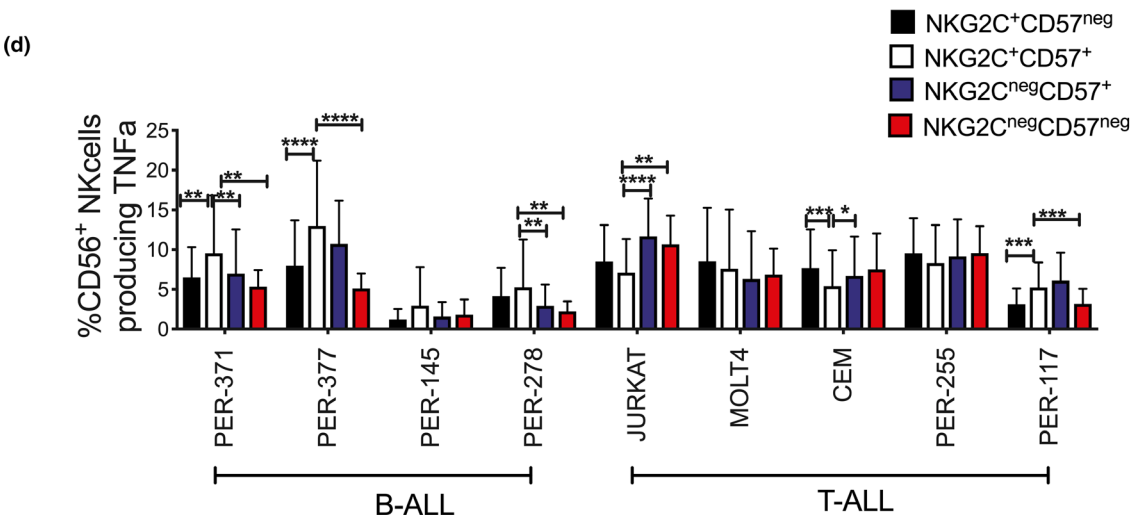


Figure 1. NK cells from CMV$^+$ and CMV^{neg} donors are effective against B- and T-ALL with adaptive NK cells from CMV$^+$ donors better against B-ALL. Donors were divided into two groups CMV$^+$ (n = 32) and CMV$^-$ (n = 17) prior to incubation with either media alone (data not shown) or against a panel of leukaemic cell targets. After incubation, CD107a expression **(a)** and TNFα production **(b)** were measured on CD56$^+$ CD3$^-$ NK cells. **(c)** The percentage of NK cells expressing NKG2C and CD57 from CMV$^+$ donors was correlated with the percentage of CD56$^+$ NK cells producing TNFα. Pearson's r and the P-value for each pair were calculated. **(d)** CD56$^+$ NK cells from CMV$^+$ donors were divided into four groups: NKG2C$^+$CD57^{neg}, NKG2C$^+$CD57$^+$, NKG2C^{neg}CD57$^+$ and NKG2C^{neg}CD57^{neg}. TNFα production was measured after 5 h of incubation with leukaemic cell targets. Bars represent the means ± SEM. NKG2C$^+$CD57$^+$ NK cells were compared to the other subsets using the paired Student's t-test, *$P \leq 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.001$.

next assessed the surface expression of a subset of NKG2D (MICA/B) and DNAM-1 (CD112 and CD155) ligands (Figure 3c). Despite similar mRNA expression, surface expression differed greatly amongst the ALL cells. Overall B-ALL cells expressed slightly lower levels of MICA/B which may partly explain the difference in NKG2D binding. Interestingly, while most ALL cells

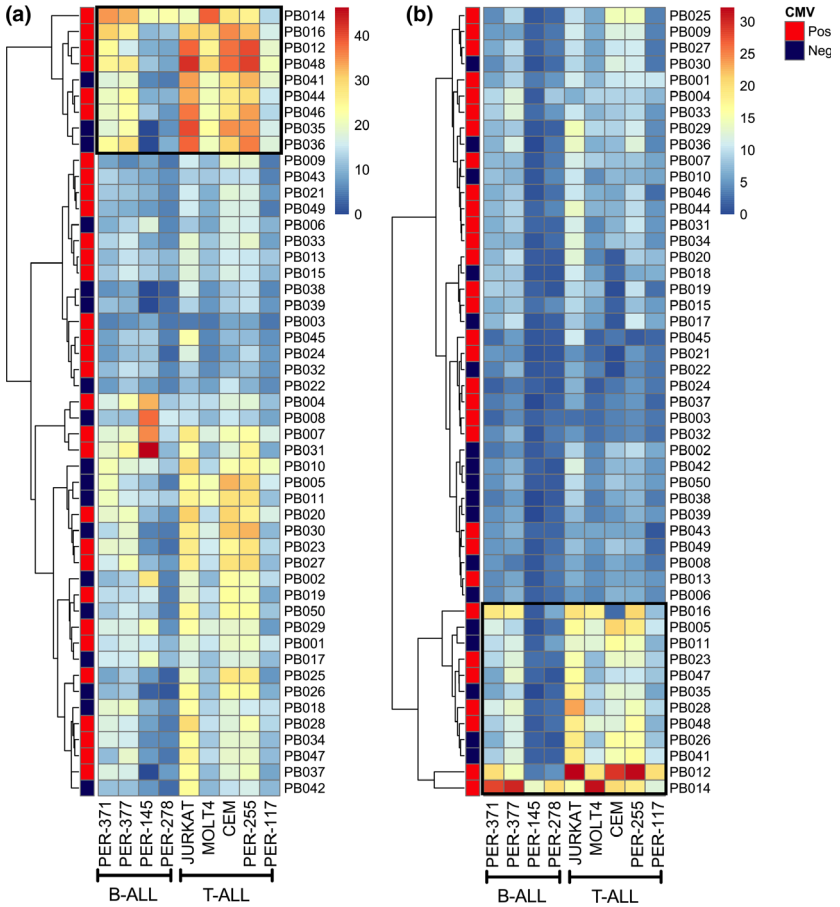


Figure 2. A cluster of donors dominate against several ALL cells. Unsupervised hierarchical clustering analysis based on the percentage of $CD56^+$ NK cells expressing CD107a **(a)** or producing TNFα **(b)**. This analysis clustered donors (indicated by PB number) based on their similar responses against the 9 ALL cells. The cluster of donors with the highest activity against the most ALL cells are highlighted in black. The colour scale represents the percentage of NK cells expressing either CD107a or TNFα.

expressed similar levels of CD112 and CD155, only PER-117 expressed very high levels of CD155 and was the only ALL cell to respond to blocking DNAM-1. Taken together, these results suggest that additional receptors are involved in the recognition of B-ALL, and thus, we investigated additional activating receptors including NKG2C, NKp30, NKp46 and 2B4. Of these additional receptors, only 2B4 appeared to play a role in NK cell recognition, as blocking 2B4 significantly decreased both CD107a expression and TNFα production against two of the B-ALL cells, PER-371 and PER-278 (Figure 3d). Despite PER-377 also expressing equivalent levels of CD48, the ligand for 2B4 (Supplementary figure 2a) there was no effect of blocking 2B4. Similarly, blocking NKp30, NKp46 or HLA-E (ligand for NKG2C) failed to suppress NK cell function (Supplementary figure 2b and c). Collectively, this demonstrates that our superior donors recognise B- and T-ALL through distinct mechanisms.

CD57 expression demarcates NK cell subsets with enhanced effector function against both B- and T-ALL

We next sought to determine whether there were differences in the phenotype of the NK cell subset (s) that had the highest effector function against each of the ALL cells. $CD107a^+$ or $TNF\alpha^+$ NK cells from the top 20 donors against each ALL cell were divided into 29 unique subsets based on CD158a, CD158b, KIR3DL1, NKG2C and CD57 expression (Figure 4 CD107a, Supplementary figure 3 TNFα). While there was a large range in responses, the most functional NK cells all expressed CD57, suggesting that NK cells expressing CD57 are the most effective against paediatric B- and T-ALL.

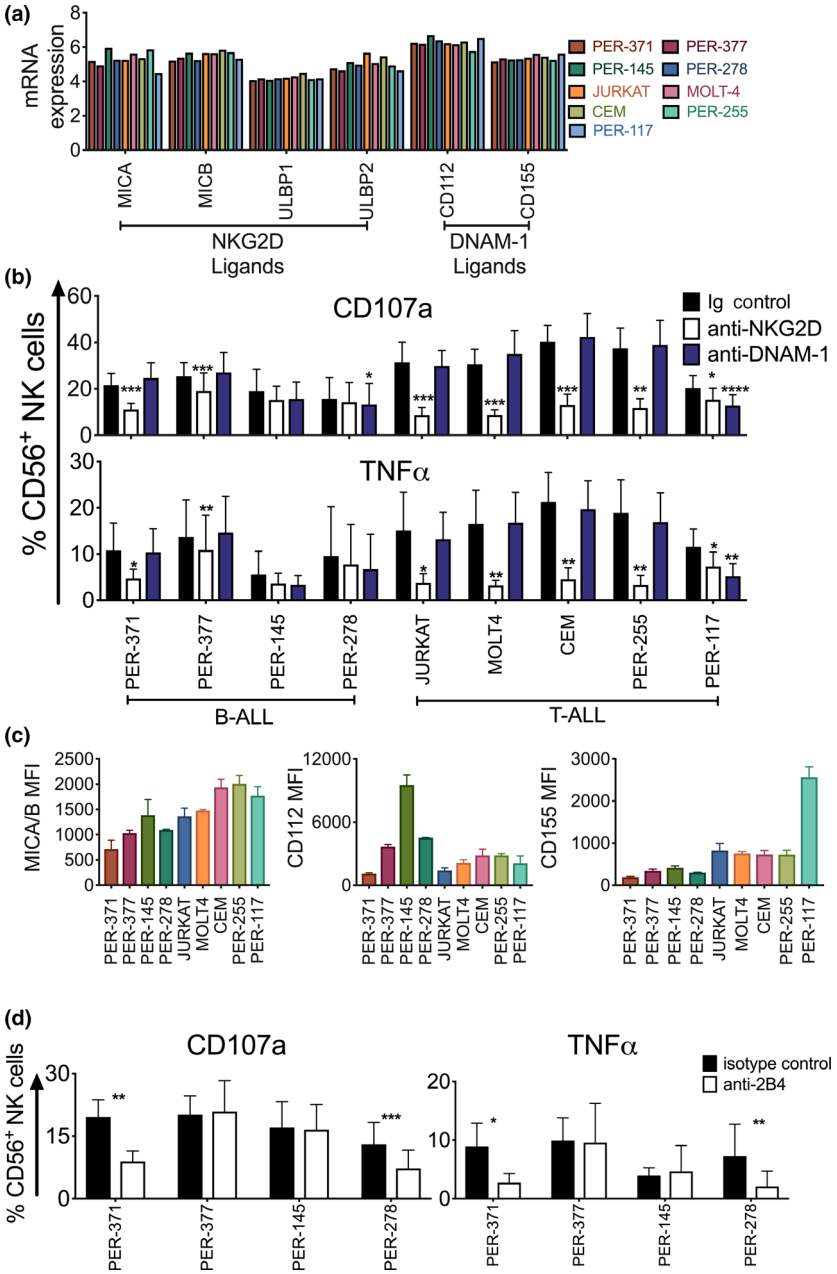


Figure 3. NK cells predominately recognise T-ALL through NKG2D and B-ALL through 2B4. **(a)** mRNA expression of known ligands of the activating receptors NKG2D and DNAM-1 on the panel of leukaemic cell targets. **(b)** Resting PBMCs from the top donors (n = 5–7) against each leukaemic cell were pre-incubated with either 10 μg mL^{-1} isotype control or 10 μg mL^{-1} anti-NKG2D or anti-DNAM-1 prior to incubation with each leukaemic cell target for 5 h. After incubation, CD107a expression (top panel) and TNFα production (bottom panel) were measured on CD56^{+} CD3^{-} NK cells. Bars represent the means ± SEM. PBMCs blocked with anti-NKG2D or anti-DNAM-1 were compared against the isotype control using the paired Student's t-test, *$P \leq 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$. **(c)** Surface expression of MICA/B, CD112 and CD155 (measured as MFI) was determined by flow cytometry. **(d)** Resting PBMCs from the top donors (n = 5 or 6) against each B-ALL cell were pre-incubated with either 10 μg mL^{-1} isotype control or 10 μg mL^{-1} anti-2B4 prior to incubation with each leukaemic cell target for 5 h. After incubation, CD107a expression (left panel) and TNFα production (right panel) were measured on CD56^{+} CD3^{-} NK cells. Bars represent the means ± SEM. PBMCs blocked with anti-2B4 were compared against the isotype control using the paired Student's t-test, *$P \leq 0.05$ and **$P < 0.01$.

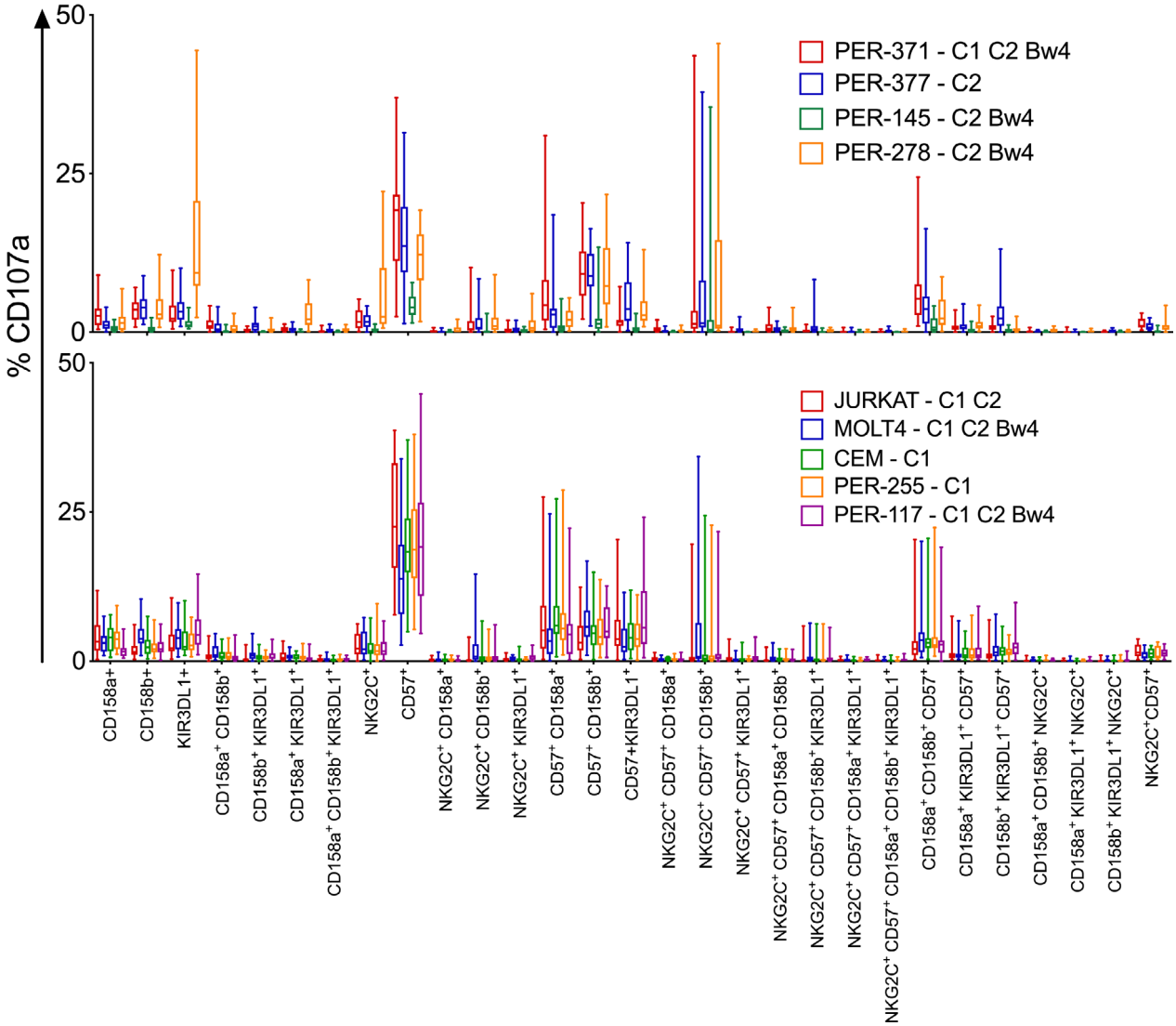


Figure 4. CD57-expressing NK cell subsets dominate against both B- and T-ALL cells regardless of ALL HLA expression. Each donor was ranked based on the percentage of $CD56^+$ $TNF\alpha^+$ and $CD56^+$ $CD107a^+$ NK cells against each ALL cell. The top 20 donors were identified, and the $CD56^+$ NK cells expressing CD107a were further subdivided into distinct NK cell subsets based on CD158a, CD158b, KIR3DL1, NKG2C and CD57 staining.

These $CD57^+$ NK cells expressed either no KIR or a single dominant KIR. Despite a strong trend towards $CD57^+$ NK cells being highly functional, NK cells co-expressing CD57 were not universally strong effectors as there were subsets co-expressing CD57 with poor effector function.

Distinct NK cell subsets dominate across both B- and T-ALL regardless of HLA expression

While $CD57^+$ NK cells dominated against both B- and T-ALL, the range of responses for many of the subsets varied greatly. To gain a better understanding into these varied responses, we next focused our analysis on individual donors and their responses against each of the ALL cells. We first selected two superior donors, PB014 and PB012 with differing target efficacies: PB014 had a higher propensity for B-ALL compared to T-ALL, whereas the opposite was true for PB012. Interestingly, we observed that distinct subsets dominated across all ALL cells and differed between the two donors (Figure 5). Despite being both CMV^+, only PB014 relied on adaptive NK cells expressing NKG2C and CD57 as their most

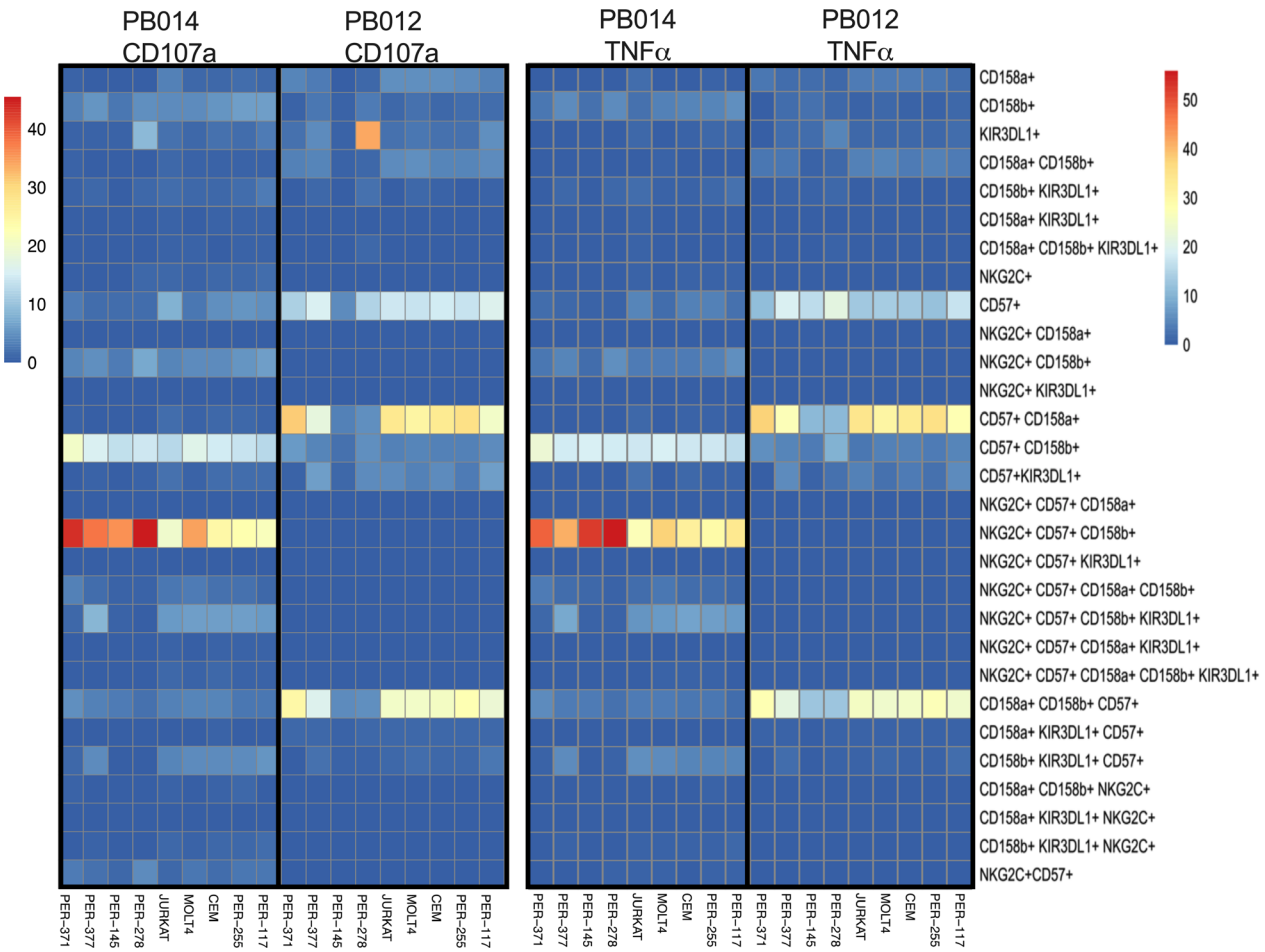


Figure 5. Distinct NK cell subsets exhibit ALL recognition activity from PB014 and PB012. $CD56^+$ NK cells expressing CD107a or producing TNFα were further subdivided into distinct NK cell subsets based on CD158a, CD158b, KIR3DL1, NKG2C and CD57 staining. The scale represents the degree to which each subset (individual squares) contributes to the $CD107a^+$ or $TNF\alpha^+$ population of NK cells.

effective subset, with this subset also expressing CD158b, while for PB012, NK cells expressing CD57 and CD158a were the most effective.

Surprisingly, despite the leukaemic cells expressing different combinations of inhibitory KIR ligands (Table 1) this had little effect on the subsets with the greatest effector function. Rather, the same subsets dominated even if the leukaemic cells were expected to express an HLA that would be predicted to inhibit the function of these NK cells. Of particular note, 6/9 ALL cells expressed KIR ligand C1, and 7/9 ALL cells expressed KIR ligand C2 (Table 1), yet $CD158b^+$ NK cells dominated for PB014 and $CD158a^+$ dominated for PB012. Upon analysis of additional donors with both high responses against B- and T-ALL cells, we observed the same pattern of distinct subsets dominating across all ALL cells regardless of the HLA expressed by the ALL cell (Supplementary figure 4 and data not shown).

Self-KIR-expressing NK cells are effective in the presence of their cognate ligand

The observation that distinct NK cell subsets dominate across our ALL panel irrespective of NK inhibitory ligand expression is highly relevant for selecting appropriate donors for NK cell adoptive therapy. For most clinical trials, these donors are selected using KIR ligand mismatch models, which predicts a donor's response based on the presence or absence of KIR ligands on the leukaemic cells. Here, we can show that amongst our top donors there are clearly highly effective NK cell responses

in the presence of their cognate ligand. ALL cells are well known for their high expression of class I HLA[26,27] so the dominance of NK cells in the presence of their cognate ligand was unexpected. Analysis of microarray data on these cells confirmed that all ALL cells in our panel had similar mRNA expression of class I HLA (Figure 6a). While cell surface density of pan-HLA class I (Figure 6b) of each cell varied, all cell lines expressed at least 9-fold higher levels of HLA class I compared to the class I-negative cell line K562. Since we had observed these discordant responses for our top donors, we broadened our analysis to all donors to determine whether discordant responses were seen for the whole cohort. Self-KIR-expressing cells from each donor were divided into 6 subsets based on CD158a, CD158b and KIR3DL1 expression and the presence of their cognate ligand in their donor (Figure 6c). For PER-377, CEM and PER-255, self-KIR-expressing NK cells were predicted to be the most effective (indicated in green); however, there were still high discordant responses from some self-KIR-expressing NK cells failing to meet these prediction criteria. Similar responses were observed for TNFα production (data not shown). This trend was particularly noticeable against the leukaemic cells expressing the three main inhibitory ligands, C1 C2 and Bw4 (PER-371, MOLT-4 and PER-117) with high responses from NK cells expressing self-KIR in the presence of their cognate ligand. Interestingly, when HLA class I was blocked with a pan-HLA class I antibody, overall NK cell function was increased, and the same subsets dominated (Supplementary figure 5 and data not shown) suggesting that while the ALL cells in our panel express levels of HLA class I capable of inhibiting NK cell function, NK cells from our top donors have the capacity to overcome this inhibition.

In vitro screen identifies the top donors for adoptive NK cell therapy

Our *in vitro* screen has clearly identified potential donors, with highly potent NK cells, that would have been excluded based on the current prediction models.[9,28] We next evaluated the capacity of NK cells from these donors to treat leukaemia *in vivo*. Donors PB014 (CMV^+) and PB012 (CMV^+) were selected as the top donors against B-ALL PER-371 and T-ALL Jurkat, respectively. We also selected two donors PB008 (CMV^{neg}) and PB002 (CMV^{neg}) with moderate anti-tumor efficacy against the chosen ALL cells. NSG mice were inoculated with either PER-371 (Figure 7a) or Jurkat (Figure 7b) leukaemic cells followed by adoptive transfer of activated NK cells. For PER-371, adoptive transfer of NK cells from PB014 significantly increased survival compared to no treatment with one mouse surviving for over 100 days (Figure 7c top panel). Similarly, mice with T-ALL treated with adoptive transfer of NK cells from PB012 lived significantly longer than untreated mice (Figure 7c bottom panel). More importantly, both our top donors were better at controlling leukaemia progression than NK cells from our moderate donors, providing proof-of-principal evidence that our *in vitro* screen can identify optimal donors for NK cell-based cellular therapies.

DISCUSSION

NK cells are critical mediators of the elimination of leukaemic cells making them ideal candidates for cellular therapy. Characteristics of superior donors or NK cell subset(s) for adoptive NK cell therapy, however, are poorly understood. Here, we demonstrate in a population of 49 healthy donors, there is a potential pool of donors with superior responses against paediatric ALL, which could represent a source of effective, functional NK cells for cellular therapy. These donors harboured individually distinct NK cell subsets that were effective against all ALL cells regardless of the HLA expressed on the leukaemic cells or the way they recognised each type of ALL. Collectively, our data highlight the importance of *in vitro* functional testing of potential donors. Many of the superior donors we identified may not have been selected using currently employed KIR ligand mismatch selection methods.

Understanding why some donors harbour NK cells with superior responses against paediatric ALL remains to be further explored. Based on the association between CMV reactivation and leukaemic relapse[20] and the enhanced effector function of NK cells from CMV^+ donors,[12–16] there has been a recent interest to develop cellular therapies from these NK cells.[23,24] Liu and colleagues demonstrated that expanded activated adaptive NK cells from CMV^+ donors efficiently eliminate HLA-mismatched ALL blasts.[24] Here, we

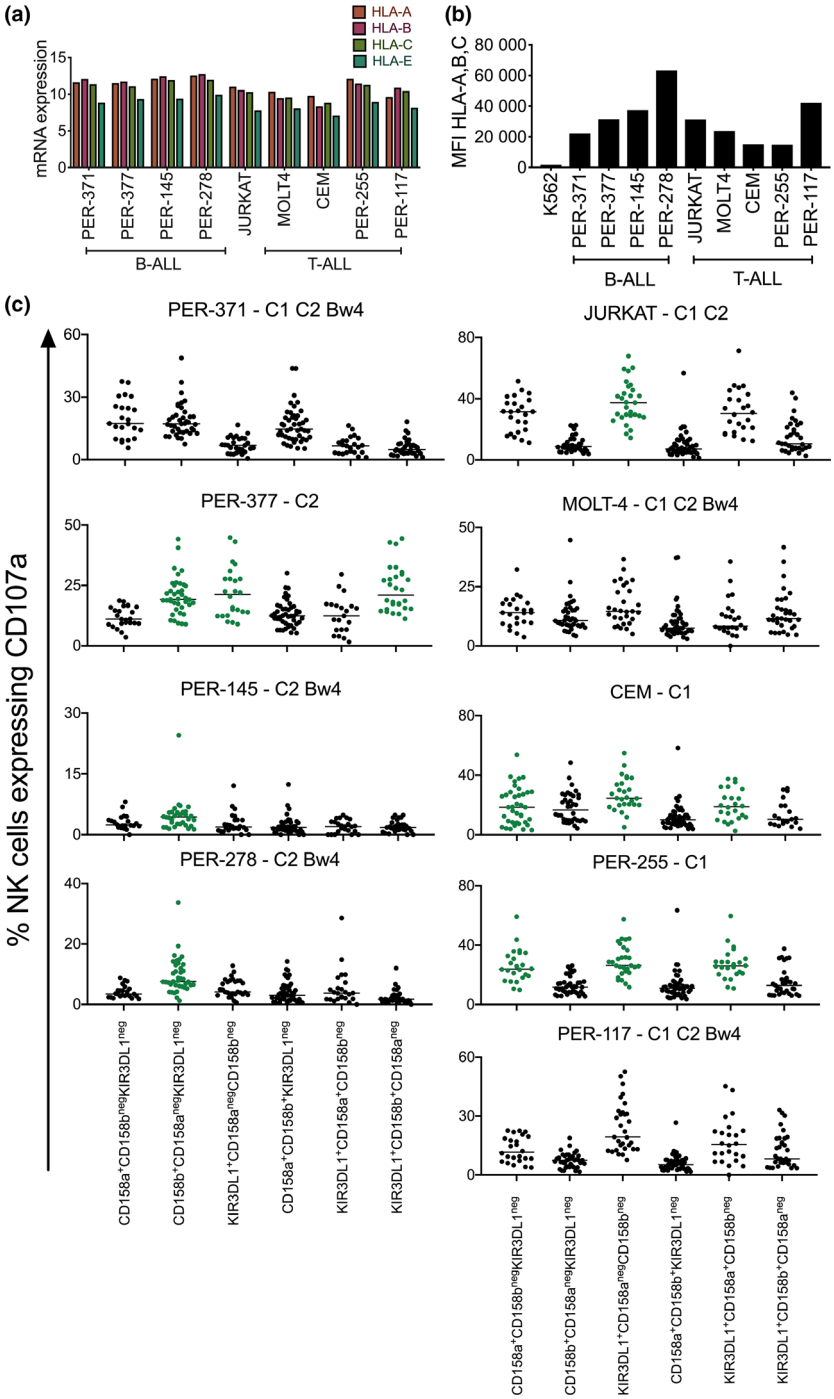


Figure 6. Self-KIR-expressing NK cells are effective in the presence of their cognate ligand. Expression of HLA class I was determined by mRNA **(a)** or surface expression **(b)** measured as MFI using flow cytometry. **(c)** Self-KIR-expressing NK cells from each donor (n = 49) were divided into 6 subsets based on CD158a, CD158b and KIR3DL1 expression and the presence of their cognate ligand in their respective donor. KIR subsets predicted to respond in the absence of their cognate ligand on the ALL cell are marked in green.

confirm that adaptive NK cells from CMV^{+} donors can recognise ALL, but that these NK cells have a higher propensity for B-ALL over T-ALL. Furthermore, while the majority of top donors were CMV^{+}, there were clearly highly effective NK cells from CMV^{neg} donors. CMV has been shown

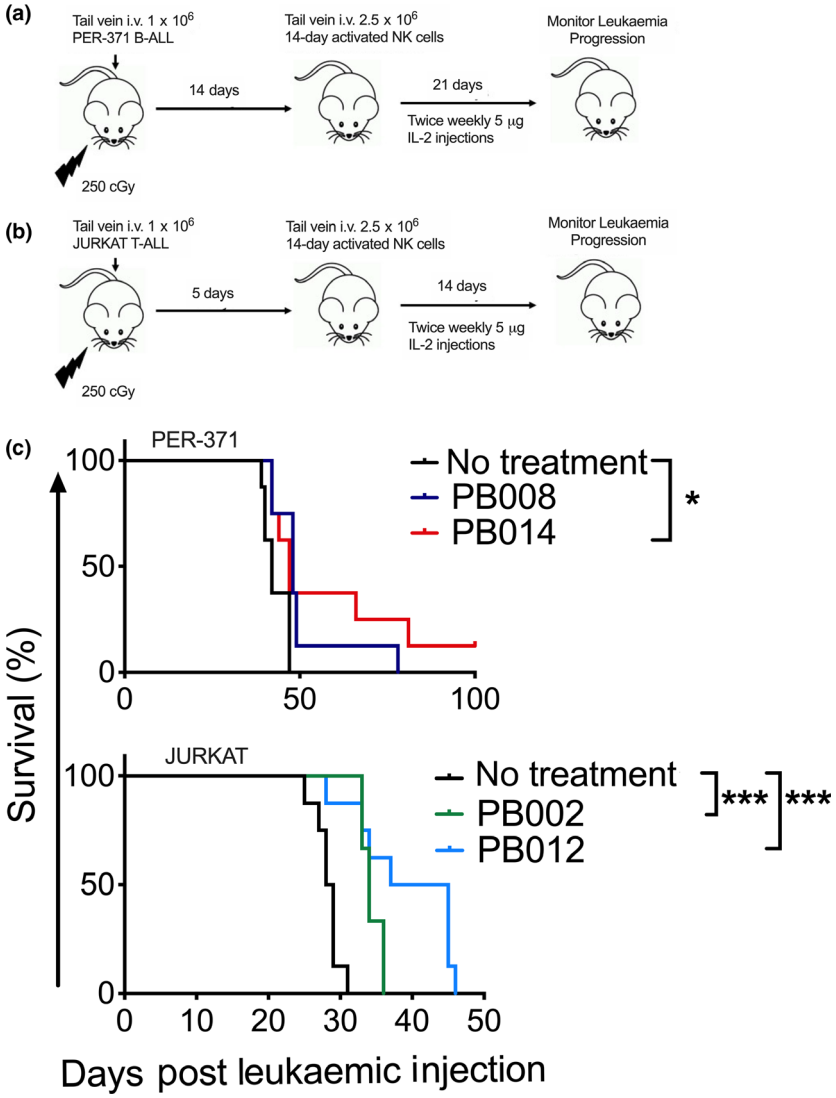


Figure 7. Adoptive transfer of NK cells improves survival of both B-ALL and T-ALL. NSG mice were irradiated with 250cGy followed by tail vein injection of either 1×10^6 PER-371 B-ALL **(a)** or Jurkat T-ALL **(b)**. Mice were either left untreated or treated with 14-day-activated NK cells, followed by twice-weekly injections of IL-2. **(c)** Kaplan–Meier curves summarise the survival of mice treated. A total of 6–8 animals were used per group, and each experiment was performed independently two times. Groups without treatment were compared with treatment groups using the log-rank (Mantel–Cox) test, $*P \leq 0.05$ and $***P < 0.001$.

to expand subsets of NK cells that have undergone profound epigenetic reprogramming resulting in NK cells that can respond more efficiently.[16,19,29] Whether similar changes occur in our superior CMV^{neg} donors as a result of exposure to other pathogens or other environmental exposures remains unclear. Furthermore, it is not clear whether past exposure to CMV is the main mechanism in driving heightened effector responses against paediatric ALL in our superior CMV^{+} donors. Nevertheless, our data clearly demonstrate that effective NK cells can be identified from both CMV^{+} and CMV^{neg} donors and future studies to understand the mechanisms that drive these superior NK cell responses are warranted for the design of optimal adoptive NK cell therapeutic products.

Here, we show that NK cells expressing only CD57 or co-expressing CD57 were the most effective. NK cells acquire CD57 as they functionally mature, and CD57 defines NK cells that are highly differentiated.[18] High frequencies

of $CD57^+$ NK cells have been associated with reduced relapse rates and overall improved survival in a range of different cancers including leukaemia.[30] However, not all NK cells that expressed CD57 had heightened responses against ALL in our panel, and thus, CD57 expression alone is not sufficient to predict superior donors. While it is not surprising that CD57 NK cells lacking expression of the three major inhibitory KIRs (CD158a, CD158b and KIR3DL1) were the most effective subsets in the majority of donors, the high responses from KIR-expressing NK cell subsets in the presence of their cognate inhibitory ligand were surprising. In addition, despite the ALL cells in our panel expressing different combinations of the three HLA class I KIR ligands C1, C2 and Bw4, the same NK cell subsets from each individual donor dominated across all of the ALL cells. It is possible that a proportion of these NK cells may express activating KIR which when bound to their cognate ligand (HLA-C) activates these particular NK cells. While we did not perform an exhaustive analysis of activating KIR, our data so far suggest that at least against the T-ALL Jurkat, activating KIRs are not the main receptors involved in the recognition of leukaemia (data not shown). Collectively, these findings contrast with the currently used method to select potential donors in the clinic, which uses the prediction of whether a potential donor's NK cells will be inhibited by the HLA expressed on the leukaemic cell. Relying on this prediction, we would have failed to identify the most efficacious donors analysed in this study. It is possible that the level of HLA class I required for sufficient inhibition differs amongst different leukaemic cells. This may result in the capacity for some subsets of NK cells to mount an effective response even in the presence of their cognate inhibitory ligand. These findings also raise the possibility that strong activating signals may be overriding inhibitory signals on these NK cells, allowing the NK cell to eliminate the target as we and others have previously reported.[9,31,32] Collectively, based on our data we would argue that different subsets may be more effective from different donors and there is not an overall subset that is necessarily more effective over another. Determining the optimal way to identify superior donors, and their relevant effective subsets, is certainly warranted to develop improved NK cell therapies for ALL.

While we observed that distinct subsets of NK cells show efficacy against all ALL cells, the mechanism of recognition of B- versus T-ALL varied greatly. Despite previous reports observing a role for DNAM-1 in recognition of ALL,[25,33] DNAM-1 was only involved in the recognition of the T-ALL, PER-117. This recognition is likely driven by the high expression of CD155 on PER-117, which was expressed at lower levels on all other cells. Furthermore, in contrast to a previous study by Toreli and colleagues[29] who reported a role for CD112 in the recognition of paediatric ALL, despite expression of CD112 on nearly ALL cells it does not appear that DNAM-1 recognition of CD112 is involved in enhanced NK cell effector function against our panel of ALL cells. NKG2D, however, was the predominant receptor involved in the recognition of T-ALL. In contrast, NKG2D was only partially involved in the recognition of B-ALL in our panel. Despite testing multiple other receptors, only the SLAM family member 2B4 was identified as being involved in the recognition of B-ALL, which was observed for two of the B-ALL cells despite high surface expression of CD48 also on PER-377. These two B-ALLs (PER-371 and PER-278) were the most closely related leukaemias based on our microarray data,[34] raising the possibility that 2B4 is involved in the recognition of a subset of B cell leukaemias. In addition, we cannot conclude why superior NK cells responded better than other NK cells in recognising ALL. We performed additional phenotyping to see whether we could detect a difference between superior and non-superior donors in respect to expression of NKG2D, 2B4 or DNAM-1 yet we found no significant differences between the groups (data not shown). Our results suggest that NK cell recognition of paediatric ALL is far more heterogeneous than previously thought, and identification of the crucial receptors and pathways involved will greatly improve the selection of efficacious NK cells to treat B- and T-ALL.

While treatment success rates for paediatric ALL have risen dramatically, children diagnosed with poor prognosis ALL and patients with relapsed ALL urgently require improved treatment options. Cellular therapies derived from the NK cells of healthy donors provide alternative immune-based options for these patients. Using our *in vitro* screen, we have identified a pool of superior donors that can be used to develop 'off the shelf' cellular therapies to treat these patients. Adoptive transfer of these superior NK cells significantly prolonged the survival of mice with either B- or T-

ALL. While these NK cells alone were not curative, there are multiple approaches that can be explored to further enhance the ability of these superior NK cells to improve the control of leukaemia. Enriching for particular subsets, such as $CD57^+$ NK cells, is one approach to improve therapeutic efficacy. Although $CD57^+$ NK cells are typically less proliferative compared to $CD57^{neg}$, Cichocki and colleagues have demonstrated that the addition of the small molecule GSK3 inhibitor, CHIR99021, aids in the enrichment of $CD57^+$ NK cells.[23] Another approach may involve incorporating chimeric antigen receptors (CAR) into the most effective NK cells.[35] Whether this involves a CD19 CAR similar to that in use for CAR T-cell therapy,[35,36] a CD5 CAR for T-ALL[37] or a CAR that utilises NK cell activating receptors,[38] advancements in our ability to engineer NK cells have greatly improved the potential to further enhance NK cells for adoptive cell therapy. Regardless of which approach or combination of approaches is selected for further development of an NK cell-based cellular therapy product, it is critical that donors with the most effective NK cells are selected. We have identified superior NK cells against both B- and T-ALL. Future studies to understand the mechanisms that drive the enhanced effector function of these superior NK cells against not just ALL but other leukaemias and potentially solid tumors will lead to the design of robust cellular therapy protocols that maximise the potential of these superior NK cells to efficiently eliminate cancer.

METHODS

Donors

Blood was obtained from 49 blood donors attending The Australian Red Cross Blood Service, Western Australia, with informed consent obtained in accordance with the Declaration of Helsinki. Written approval to use blood samples was obtained from the University of Western Australia (RA/4/1/7311). Peripheral blood mononuclear cells (PBMCs) were purified by density centrifugation using Lymphoprep (StemCell Technologies, Vancouver, Canada) and cryopreserved. Before analysis for degranulation or production of intracellular cytokines, the thawed cells were incubated overnight at 37°C in complete media (R10) without exogenous cytokines [RPMI (Life Technologies, Scoresby, Australia) supplemented with 10% foetal calf serum (Sigma, Castle Hill, Australia), 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 2 mM GlutaMax, 24 μM 2-ME, 1% sodium pyruvate and 1% non-essential amino acids (all Life Technologies)]. HLA class I typing was performed for each donor by next generation sequencing of exons 2 and 3 of HLA-A, HLA-B and HLA-C by the Institute of Immunology and Infectious Diseases, Murdoch University, Australia.

Cell lines

Patient-derived cell lines PER-371, PER-377, PER-145, PER-278, PER-255 and PER-117 were established from the bone marrow of paediatric patients being treated at the former Princess Margaret Hospital for Children in Perth, Australia (Table 1), as previously reported.[34] Commercial cell lines JURKAT, MOLT-4, CEM and the class I-negative cell line K562 were obtained from the American Type Tissue Culture (ATCC) (Manassas, VA). All cell lines were cultured in R10 and used in experiments within three weeks after thawing. Each cell line was determined to be mycoplasma free using the MycoAlert Mycoplasma Detection Kit (Lonza, Basel, Switzerland). The ALL cells used in this study have been previously analysed on a cDNA microarray measuring 13,286 human cDNAs.[34] Data relevant to this project were extracted from this published data set. HLA class I typing of each ALL cell was performed by next generation sequencing of exons 2 and 3 of HLA-A, HLA-B and HLA-C. Surface HLA class I expression was determined by flow cytometry using unconjugated anti-HLA-A,B,C (W6/32; BioLegend, San Diego, CA) followed by staining with Alexa Fluor 647-conjugated rat anti-mouse IgG2a (BioLegend). Surface expression of NK ligands was determined using MICA/B (6D4), CD112 (TX31), CD155 (SKII.4) and CD48 (BJ40) antibodies (all BioLegend). Cells were analysed on a BD LSR Fortessa (BD Biosciences, San Jose, CA) and median fluorescence intensity (MFI) calculated using FlowJo Version 10 software (TreeStar, Ashland, OR).

Determination of CMV status

Cytomegalovirus serostatus was determined by testing performed by the Australian Red Cross Blood Service where possible. For donors where the CMV serostatus was unknown, PBMCs were stimulated with PepMix HCMVA (pp65) (JPT Peptide Technologies, Berlin, Germany) overnight before CMV-specific T cells were determined using an IFNγ ELISPOT as previously described.[39,40]

Functional flow assay

Expression of CD107a and production of TNFα were measured as described previously.[41] Briefly, PBMCs were incubated with B-ALL, T-ALL or K562 target cells at an effector-to-target ratio of 2:1 for 5 h. For some experiments, PBMCs were pre-incubated with either 10 μg mL^{-1} of isotype control or 10 μg mL^{-1} of anti-HLA-A, B,C, anti-HLA-E (3D12), anti-NKG2D (1D11), anti-DNAM-1 (11A8), anti-NKp30 (P30-15), anti-NKp46 (9E2) and anti-2B4 (C1.7) (all BioLegend), for 20 min at 37°C. Brefeldin A and monensin (both BD Biosciences) were added after 1 h. The following antibodies were used: anti-NKG2C (134591; R&D Systems, Minneapolis, MN), anti-KIR3DL1 (DX9; BioLegend), anti-CD56 (clone B159), anti-CD3 (SK7), anti-CD57 (NK-1), anti-CD158a (HP-3E4), anti-CD158b (CH-L), anti-CD107a (H4A3), anti-TNFα (MAb11) and fixable viability stain FV575

(all BD Biosciences). Cells were analysed on an BD LSR Fortessa and using FlowJo Version 10 software.

NK cell expansion

$CD3^+$ cells were depleted from each donor's PBMC using $CD3^+$ magnetic bead selection (StemCell Technologies) prior to expansion with irradiated K562 cells at a 6:1 ratio in R10 supplemented with 10 IU mL^{-1} (increasing to 100 IU mL^{-1} on day 7) recombinant human IL-2 (Life Technologies) and 5 ng mL^{-1} recombinant human IL-15 (PeproTech, Rocky Hill, NJ), replacing half the media every 2–3 days and splitting 1:4 on day 7. On day 14, cells were counted and washed three times in PBS in preparation for adoptive transfer.

In vivo adoptive transfer

Eight- to ten-week-old NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were purchased from the Animal Research Centre, Perth, Australia. Animals were housed under specific pathogen-free conditions, and all studies were approved by the Animal Ethics Committee, Telethon Kids Institute, Perth, Australia. Mice received one dose of 250cGy irradiation followed by transfer of 1×10^6 PER-371 or Jurkat cells. Following engraftment of leukaemic cells, mice received adoptive transfer of 2.5×10^6 activated NK cells. Mice received twice-weekly (for 2–3 weeks as indicated) 5 μg injections of recombinant human IL-2 (Life Technologies). Mice were monitored for disease progression, and once symptoms of leukaemia developed, mice were euthanised. Bone marrow, spleen and blood were analysed by flow cytometry for the presence of leukaemic cells.

Statistics

Data were summarised with mean and standard error of the mean (mean ± SEM). For comparisons between independent samples, the Student's *t*-test was used. For comparisons of matched samples, the paired *t*-test was used. For *in vivo* experiments, length of survival between groups was compared using the log-rank (Mantel–Cox) test. Statistical significance was indicated as $*P \leq 0.05$, $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$. Statistical analyses were performed using Prism 7 (GraphPad Software, San Diego, CA, USA).

ACKNOWLEDGMENTS

This work was supported by funding from Cancer Australia, Cure Cancer Australia, the Raine Medical Research Foundation, the National Health and Medical Research Council, the Government of Western Australia Department of Health, Cancer Council WA, Brady Cancer Support Foundation, The Pirate Ship Foundation, and the Children's Leukaemia and Cancer Research Foundation. We thank the Telethon Kids Institute Bioresources team for their excellent animal care and the Telethon Kids Institute Flow Facility for assistance.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest or any competing financial interests in relation to the work described.

AUTHOR CONTRIBUTIONS

Bree Foley: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Validation; Visualization; Writing-original draft; Writing-review & editing. **Clara Ta:** Data curation; Investigation; Methodology. **Samantha Barnes:** Data curation; Investigation; Methodology; Writing-review & editing. **Emma de Jong:** Formal analysis; Visualization; Writing-original draft; Writing-review & editing. **Michelle Nguyen:** Data curation. **Laurence Cheung:** Methodology; Resources; Writing-review & editing. **Anthony Buzzai:** Data curation; Writing-original draft; Writing-review & editing. **Teagan Wagner:** Data curation; Writing-original draft; Writing-review & editing. **Ben Wylie:** Writing-original draft; Writing-review & editing. **Sonia Fernandez:** Writing-original draft; Writing-review & editing. **Mark Cruickshank:** Writing-original draft; Writing-review & editing. **Raelene Endersby:** Funding acquisition; Writing-original draft; Writing-review & editing. **Ursula Kees:** Conceptualization; Funding acquisition; Resources; Writing-original draft; Writing-review & editing. **Jason Waithman:** Conceptualization; Formal analysis; Funding acquisition; Methodology; Resources; Supervision; Writing-original draft; Writing-review & editing.

REFERENCES

1. Burns LJ, Weisdorf DJ, DeFor TE *et al*. IL-2-based immunotherapy after autologous transplantation for lymphoma and breast cancer induces immune activation and cytokine release: a phase I/II trial. *Bone Marrow Transplant* 2003; **32**: 177–186.
2. Miller JS, Tessmer-Tuck J, Pierson BA *et al*. Low dose subcutaneous interleukin-2 after autologous transplantation generates sustained *in vivo* natural killer cell activity. *Biol Blood Marrow Transplant* 1997; **3**: 34–44.
3. Rosenberg SA, Lotze MT, Muul LM *et al*. A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. *N Engl J Med* 1987; **316**: 889–897.
4. Lanier LL. NK cell receptors. *Annu Rev Immunol* 1998; **16**: 359–393.
5. Moretta A, Bottino C, Vitale M *et al*. Receptors for HLA class-I molecules in human natural killer cells. *Annu Rev Immunol* 1996; **14**: 619–648.
6. Ruggeri L, Capanni M, Urbani E *et al*. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* 2002; **295**: 2097–2100.
7. Miller JS, Soignier Y, Panoskaltsis-Mortari A *et al*. Successful adoptive transfer and *in vivo* expansion of human haploidentical NK cells in patients with cancer. *Blood* 2005; **105**: 3051–3057.

8. Leung W, Iyengar R, Turner V *et al*. Determinants of antileukemia effects of allogeneic NK cells. *J Immunol* 2004; **172**: 644–650.
9. Pende D, Marcenaro S, Falco M *et al*. Anti-leukemia activity of alloreactive NK cells in KIR ligand-mismatched haploidentical HSCT for pediatric patients: evaluation of the functional role of activating KIR and redefinition of inhibitory KIR specificity. *Blood* 2009; **113**: 3119–3129.
10. Ruggeri L, Parisi S, Urbani E, Curti A. Alloreactive natural killer cells for the treatment of acute myeloid leukemia: from stem cell transplantation to adoptive immunotherapy. *Front Immunol* 2015; **6**.
11. Guma M, Cabrera C, Erkizia I *et al*. Human cytomegalovirus infection is associated with increased proportions of NK cells that express the CD94/NKG2C receptor in aviremic HIV-1-positive patients. *J Infect Dis* 2006; **194**: 38–41.
12. Lopez-Verges S, Milush JM, Schwartz BS *et al*. Expansion of a unique $CD57^{+}NKG2C^{hi}$ natural killer cell subset during acute human cytomegalovirus infection. *Proc Natl Acad Sci USA* 2011; **108**: 14725–14732.
13. Foley B, Cooley S, Verneris MR *et al*. Cytomegalovirus reactivation after allogeneic transplantation promotes a lasting increase in educated $NKG2C^{+}$ natural killer cells with potent function. *Blood* 2012; **119**: 2665–2674.
14. Beziat V, Liu LL, Malmberg JA *et al*. NK cell responses to cytomegalovirus infection lead to stable imprints in the human KIR repertoire and involve activating KIRs. *Blood* 2013; **121**: 2678–2688.
15. Foley B, Cooley S, Verneris MR *et al*. Human cytomegalovirus (CMV)-induced memory-like $NKG2C^{+}$ NK cells are transplantable and expand *in vivo* in response to recipient CMV antigen. *J Immunol* 2012; **189**: 5082–5088.
16. Schlums H, Cichocki F, Tesi B *et al*. Cytomegalovirus infection drives adaptive epigenetic diversification of NK cells with altered signaling and effector function. *Immunity* 2015; **42**: 443–456.
17. Sun JC, Beilke JN, Lanier LL. Adaptive immune features of natural killer cells. *Nature* 2009; **457**: 557–561.
18. Lopez-Verges S, Milush JM, Pandey S *et al*. CD57 defines a functionally distinct population of mature NK cells in the human $CD56^{dim}CD16^{+}$ NK-cell subset. *Blood* 2010; **116**: 3865–3874.
19. Luetke-Eversloh M, Hammer Q, Durek P *et al*. Human cytomegalovirus drives epigenetic imprinting of the IFNG locus in $NKG2C^{hi}$ natural killer cells. *PLoS Pathog* 2014; **10**: e1004441.
20. Elmaagacli AH, Steckel NK, Koldehoff M *et al*. Early human cytomegalovirus replication after transplantation is associated with a decreased relapse risk: evidence for a putative virus-versus-leukemia effect in acute myeloid leukemia patients. *Blood* 2011; **118**: 1402–1412.
21. Cichocki F, Cooley S, Davis Z *et al*. $CD56^{dim}CD57^{+}NKG2C^{+}$ NK cell expansion is associated with reduced leukemia relapse after reduced intensity HCT. *Leukemia* 2016; **30**: 456–463.
22. Cichocki F, Taras E, Chiuppesi F *et al*. Adaptive NK cell reconstitution is associated with better clinical outcomes. *JCI Insight* 2019; **4**.
23. Cichocki F, Valamehr B, Bjordahl R *et al*. GSK3 inhibition drives maturation of NK cells and enhances their antitumor activity. *Cancer Res* 2017; **77**: 5664–5675.
24. Liu LL, Beziat V, Oei VYS *et al*. *Ex vivo* expanded adaptive NK cells effectively kill primary acute lymphoblastic leukemia cells. *Cancer Immunol Res* 2017; **5**: 654–665.
25. Torelli GF, Peragine N, Raponi S *et al*. Recognition of adult and pediatric acute lymphoblastic leukemia blasts by natural killer cells. *Haematologica* 2014; **99**: 1248–1254.
26. Godal R, Bachanova V, Gleason M *et al*. Natural killer cell killing of acute myelogenous leukemia and acute lymphoblastic leukemia blasts by killer cell immunoglobulin-like receptor-negative natural killer cells after NKG2A and LIR-1 blockade. *Biol Blood Marrow Transplant* 2010; **16**: 612–621.
27. Pfeiffer M, Schumm M, Feuchtinger T *et al*. Intensity of HLA class I expression and KIR-mismatch determine NK-cell mediated lysis of leukaemic blasts from children with acute lymphatic leukaemia. *Br J Haematol* 2007; **138**: 97–100.
28. Velardi A. Natural killer cell alloreactivity 10 years later. *Curr Opin Hematol* 2012; **19**: 421–426.
29. Lee J, Zhang T, Hwang I *et al*. Epigenetic modification and antibody-dependent expansion of memory-like NK cells in human cytomegalovirus-infected individuals. *Immunity* 2015; **42**: 431–442.
30. Nielsen CM, White MJ, Goodier MR, Riley EM. Functional significance of CD57 expression on human NK cells and relevance to disease. *Front Immunol* 2013; **4**: 422.
31. Bauer S, Groh V, Wu J *et al*. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* 1999; **285**: 727–729.
32. Foley B, De Santis D, Lathbury L, Christiansen F, Witt C. KIR2DS1-mediated activation overrides NKG2A-mediated inhibition in HLA-C C2-negative individuals. *Int Immunol* 2008; **20**: 555–563.
33. Pende D, Spaggiari GM, Marcenaro S *et al*. Analysis of the receptor-ligand interactions in the natural killer-mediated lysis of freshly isolated myeloid or lymphoblastic leukemias: evidence for the involvement of the Poliovirus receptor (CD155) and Nectin-2 (CD112). *Blood* 2005; **105**: 2066–2073.
34. Kees UR, Ford J, Watson M *et al*. Gene expression profiles in a panel of childhood leukemia cell lines mirror critical features of the disease. *Mol Cancer Ther* 2003; **2**: 671–677.
35. Imai C, Iwamoto S, Campana D. Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. *Blood* 2005; **106**: 376–383.
36. Maude SL, Laetsch TW, Buechner J *et al*. Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia. *N Engl J Med* 2018; **378**: 439–448.
37. Chen KH, Wada M, Pinz KG *et al*. Preclinical targeting of aggressive T-cell malignancies using anti-CD5 chimeric antigen receptor. *Leukemia* 2017; **31**: 2151.
38. Li Y, Hermanson DL, Moriarity BS, Kaufman DS. Human iPSC-derived natural killer cells engineered with chimeric antigen receptors enhance anti-tumor activity. *Cell Stem Cell* 2018; **23**: 181–192. e185.

39. Fernandez S, French MA, Price P. Immunosenescent $CD57^{+}CD4^{+}$ T-cells accumulate and contribute to interferon-gamma responses in HIV patients responding stably to ART. *Dis Markers* 2011; **31**: 337–342.
40. Keane NM, Price P, Stone SF *et al*. Assessment of immune function by lymphoproliferation underestimates lymphocyte functional capacity in HIV patients treated with highly active antiretroviral therapy. *AIDS Res Hum Retroviruses* 2000; **16**: 1991–1996.
41. Foley B, Cooley S, Verneris MR *et al*. NK cell education after allogeneic transplantation: dissociation between recovery of cytokine-producing and cytotoxic functions. *Blood* 2011; **118**: 2784–2792.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.