Natural Killer cells differentiated *in vitro* from CB CD34$^+$ cells are more advantageous for use as an Immunotherapy over Peripheral Blood and Cord Blood Natural Killer cells

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Running title: The use of CBCD34$^+$-NK cells for clinical immunotherapy

Abstract

Background

Natural Killer (NK) cells have the potential to become a successful immunotherapy as they can target malignant cells without being direct effectors of graft-versus-host disease. Our group has previously shown that large numbers of functional NK cells can be differentiated *in vitro* from umbilical cord blood (CB) $CD34^+$ cells. In order to produce a clinically relevant and effective therapy we hypothesise that it is dependent on the NK cells being able to proliferate and persist *in vivo* whilst still maintaining an optimum activation status.

Methods

Here, we evaluated the proliferation capacity, telomere length and terminal differentiation markers of NK cells differentiated *in vitro*. As well as identifying how their cytotoxicity compared against PBNK cells and CBNK cells when targeting patient AML blasts and solid tumor cell lines.

Results

We found that the differentiated NK cells could respond to interleukin-2 and proliferate *in vitro* and that telomere length was significantly increased whilst CD57 expression was significantly reduced in comparison to PBNK cells. The cytotoxicity of the differentiated NK cells was equivalent to that of the PBNK and CBNK cell controls and priming consistently led to higher levels of killing of patient leukemic blasts and solid tumour cell lines *in vitro*. Interestingly this activation step was not required to observe killing of patient AML blasts *in vivo.*

Conclusions

We are therefore able to generate NK cells from CB $CD34^+$ cells in high numbers allowing for multiple infusions of highly cytotoxic NK cells that have potential to further proliferate *in vivo*.

Abbreviations

AML = acute myeloid leukemia, CB = cord blood, $CBCD34^+$-NK cells = Natural Killer cells differentiated from CB $CD34^+$ cells, CBNK cells = cord blood Natural Killer cells, GvHD = graft versus host, GvL = Graft versus Leukemia, HSCT = hematopoetic stem cell transplantation, NK = Natural Killer, NSG = NOD/SCID IL-2Rγnull, PBNK cells = peripheral blood Natural Killer

Introduction

Natural Killer (NK) cells are lymphocytes of the innate immune capable of cytotoxicity without prior sensitization. They are defined by the surface expression of CD56 and absence of CD3. NK cells have been shown to be effectors of Graft versus Leukemia (GvL) without causing graft versus host (GvHD) following hematopoetic stem cell transplantation (HSCT)[1] but also as an immunotherapy alone[2]. NK cells can be isolated directly from peripheral blood (PB) or umbilical cord blood (CB) however, as NK cells make up only 10-15% of circulating lymphocytes in PB[3] and 15-30% in CB[4] the number of cells obtained can be limited and could potentially prevent the option for multiple infusions. The half-life of an NK cell is reported to be around 7 days[5], therefore multiple infusions would be necessary if longer lasting therapeutic results are required for the patient.

Our group has previously shown that large cell numbers of NK cells capable of secreting high levels of IFN-γ and able to kill leukemic cells *in vitro* and *in vivo* can be differentiated from frozen CBCD34^{+} cells[6]. Further it has also been proven that these cells maintain function post cryopreservation paving the way for an off the shelf cellular product that can be used for multiple infusions to prevent infection or treat relapse[7].

To tackle the limitations posed by obtaining low cell numbers from PB and CB numerous clinical trials have been performed post expansion of the isolated NK cells to obtain higher cell numbers [8-12]. These products have been shown to be safe and some efficacy observed however consistent clinical effects are yet to be seen. It can be hypothesized that the lack of consistent results may be the result of an NK cell product which is unable to proliferate once infused into a patient following long term cell

expansion possibly leading to a senescent population. We therefore postulate that proliferation, persistence and activation of NK cells *in vivo* is fundamental for the development of a clinically relevant cellular product that will be able to target tumor cells efficiently in patients. The aims of this study are therefore to identify if CBCD34^{+}NK cells can still proliferate *in vitro* following additional cytokine stimulation, assess the "age" of the differentiated NK cells, evaluate how priming and IL-2 stimulation compares as a method of NK cell activation and elucidate if the resting and activated NK cells can lyse patient AML blasts and solid tumor cell lines.

Material and Methods

HSC samples and cell lines

CB samples were obtained from the Anthony Nolan Cord Blood Bank with prior written consent from pregnant mothers and ethical committee approval (Research Ethics Committee reference 10/H0405/27). Patient AML samples were kindly supplied by Professor M. Lowdell (University College London) all of which were obtained with written informed consent for research into innate immunity to leukaemia. 1520, A478, CTV-1, HT-29, K562, MCF-7 and RAJI cell lines were cultured at 37ºC/5 % CO_2 with 95% humidity in RPMI-1640 culture media (Lonza) supplemented with 10% FBS (Lonza) and 1% penicillin-streptomycin (Lonza). EL08.1D2 cells were cultured as previously described[13].

HSC differentiation into NK cells

CB mononuclear cells were separated from whole blood by density centrifugation using Ficoll-Paque™ premium (GE Healthcare). HSC were isolated using the $CD34^+$ MicroBead kit (Miltenyi Biotec) as previously described[14] and frozen in FBS + 10% DMSO at 1 x 10^6 cells/mL for future use. The mean purity (%) ± SD of all isolations carried out was 92.43 ± 3.13 %. Thawed $CD34^+$ cells were plated on irradiated EL08.1D2 cells and cultured as described by Grzywacz *2006*[13] except for the addition of 50 ng/mL IL-15 alone for the last two weeks[6].

NK cell isolation and priming

NK cells were isolated from PB or CB mononuclear cells using the NK Cell Negative Isolation Kit (Miltenyi Biotec) following the manufacturers recommendations. The mean purities (%) ± SD of the NK cell isolations were 92.27

± 4.5 % for CB and 90 ± 2.2 % for PB. NK cell priming was performed using a lysate produced from CTV-1 cells that were kindly supplied by Professor M. Lowdell (University College London). NK cells and CTV-1 lysates were incubated at an NK cell-to-lysate ratio of 1:2 in complete media overnight.

Flow Cytometry

The following monoclonal antibodies were purchased from BD Biosciences: CD3 (SK7), CD16 (3G8), CD56 (B159), and CD57 (NK-1). The following blocking antibodies were obtained from Biolegend: anti-CD16 (3G8), anti-CD178 (FasL) (NOK-1), anti-CD253 (TRAIL) (RIK-2), anti-CD314 (NKG2D) (MOPC-21). Surface staining was performed by incubation in the dark at 4 ^{o}C for 10 min with fluorochrome-conjugated mAbs, washed and re-suspended in FACS buffer. DAPI (Life Technologies) was added to each sample at a concentration of 1/200 prior to acquisition. Cells were blocked by incubation for 30 minutes at RT with optimised blocking antibody concentration, alongside isotype control. The LSR Fortessa (Becton Dickinson) cell analyser was used to acquire data. FlowJo software (Tree Star) was used for data analysis.

Cytotoxicity assays

Target cells were labelled with PKH26 Red Fluorescent Cell Linker Kit (Sigma Aldrich) as per manufacturers instructions. Target and effector cells were seeded in FACS tubes at a target-to-effector ratio of 1:5 in triplicate and incubated for 4 h. Samples were labelled with the viability dye TO-PRO-3 iodide (Invitrogen) and analysed by flow cytometry.

Proliferation assay

NK cells were labeled with 2 μmol/L carboxyfluorescein succinimidyl ester (CFSE) per 1×10^6 cells (Invitrogen). $CBCD34^+$ and PBNK cells were then stimulated with 200 IU IL-2 (Prospec) and CBNK cells were stimulated with 1000 IU IL-2. Comparatively cells were also stimulated with 20 ng/mL IL-15 (Prospec) or primed with CTV-1 lysate. Proliferation was assessed on days 2, 5 and 7 post stimulation by flow cytometry.

Telomere Length Analysis

Telomere length analysis was performed in line with manufacturers instructions using the Telomere PNA kit/FITC (Dako). Denatured samples and control cells were incubated with a fluorescein-conjugated peptide nucleic acid (PNA) probe. After incubation with a DNA staining solution cells were analysed by flow cytometry. Telomere length is then calculated relative to the 1301 internal control cells.

***In vivo* Experiments**

NOD/SCID IL-2Rγnull (NSG) mice (8-10 weeks old) were irradiated at 3.75 Gy and injected intravenously with 1×10^6 PKH26 labelled patient AML blasts followed by 20×10^6 resting or primed $CBCD34^+$-NK cells 24 h later. Control mice were injected with PKH26 labelled AML cells only. All mice were culled 48 h post injection of PKH26 labelled AML cells and then the presence of the PKH26 labelled AML cells and NK cells was assessed in different tissues. All experiments were performed in agreement with Home Office regulations (project license 80/1293).

Statistics

All statistical analysis was carried out using GraphPad Prism (GraphPad Software, La Jolla, CA) using the non-parametric Mann-Whitney test. Results are presented as median ± range, p values < 0.05 were considered statistically significant.

Results

CBCD34$^+$-NK cells can still proliferate following additional cytokine stimulation

First we analysed if NK cells differentiated *in vitro* from CB CD34$^+$ cells could further respond to cytokine stimulation. Extensive long-term culture of NK cells has been associated with senescence[15] resulting in lack of response to additional cytokine stimulation and poor proliferation. Previously it has been shown that PBNK cells and CBNK cells respond to different concentrations of IL-2[16], therefore we tested the response of CBCD34$^+$-NK cells to 200 IU and 1000 IU of IL-2 the optimum concentration for PBNK cell and CBNK cell activation respectively. **Supplementary figure 1** shows that the cells could respond to both concentrations of IL-2 to the same degree. CBCD34$^+$-NK cells can therefore be stimulated with the same concentration of cytokine as PBNK cells henceforth 200 IU IL-2 was used to activate NK cells differentiated *in vitro* for the rest of the study.

NK cell proliferation following IL-15 stimulation was also assessed being a key cytokine promoting NK cell differentiation and proliferation[17]. In addition, we previously showed that IL-15 led to a better activation and proliferation of CBNK cells than IL-2 in vitro[18]. **Figure 1** shows the proliferation of CBCD34$^+$-NK cells following stimulation with IL-2 and IL-15 compared with PBNK cells and CBNK cells (**Figure 1A-C)**. We found no difference in the proliferative response of CBCD34$^+$-NK cells, PBNK cells and CBNK cells after stimulation with either IL-2 or IL-15. Taken together, this suggests that CBCD34$^+$-NK cells can respond to cytokines as efficiently as freshly isolated PBNK cells and CBNK cells.

CBCD34$^+$-NK cells have a less exhausted phenotype over PBNK cells and CBNK cells

Long-term culture of NK cells could result in exhaustion and therefore reduced anti-tumour activity of the cellular population. Eomes and T-bet have been identified as key molecular markers of cellular exhaustion[19]. A comparison of their expression in CBCD34$^+$-NK cells, PBNK cells and CBNK cells was carried out via RT-PCR. **Figure 2A** shows no significant difference in the expression of these markers by CBCD34$^+$-NK cells and resting PBNK cells and CBNK cells therefore none of these cells types show an exhausted phenotype. As a cell matures telomere length is reduced[20], therefore comparing the length of CBCD34$^+$-NK cells, PBNK cells and CBNK cells can give an accurate assessment of cell age. It can be seen in **Figure 2B** that CBCD34$^+$-NK cells had significantly longer telomeres than PBNK cells ($p < 0.001$) and although there is no significance this trend was also observed with CBNK cells. CBCD34$^+$-NK cells therefore have a younger cell age than NK cells from other sources, this would imply an ability to survive and persist for longer *in vivo*. CD57 is expressed at the last stage of maturation of NK cells and has been associated with cellular exhaustion[21]. The expression level of this marker therefore further characterizes the life stage of the cells. **Figure 2C** shows the percentage expression of CD57 showing that PBNK cells had a significantly highly expression over CBCD34$^+$-NK cells ($p < 0.001$) and CBNK cells ($p < 0.001$). CBNK cells also had a significantly higher CD57 expression over CBCD34$^+$-NK cells ($p < 0.001$) further supporting that CBCD34$^+$-NK cells show a less exhausted phenotype over PBNK cells and CBNK cells.

Resting CBCD34$^+$ and PBNK cells are able to target K562 cells *in vitro* but not patient AML blasts

It is fundamental to identify if CBCD34^{+}-NK cells are capable of demonstrating a cytotoxic effect against tumor cells *in vitro* as this is a key function required for a successful immunotherapy allowing for the targeting of malignancies and preventing relapse in patients. Cytotoxicity was assessed by a flow cytometry based method as primary cells are poor at up taking Cr^{51}. Due to the absence of MHC class I on K562 cells, they are highly susceptible to NK cell-mediated lysis and are therefore considered the gold standard target for assessing NK cell cytotoxic function. Resting CBCD34^{+}-NK cells and PBNK cells were capable of targeting the NK cell susceptible cell line K562 to the same degree showing that NK cells from both cell sources are fully functional *in vitro* (**Figure 3A**). In order to produce a successful clinical cell therapy NK cells must also be able to kill patient samples, cytotoxicity was therefore assessed against AML samples varying in severity from M1-M4. It can be seen in **Figure 3B** that there was some degree of killing observed by both CBCD34^{+}-NK cells and PBNK cells however these results are inconsistent. There was no significance observed by either source cell so therefore further activation of NK cells is required.

Priming CBCD34^{+} NK cells leads to enhanced cytotoxicity against K562 cells and successful targeting of patient AML blasts *in vitro*

As a result of the lack of clinically reproducible data following cytokine activation of NK cells it has to be considered if there is an alternative activation method. North and colleagues [22, 23] have previously developed a method for stimulating NK cells based on a two-step activation process known as priming and triggering. A lysate has been shown to prime resting PBNK cells enabling significantly up regulated cytotoxicity against numerous targets that were previously resistant to NK cell lysis. Primed CBCD34^{+}NK cells could therefore potentially produce a more robust and clinically

effective therapy. Primed CBCD34$^+$-NK cells showed a significantly enhanced ability to target K562 cells as compared to resting CBCD34$^+$-NK cells ($p < 0.001$) (**Figure 4A**). More importantly, **Figure 4B** shows that primed, as opposite to resting, CBCD34$^+$-NK cells are able to consistently target patient AML blasts *in* vitro ($p < 0.0001$) (8.067 % (0-16.49) to 45.04 % (27.02-61.15)).

Priming is superior to IL-2 stimulation as a method of NK cell activation

IL-2 is currently used routinely in numerous clinical trials to activate NK cells, therefore a comparison with priming was carried out against patient AML blasts, K562 cells and the NK resistant cell line RAJI with CBCD34$^+$-NK cells, PBNK cells and CBNK cells. **Figure 5** shows the specific lysis of patient AML blasts, K562 cells and RAJI cells by resting, primed and IL-2 stimulated CBCD34$^+$-NK cells. It can be observed that primed NK cells, whatever the source of NK cells considered, had a significantly improved cytotoxicity against AML ($p< 0.001$), K562 ($p < 0.01$) and RAJI cells ($p < 0.01$) (**Figure 5A-C**). When comparing the level of specific lysis by primed CBCD34$^+$-NK cells, PBNK cells and CBNK cells against patient AML blasts (**Supplementary Figure 2**) it can be seen that CBCD34$^+$-NK cells were significantly less cytotoxic than PBNK cells ($p < 0.05$) and CBNK cells ($p < 0.01$). This difference however is only 2.394 % less than PBNK cells and 7.164 % less than CBNK cells and is unlikely to have any improved clinical implications. It can therefore be concluded that priming NK cells overnight is a significantly better method of activation over IL-2 stimulation.

Priming CBCD34$^+$-NK cells is not required to observe cytotoxicity against patient AML blasts *in vivo*

We then analysed if primed CBCD34$^+$-NK cells could kill patient AML blasts *in vivo.* For this irradiated NSG mice were injected with 1 x 10^6 PKH26 labelled AML blasts, followed by resting or primed CBCD34$^+$-NK cells 24 h later. **Figure 6A** shows that PKH26 labelled AML blasts could be detected in the BM, liver, lungs and spleen 48 h post infusion. Interestingly, killing of patient AML blasts was observed in the liver, lungs and spleen of the mice injected with both resting and primed CBCD34$^+$-NK cells ($p < 0.01$). There was no reduction in the percentage of PKH26 labelled AML cells in the bone marrow. Resting and primed CBCD34$^+$-NK cells could also be detected in all of the organs analysed (**Figure 6B**). Therefore, it can be concluded that CBCD34$^+$-NK cells might not require prior activation to have a cytotoxic impact on patient AML blasts *in vivo*.

Cytokine stimulated CBCD34$^+$-NK cells can proliferate significantly more than primed cells

In order to identify why CBCD34$^+$-NK cells do not require prior activation to observe a cytotoxic effect *in vivo* we compared the difference of proliferation following cytokine stimulation and priming. It was observed that CBCD34$^+$-NK cells proliferated significantly more when activated with cytokines over priming (**Supplementary Figure 3**) ($p < 0.01$). This difference was not observed with PBNK cells, which could account for why it might not be necessary to prime CBCD34$^+$-NK cells to achieve cytotoxicity *in vivo*.

Priming leads to significantly improved cytotoxicity against solid tumor cell lines *in vitro*

Solid tumours are notoriously difficult to target by immunotherapy so therefore it was assessed if resting, primed or IL-2 stimulated CBCD34$^+$-NK cells, PBNK cells and CBNK cells could target solid tumor cell lines *in vitro*. Analysis was carried out against the breast adenocarcinoma MCF7 (**Figure 7A**), colon cancer HT29 (**Figure 7B**), melanoma 1520 (**Figure 7C**) and renal cell carcinoma A478 (**Figure 7D**). These data support what has been observed previously in that primed NK cells of any source were highly superior at targeting tumor cells over resting and cytokine stimulated NK cells ($p < 0.01$). Further it is interesting to note that resting and cytokine stimulated CBCD34$^+$-NK cells were significantly more cytotoxic than PBNK cells and CBNK cells ($p < 0.05$) when targeting the colon cancer cell line HT29 (**Figure 7B**).

CD16 is a key pathway required for NK cells to target resistant cell lines after priming

To study which mechanism might be involved in targeting NK cell resistant cells a blocking experiment was carried out before performing an *in vitro* cytotoxicity assay against the RAJI cell line. We assessed the level of cytotoxicity after blocking of TRAIL and FasL, death receptors that can directly eliminate targets post engagement[24], the co-engagement activating receptor NKG2D and the mediator of antibody-dependent cellular cytotoxicity, CD16[25]. The percentage expression of these receptors was unchanged subsequent to priming (data not shown). After blocking (**Figure 8A**), FasL (**Figure 8B**) and NKG2D (**Figure 8C**) there was no difference observed in the specific lysis of RAJI cells in comparison to the unblocked controls. This indicates that primed CBCD34$^+$-NK cells, PBNK cells or CBNK cells do not involve these receptors in the killing of NK cell resistant target cells. Blocking of CD16 (**Figure 8D**) however showed significantly reduced cytotoxicity against RAJI cells

when compared to the unblocked control of any NK source, CBCD34^{+} cells ($p < 0.001$), PBNK cells ($p < 0.01$) and CBNK cells ($p < 0.001$). CD16 is therefore a key pathway required for primed NK cells to target resistant cell lines.

Discussion

The differentiation of NK cells from CB $CD34^+$ cells offer a rich source of highly functional cells that maintain function post cryopreservation allowing for the development of an of-the-shelf cell therapy. It is therefore necessary to further characterize the phenotype and function of these cells in order to elucidate how their biology compares to PBNK cells and CBNK cells and identify if they could be a more attractive source of cells for clinical immunotherapy.

The capability of CB $CD34^+$-NK cells to proliferate post infusion is key for the development of a successful immunotherapy however long-term culture of lymphocytes prior to clinical application has been associated with poor proliferation *in vivo[15]*. Here we observed that CB $CD34^+$-NK cells could proliferate following additional cytokine stimulation *in vitro* allowing for expansion once infused into a patient. Further it has been identified that as normal hematopoetic cells divide *in vitro* or *in vivo* telomere length is reduced providing a molecular clock that triggers senescence [26]. Here it is shown that CB $CD34^+$-NK cells have telomeres longer than PBNK cells and CBNK cells demonstrating a more youthful molecular phenotype. In addition the expression of the NK cell maturation marker CD57 is significantly lower in CB $CD34^+$-NK cells than in PBNK cells and CBNK cells. Previous work has suggested that the expression of CD57 is a marker of NK cell maturation and enhanced cytotoxic capacity instead of an indicator of cell anergy[27]. However here we show that CB$CD34^+$-NK cells have a significantly lower expression of CD57 in comparison to PBNK cells and CBNK cells without any variation in cytotoxic effect observed irrelevant of the cell source considered against a variety of different targets. It can therefore be hypothesized that a CD57 threshold is to be reached to demonstrate full

NK cell maturation but as expression increases past this threshold cell senescence can be correlated. Finally the molecular expression of EOMES and TBET is also equivalent to resting PBNK and CBNK cell controls further supporting that the cells have not reached an exhausted phenotype and resultant impaired function that is associated with a limited ability to target tumor cells in mice[19] and humans[28].

The cytotoxicity of the resting, primed and IL-2 stimulated cells is equal to both PBNK and CBNK cell controls when targeting K562 cells, patient AML blasts, RAJI cells and solid tumor cell lines. Demonstrating that this key function necessary to target patient tumors is uncompromised whether NK cells are differentiated *in vitro* or *in vivo*. Further blocking shows that killing by primed CBCD34$^+$ and CBNK cells utilizes the same biological pathway as PBNK cells supporting previous literature[23].

Interestingly priming was not necessary to observe killing *in vivo* of patient AML blasts. This could be the result of significantly reduced proliferation by primed CBCD34$^+$-NK cells in comparison to cytokine stimulation that was not observed in PBNK cells. Even though there is no difference observed in the IL-2 and IL-15 receptor expression by CBCD34$^+$-NK cells and PBNK cells there could still be a fundamental difference between the cells that could result in CBCD34$^+$-NK cells responding preferentially to cytokine stimulation *in vivo*. Further an increased expression of CD57 has also been associated with a reduced sensitivity to cytokines[21] which could support previous literature that CBCD34$^+$-NK cells can survive and persist for longer *in vivo over* other NK cell sources[6].

The CD34$^+$ cells used to differentiate NK cells *in vitro* are collected by a non-invasive procedure from CB units that are not deemed suitable for transplantation. The use of

these cells to produce NK cells for immunotherapy therefore further optimizes the use of clinical grade banked CB units, making the process more economically viable.

Furthermore, the cells demonstrate a younger cell profile without loss of function implying an enhanced ability to survive and proliferate when used as a cell therapy in the clinic. We therefore conclude that NK cells differentiated from CB $CD34^+$ cells are a more desirable source of cells for NK immunotherapy over PBNK and CBNK cells.

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Conflict of interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1: Proliferation of CBCD34$^+$ NK cells, PBNK cells and CBNK cells post cytokine stimulation. Proliferation of CBCD34$^+$-NK cells (A), PBNK cells (B) and CBNK cells (C) was assessed by CFSE analysis after stimulation with 200 IU of IL-2 for CBCD34$^+$ and PBNK cells and 1000 IU of IL-2 for CBNK cells or 20 ng/mL IL-15. The median MFI of CFSE (± range) CBCD34$^+$-NK cells (n=4), PBNK cells (n=4) and CBNK cells (n=4) is shown.

Figure 2: Expression of exhaustion markers by CBCD34$^+$ NK cells, PBNK cells and CBNK cells. A) Expression of EOMES and T-bet was analysed by RT-PCR. The median relative expression (± range) of CBCD34$^+$-NK cells (n = 6), PBNK cells (n = 6) and CBNK cells (n = 6) is shown. B) Telomere length analysis was carried out using a telomere PNA kit. Median relative telomere length (± range) of CBCD34$^+$-NK cells (n = 6), PBNK cells (n = 6) and CBNK cells (n = 6) is shown. C) Percentage CD57 expression (± range) of CBCD34$^+$-NK cells (n = 9), PBNK cells (n = 9) and CBNK cells (n = 9) is shown. ** $p < 0.01$, *** $p < 0.001$.

Figure 3: Killing of AML and K562 cells *in vitro.* *In vitro* killing was analysed by a flow cytometry based cytotoxicity assay A) Median specific K562 lysis (± range) by CBCD34$^+$-NK cells (n = 8) and PBNK cells (n = 8). B) Median specific AML lysis (± range) by CBCD34$^+$-NK cells (n = 18) and PBNK cells (n = 18) *in vitro*.

Figure 4: CTV-1 lysate primes resting NK cells to lyse AML and K562 cells *in vitro*. *In vitro* killing was analysed by a flow cytometry based cytotoxicity assay A) Median specific K562 lysis (± range) by resting CBCD34$^+$-NK cells (n = 10) and

primed CBCD34$^+$-NK cells (n = 10). B) Median specific AML lysis (± range) by resting CBCD34$^+$-NK cells (n = 27) and primed CBCD34$^+$-NK cells (n = 27). Statistical analysis was performed using Mann-Whitney test. **** $p < 0.0001$.

Figure 5: Killing of patient AML blasts, K562 cells and RAJI cells by resting, primed and IL-2 stimulated CBCD34$^+$-NK cells, PBNK cells, CBNK cells. *In vitro* killing was analysed by a flow cytometry based cytotoxicity assay. Median specific lysis (± range) of AML (n = 15), K562 cells (n = 5) and RAJI cells (n = 5) by CBCD34$^+$-NK cells (A), PBNK cells (B) and CBNK cells (C) is shown. Statistical analysis was performed using Mann-Whitney test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 6: Killing of AML cells *in vivo* by resting and primed CBCD34+-NK cells A) Median percentage of PKH26 labelled AML cells (± range) detected in the BM, liver, lungs and spleen (n = 6). B) Median percentage of NK cells (± range) detected in the bone marrow, liver, lungs and spleen (n = 6). Statistical analysis was performed using Mann-Whitney test ** $p < 0.01$.

Figure 7: Killing of solid tumour cell lines by resting, primed and IL-2 stimulated CBCD34$^+$-NK cells, PBNK cells, CBNK cells. *In vitro* killing was analysed by a flow cytometry based cytotoxicity assay. A) Median specific lysis (± range) of MCF-7 by resting, primed and IL-2 stimulated CBCD34$^+$ (n = 6), PBNK (n = 6) and CBNK cells (n = 6). B) Median specific lysis (± range) of HT-29 by resting, primed and IL-2 stimulated CBCD34$^+$(n = 6), PBNK (n = 6) and CBNK cells (n = 6). C) Median specific lysis (± range) of 1520 by resting, primed and IL-2 stimulated CBCD34$^+$(n = 6), PBNK (n = 6) and CBNK cells (n = 6). D) Median specific lysis (± range) of A478 by resting,

primed and IL-2 stimulated CBCD34$^+$(n = 6), PBNK (n = 6) and CBNK cells (n = 6). Statistical analysis was performed using Mann-Whitney test * $p < 0.05$ ** $p < 0.01$.

Figure 8: Blocking of TRAIL, FasL, NKG2D and CD16 on CBCD34$^+$, PBNK and CBNK cells. Receptor involvement was analysed by blocking prior to priming before performing an *in vitro* killing assay against the NK resistant cell line RAJI. A) Median specific lysis (± range) by resting, primed isotype, primed and primed TRAIL blocked CBCD34$^+$(n = 6), PBNK (n = 6) and CBNK cells (n = 6). B) Median specific lysis (± range) by resting, primed isotype, primed and primed FasL blocked CBCD34$^+$(n = 6), PBNK (n = 6) and CBNK cells (n = 6). C) Median specific lysis (± range) by resting, primed isotype, primed and primed NKG2D blocked CBCD34$^+$(n = 6), PBNK (n = 6) and CBNK cells (n = 6). D) Median specific lysis (± range) by resting, primed isotype, primed and primed CD16 blocked CBCD34$^+$(n = 6), PBNK (n = 6) and CBNK cells (n = 6).

Supplementary Figure Legends

Supplementary Figure 1: CBCD34+-NK cells can be activated by 200 IU of IL-2. Proliferation was assessed by CFSE analysis after stimulation with 200 (n = 3) or 1000 IU (n = 3) of IL-2. Median MFI CFSE (± range) at D2, 5 and 7 post activation by CBCD34$^+$-NK cells is shown.

Supplementary Figure 2: Comparing the cytotoxicity of CBCD34$^+$, PBNK and CBNK cells against patient AML blasts. *In vitro* killing was analysed by a flow cytometry based cytotoxicity assay. Median specific lysis (± range) by CBCD34$^+$(n = 15), PBNK (n = 15) and CBNK cells (n = 15) is shown. Statistical analysis was performed using Mann-Whitney test * $p < 0.05$.

Supplementary Figure 3: Cytokine stimulated CBCD34$^+$-NK cells are able to proliferate significantly more than primed CBCD34$^+$-NK cells. Proliferation was assessed by CFSE analysis after stimulation with 200 IU IL-2, 20 ng/mL IL-15 or priming with CTV-1 lysate. A) Median MFI CFSE (± range) at D2, 5 and 7 post activation of CBCD34$^+$-NK cells (n = 4) is shown. B) Median MFI CFSE (± range) at D2, 5 and 7 post activation of PBNK cells (n = 4) is shown. Statistical analysis was performed using Mann-Whitney test * $p < 0.05$.