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Introduction

Natural Killer (NK) cells are CD3$^-$CD56$^+$ lymphocytes that exert innate immunity against cancer and viral infections [1]. Recognition and subsequent killing of virus-infected and transformed cells by NK cells is regulated through the balance of signals from inhibitory and activating receptors [1]. Due to their strong ability to target tumor cells, NK cells have been described as promising effectors for adoptive immunotherapy against cancer [2]. It has been well demonstrated that NK cell alloreactivity can control relapse of acute myeloid leukemia (AML) without causing graft-versus-host disease (GVHD) in the setting of haploidentical stem cell transplantation (SCT) [3]. Moreover, haploidentical NK cell infusions in adult and childhood AML following lymphocyte depleting chemotherapy have provided encouraging results [4,5]. However, only a few trials investigating adoptive NK cell infusions in patients with cancer have been conducted to date. A major obstacle is that relative small numbers of NK cells can be isolated from a regular leukapheresis products. This hampers clinical trials evaluating for NK-cell dose dependent anti-tumor responses in humans with cancer [6–11]. Therefore, protocols for ex vivo expansion and activation of NK cells are under investigation enabling clinical trials at higher NK cell dosages and to permit multiple NK cell infusions [12–16]. However, most protocols still deal with technical disadvantages by using supportive feeder cell lines that could lead to regulatory problems producing NK cell products for large-scale and multi-center trials. Interestingly, a recent study by Suthu et al. reported that large amounts of highly active NK cells can be produced from peripheral blood in a closed, automated bioreactor under feeder-free conditions. [17].

Recently, we have described an alternative cytokine-based culture method with the capability of generating clinically relevant NK cell products using bioreactors with a mean expansion of more than 2,000 fold and a purity of >90%. Moreover, expansion in the bioreactor yielded a clinically relevant dose of NK cells (mean: 2 $\times$ 10$^9$ NK cells), which display high expression of activating NK receptors and cytolytic activity against K562. Finally, we established a versatile closed washing procedure resulting in optimal reduction of medium, serum and cytokines used in the cell culture process without changes in phenotype and cytotoxic activity. These results demonstrate that large numbers of UCB stem cell-derived NK cell products for adoptive immunotherapy can be produced in closed, large-scale bioreactors for the use in clinical trials.
multitude of therapeutic cell products including NK cells [18–20]. An optimal procedure for the clinical-grade generation of UCB progenitor cell-derived NK cells must include a GMP-compatible HSC enrichment procedures as well as a closed-system culture system free of animal products and feeder cells. In the present study, we have investigated the feasibility of large scale NK cell generation using cryopreserved UCB units as progenitor cell source. We have optimized the enrichment of CD34+ cells from thawed UCB units using the CliniMACS system. Furthermore, we have evaluated CD34+ cells-derived NK cell generation in static cell culture bags and an automated bioreactor, with the aim of optimizing fully closed, large-scale production of highly active and functional NK cells for the use in a phase I dose-finding trial in elderly AML patients not eligible for allogeneic SCT.

Results

Efficient enrichment of CD34+ cells from cryopreserved umbilical cord blood

The overall aim of this study was to develop a closed system for the expansion and differentiation of CD34+ UCB cells into NK cells followed by the subsequent log-scale generation of CD56+CD3− NK cells. As the initiation of our culture process requires hematopoietic progenitor cells, we optimized the CD34+ enrichment procedure from cryopreserved UCB units using the CliniMACS system. Prior to banking in liquid nitrogen the collected UCB units used for this study (n = 16) have been reduced for red blood cells and volume using EloHAES® separation. The mean volume of 111±34 ml (range 72–175 ml) and mean WBC count of 1,503±455×10^6 cells (range 772–2,380×10^6) was reduced to 25 ml with a WBC count of 1,065±357×10^6 cells (range 600–1,721×10^6) containing 3.78±1.95×10^6 CD34+ cells (range 1.73–8.72×10^6) (Table 1). Cryopreserved UCB units were thawed and prepared for CD34+ selection using CliniMACS buffer containing clinical-grade DNAse. The recovery of CD34+ cells after thawing was 76%±16%, which resulted in a total yield of 2.79±1.59×10^6 CD34+ cells (range 1.43–8.12×10^6) for the selected UCB units (Table 1). Next, CD34+ cells were enriched using the CliniMACS cell separator resulting in a mean recovery of 71%±11% (range 50–91%) (Table 2). The purity of the enriched CD34+ product was 67%±14% (range 44–92%). Total recovery after thawing and CD34+ enrichment was 53%±15% (range 33–82%) with a mean CD34+ cell number of 1.96±1.27×10^6 (range 0.89–6.34×10^6) (Table 2). These results demonstrate that CD34+ cells can be efficiently enriched from volume-reduced and cryopreserved UCB units providing a clinical-grade starting product for the NK cell generation and expansion culture process.

Enriched CD34+ UCB cells can be efficiently expanded using static cell culture bags

Previously, research scale experiments in 6-well plates showed that CD34+ cells, enriched from frozen UCB units, can be

| Donor 1 | 88 | 1294 | 790 | 3.90 | 368 | 2.96 | 76 |
| Donor 2 | 151 | 1857 | 1312 | 5.88 | 469 | 3.73 | 63 |
| Donor 3 | 141 | 1734 | 1378 | 4.96 | 653 | 3.23 | 65 |
| Donor 4 | 87 | 1992 | 1588 | 8.72 | 819 | 8.12 | 93 |
| Donor 5 | 119 | 1857 | 1312 | 5.88 | 583 | 2.28 | 62 |
| Donor 6 | 153 | 1775 | 1519 | 3.17 | 829 | 2.15 | 68 |
| Donor 7 | 152 | 1733 | 978 | 2.08 | 440 | 2.06 | 99 |
| Donor 8 | 72 | 1210 | 760 | 2.70 | 403 | 2.07 | 77 |
| Donor 9 | 78 | 772 | 600 | 3.96 | 248 | 1.84 | 46 |
| Donor 10 | 97 | 927 | 616 | 1.73 | 386 | 1.69 | 98 |
| Donor 11 | 81 | 1207 | 974 | 2.82 | 479 | 2.52 | 89 |
| Donor 12 | 175 | 2380 | 1721 | 6.90 | 943 | 3.96 | 57 |
| Donor 13 | 95 | 1430 | 1008 | 3.04 | 558 | 2.66 | 87 |
| Donor 14 | 77 | 857 | 680 | 1.75 | 273 | 1.43 | 82 |
| Donor 15 | 88 | 1223 | 969 | 2.40 | 563 | 2.14 | 89 |
| Donor 16 | 130 | 1829 | 1364 | 2.78 | 821 | 1.82 | 66 |

Table 1. Characteristics of the UCB units after EloHAES separation and cryopreservation.

The table summarizes the processing of 16 UCB units used for CD34+ enrichment after collection, volume reduction and thawing process. Nucleated cells (NCs) were counted with the AcT10 counter (Beckman coulter). CD34+ cells were enumerated by single platform flow cytometry analysis. Results are depicted as mean, standard deviation, median, minimum (min) and maximum (max) volume, number of cells or percentages, respectively.

doi:10.1371/journal.pone.0020740.t001
Table 2. Characteristics of the CD34+ CliniMACS separation on thawed UCB units.

<table>
<thead>
<tr>
<th>CD34-positive fraction</th>
<th>Recovery after CD34 enrichment only (%)</th>
<th>CD34+ cell content (%)</th>
<th>CD34+ cells (x10^6)</th>
<th>Recovery of CD34+ cells after processing (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor 1</td>
<td>50</td>
<td>52</td>
<td>1.47</td>
<td>38</td>
</tr>
<tr>
<td>Donor 2</td>
<td>53</td>
<td>77</td>
<td>1.99</td>
<td>34</td>
</tr>
<tr>
<td>Donor 3</td>
<td>73</td>
<td>70</td>
<td>2.36</td>
<td>48</td>
</tr>
<tr>
<td>Donor 4</td>
<td>78</td>
<td>92</td>
<td>6.34</td>
<td>73</td>
</tr>
<tr>
<td>Donor 5</td>
<td>76</td>
<td>54</td>
<td>1.74</td>
<td>47</td>
</tr>
<tr>
<td>Donor 6</td>
<td>79</td>
<td>65</td>
<td>1.70</td>
<td>54</td>
</tr>
<tr>
<td>Donor 7</td>
<td>82</td>
<td>64</td>
<td>1.70</td>
<td>82</td>
</tr>
<tr>
<td>Donor 8</td>
<td>69</td>
<td>73</td>
<td>1.42</td>
<td>53</td>
</tr>
<tr>
<td>Donor 9</td>
<td>72</td>
<td>88</td>
<td>1.32</td>
<td>33</td>
</tr>
<tr>
<td>Donor 10</td>
<td>76</td>
<td>69</td>
<td>0.89</td>
<td>51</td>
</tr>
<tr>
<td>Donor 11</td>
<td>91</td>
<td>65</td>
<td>2.29</td>
<td>81</td>
</tr>
<tr>
<td>Donor 12</td>
<td>70</td>
<td>59</td>
<td>2.79</td>
<td>40</td>
</tr>
<tr>
<td>Donor 13</td>
<td>55</td>
<td>84</td>
<td>1.47</td>
<td>48</td>
</tr>
<tr>
<td>Donor 14</td>
<td>76</td>
<td>67</td>
<td>1.09</td>
<td>62</td>
</tr>
<tr>
<td>Donor 15</td>
<td>71</td>
<td>44</td>
<td>1.52</td>
<td>63</td>
</tr>
<tr>
<td>Donor 16</td>
<td>65</td>
<td>52</td>
<td>1.19</td>
<td>43</td>
</tr>
<tr>
<td>mean</td>
<td>71</td>
<td>67</td>
<td>1.96</td>
<td>53</td>
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<tr>
<td>SD</td>
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<td>14</td>
<td>1.27</td>
<td>15</td>
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<tr>
<td>median</td>
<td>73</td>
<td>66</td>
<td>1.61</td>
<td>50</td>
</tr>
<tr>
<td>min</td>
<td>50</td>
<td>44</td>
<td>0.89</td>
<td>33</td>
</tr>
<tr>
<td>max</td>
<td>91</td>
<td>92</td>
<td>6.34</td>
<td>82</td>
</tr>
</tbody>
</table>

The table summarizes the results of the CD34+ enrichment procedure of 16 UCB units. CD34+ cells were enumerated by single platform flow cytometry analysis. Results are depicted as mean, standard deviation, median, minimal (min) and maximal (max) number of cells or percentages.

doi:10.1371/journal.pone.0020740.t002

Efficiently expanded and differentiated into the NK cell lineage using our two step ex vivo culture process [18]. To translate this protocol into a closed culture system, we have tested ex vivo expansion of CD34+ UCB cells for two weeks in static Vuelife T™ AC culture bags using CD34+ expansion medium I (day 0–9) and medium II (day 9–14). The mean total cell expansion for all experiments (n = 7) was 39±14 and 160±69 fold after 1 and 2 weeks of culture, respectively (data not shown). These results were similar to the rate of expansion of 192±82 (n = 7) obtained after 2 weeks in 6-well plates (data not shown), and indicate that selected CD34+ cells from cryopreserved UCB units can be efficiently expanded during 2 weeks of culture in disposable bags.

Efficient expansion of highly pure NK cell products using a bioreactor

Next, we investigated whether the bag-expanded CD34+ UCB cells could be differentiated and further expanded into CD56+CD5- NK cells. First, we continued the differentiation process in the same static bags as used for CD34+ cell expansion. Therefore, we added NK cell differentiation medium containing SCF, IL-7, IL-15 and IL-2 to the bag cultures from day 14 onward. The mean total cell expansion after 6 weeks of culture in the static bags was ~1,300 fold (range: 759–1,770; n = 3), generating NK cell products of 0.9-1.9x10^6 CD56+CD5- NK cells (Figure 1A and Table 3). However, ex vivo generation of CD56+CD5- NK cells in bag cultures yielded in a purity of 71%±9% (Figure 1B and Table 3). The remaining non-NK cells in the product represented CD14+ and/or CD15+ mature monocytic and myelocytic cells, but no CD34+ cells nor CD3+ T cells and CD19+ B cells could be detected (data not shown). Because differentiation of the NK cell products was sub-optimal in the bag cultures, we next tested whether differentiation of the bag-expanded CD34+ cultures into the NK cell lineage could be improved using an automated bioreactor. Therefore, in a next set of experiments expanded CD34+ UCB cells were transferred at day 14 of culture into a bioreactor system with a minimal volume of 250 ml for starting the NK cell differentiation process. Although the mean total cell expansion at 6 weeks of culture in the bioreactor cultures, which was ~2,100 fold (range 1,435–2,657; n = 4; Figure 3C and Table 3), was not significantly higher compared to the bag-expanded NK cells, the differentiation and expansion rate of NK cells was significantly better in the bioreactors (Figure 1D and E).

Importantly, ex vivo generation of CD56+CD3- NK cells in bioreactors yielded highly pure (92%±2%; n = 4) NK cell products with a total NK cell number of 1.6-5.7x10^6 CD56+CD3- NK cells (Table 3). Because of the very high NK cell purity of the bioreactor expanded products, only a small population (<5%) of mature CD14+ and/or CD15+ monocytic cells could be detected, but no CD34+ cells, CD3+ T cells or CD19+ B cells were found (data not shown). Furthermore, we compared the expression of various NK cell specific surface antigens and NK cell function in degranulation assays on static bag and bioreactor generated NK cell products (Figure S1). The bioreactor cultures show a higher expression of activating receptors such as CD314 (NKGD2) and NCRs (i.e. CD337, CD336, CD335) (Figure S1 A). This correlated with a higher degranulation of 27% of bioreactor-expanded NK cells towards K562 compared to 14–18% for NK cells from static bag cultures (Figure S1 B). These data demonstrate that the
combination of static bag cultures for progenitor cell expansion followed by efficient NK production in bioreactor systems result in a efficient production of pure NK cell products for adoptive immuno-
therapy trials.

The effect of washing on recovery, phenotype and function of expanded NK cells

After showing that CD34⁺ UCB cells could be efficiently enriched from frozen cord blood and successfully cultured into a pure NK cells product using a closed cell culture process, we optimized downstream processing using a closed system washing procedure. Therefore, final NK cell batches were washed two times with 0.5 liter CliniMACS buffer containing 0.5% HSA in transfer bags. After this washing procedure the total cell culture volume of 1 liter was reduced and exchanged to 150 ml infusion buffer. The final dilution factor of this washing procedure was between 629–1,008 fold (n = 3), with a recovery of 82% ± 5% CD56⁺CD3⁻7AAD⁻ NK cells (n = 3). Cytotoxicity and CD107a-based degranulation assays using K562 as target cells showed that the cytolytic activity of the NK cell product before and after washing was not affected (Figure 2A and B). Moreover, washing of the expanded NK cells did not negatively influence the high expression of the activating receptors NKG2D (CD314), NKR-P1 (CD161), 2B4 (CD244), NKp30 (CD337), NKp46 (CD335) and

Figure 1. Ex-vivo generation of CD56⁺ NK cells from cryopreserved CD34⁺ UCB cells. CD34-enriched UCB cells were expanded for two weeks and subsequently differentiated into NK cells for four additional weeks. Cell cultures were weekly analyzed for cell numbers and phenotype using flow cytometry. (A) Fold expansion of total cells for each donor after initial seeding of enriched CD34⁺ UCB cells during 6 weeks of culture using static Vuelife™ cell culture bags. (B) CD56⁺ cell frequency for each donor during the 6 week culture period for static bag cultures. (C) Fold expansion of total cells for each donor after initial seeding of enriched CD34⁺ UCB cell population during 6 weeks of culture using single use bioreactors. (D) CD56⁺ cell frequency for each donor during the 6 week culture period for bioreactor cultures. (E) Mean total CD56⁺ NK cell expansion during 4 weeks of differentiation using static bag (n = 3) or bioreactor cultures (n = 4). Data are depicted as mean ± SD. The asterisk (*) represents a p-value of <0.05. doi:10.1371/journal.pone.0020740.g001
NKp44 (CD336) (Figure 3). These results demonstrate that CD34+ UCB-derived NK cells (UCB-NK) for immunotherapy could be efficiently washed using a closed process without loss of functional and phenotypical characteristics of the bioreactor-expanded NK cells.

UCB-NK cell therapy products fulfill specific release, biosafety and stability tests

During the validation runs of our closed culture and washing process, we monitored purity, cell numbers, viability, phenotype, and...
activity and recovery of the UCB-NK cell products. All four validation runs in the bioreactor resulted in a final cell product containing $\geq 90\%$ viable CD56$^+$CD3$^-$7AAD$^-$ NK cells, and CD3$^+$ T cells could not be detected ($\leq 0.01\%$). Importantly, cytogenetic analysis showed that the NK cell products displayed a normal karyotype. In addition, extensive testing was performed to ensure that our process was free of bacterial, fungal, mycoplasma and endotoxin contamination (Table 4). These tests were performed at the end of the NK cell production and after the washing procedure, and were negative or below specifications in all validation runs. The presence of residual SCF, IL-7, IL-15 and IL-2, which were used in the NK cell differentiation medium, was tested by specific ELISA. After washing the NK cell products, the cytokine concentrations were below the specified range of $\leq 25$ pg/ml SCF, IL-7 and IL-15 and $\leq 1$ U/ml IL-2.

Since we intend in our phase I clinical trial to infuse freshly prepared NK cell products without cryopreservation, we determined the stability of the NK cells in order to establish a time frame for the product release testing to be finished. Therefore, we stored UCB-NK cell products in infusion buffer (i.e. 0.9% NaCl plus 5% HSA) at 4°C or RT, and tested purity and viability at 24, 48 and 72 hours. We did not detect a decrease in purity of the NK cell product over time and also detected no differences between storage at 4°C and RT (Figure 4A). Only a small decline in viability of CD56$^+$7AAD$^-$ NK cells was observed at day 2 or 3 after storage at both 4°C and RT (Figure 4B). A more detailed view on the stability tests regarding the expression of several inhibitory and activating NK cell receptors showed a stable percentage of CD159a (NKG2A) and CD314 (NKG2D) positive NK cells, but the expression of the NCRs CD337, CD336, CD335 declines over time irrespective of storage at 4°C or RT (Figure S2A–D). Our specification for NK cell infusion requires a minimum of 70% viability, and therefore we have set our expiration time for UCB-NK cells at 24 hours after final formulation.

Collectively, these results demonstrate the feasibility to generate highly pure, safe and active UCB-NK cell therapy products using a fully closed cell culture and downstream manufacturing process for evaluating in a phase I dose escalation trial in poor-prognosis patients with AML.

**Discussion**

To date only a few trials have investigated adoptive NK cell infusions in patients with cancer primarily due to difficulties in isolating high numbers of NK cells from regular leukapheresis products [6–11]. Furthermore, ex vivo expansion protocols still deal with technical disadvantages by using supportive feeder cell lines that could lead to regulatory problems producing NK cell products for large-scale and multi-center trials [12–16]. Here, we report the successful translation of our recently developed, highly efficient cell culture method for the ex vivo generation of functional NK cell products from UCB-derived hematopoietic stem and precursor cells into a clinical applicable GMP procedure. Previously, we described a highly potent culture method for the ex vivo generation of fully active NK cells efficiently targeting AML and melanoma cells [18]. This cytokine-based, feeder cell-free culture process uses only human or human recombinant proteins. To translate this NK cell bioprocess into a GMP compliant procedure, we first optimized clinical-grade enrichment of CD34$^+$ cells from cryopreserved UCB. Thereafter, we validated ex vivo generation of UCB-NK cell therapy products using a closed production process optimized for NK cell differentiation using bioreactors.

A number of studies reported previously about closed system immunomagnetic beads selection of CD34$^+$ cells from cryopreserved UCB [21–24]. Most of these studies used the Isolex300i or the ClinMACS system, but currently only the ClinMACS system
After optimizing CD34 enrichment from cryopreserved UCB, we investigated whether CliniMACS-enriched CD34⁺ UCB cells could be efficiently expanded and further differentiated into the NK cell lineage using a closed cell culture system. Recently, Sutlu et al. reported that large amounts of highly active NK cells can be produced from peripheral blood mononuclear cells (PBMCs) in a closed, automated, bioreactor under feeder-free conditions [17]. Interestingly, bioreactor cultures yielded a final product containing a clinically relevant NK cell dose (mean 9.8×10⁹ NK cells). Moreover, they observed that NK cells expanded in a bioreactor, compared to flasks and bags, displayed higher cytotoxic activity possibly attributed to a higher expression level of NKp44. In agreement with the studies of Sutlu et al., we also obtained a much better purity, functionality and significant better NK cell expansion using the Wave™ and Biostat™/bioreactor systems during the NK cell differentiation process. For efficient NK cell generation it seems, that an optimal gas exchange for the cell suspension could be provided by rocking or waving of the cell suspension. Finally, we performed safety and release tests on the NK cell end products (summarized in Table 4). The genetic stability was analyzed by karyotype analysis and all NK cell products had a normal karyotype. Furthermore, all generated products were negative for bacterial, fungal or mycoplasm contamination. After washing of the product, the volume was reduced to below 25 pg/ml. Immunophenotyping analysis demonstrated high purity, viability and activated phenotype of the NK cell product with the complete absence of CD3⁴⁺ T cells. We additionally demonstrate, that the cell culture process is safe and that the product could be further processed, stored and safely released for patients.

In conclusion, we successfully adapted our method into a closed-system bioprocess for production of allogeneic NK cell batches under GMP conditions, in order to utilize ex vivo-expanded NK cells for adoptive immunotherapy in poor-prognosis AML patients. Large-scale experiments using gas-permeable culture...

Table 4. Product release testing criteria and results of the final NK cell products.

<table>
<thead>
<tr>
<th>Test</th>
<th>Method</th>
<th>Specification</th>
<th>Donor 10</th>
<th>Donor 13</th>
<th>Donor 15</th>
<th>Donor 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK cell number</td>
<td>FCM</td>
<td>CD56⁺CD3⁺ NK cells</td>
<td>2.2×10⁶</td>
<td>2.4×10⁹</td>
<td>3.7×10⁹</td>
<td>1.6×10⁹</td>
</tr>
<tr>
<td>Purity</td>
<td>FCM</td>
<td>&gt;70% CD56⁺CD3⁺ NK cells</td>
<td>95%</td>
<td>90%</td>
<td>92%</td>
<td>92%</td>
</tr>
<tr>
<td>Viability</td>
<td>FCM</td>
<td>&gt;70% 7-AAD negative</td>
<td>n.a.</td>
<td>98%</td>
<td>97%</td>
<td>93%</td>
</tr>
<tr>
<td>Phenotype</td>
<td>FCM</td>
<td>&gt;30% positivity for CD56, CD9, NKKG2D, NCR and NKG2D.</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Karyotyping</td>
<td>Cell culture</td>
<td>Normal karyotype</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Recovery</td>
<td>FCM</td>
<td>% CD56⁺CD3⁺ negative cells.</td>
<td>n.a.</td>
<td>83%</td>
<td>86%</td>
<td>76%</td>
</tr>
<tr>
<td>Content CD19⁺ B-cells</td>
<td>FCM</td>
<td>&lt;1×10⁶ CD19⁺ B cells/kg body weight of the patient</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Sterility</td>
<td>Culture</td>
<td>Negative for bacterial and fungal contamination</td>
<td>negative</td>
<td>negative</td>
<td>negative</td>
<td>negative</td>
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<tr>
<td>Mycoplasm</td>
<td>Luminescence assay</td>
<td>Negative for mycoplasm contamination</td>
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<td>negative</td>
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<td>negative</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>LAL assay</td>
<td>&lt;0.25 EU/ml</td>
<td>0.08</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Absence of cytokines</td>
<td>ELISA</td>
<td>&lt;25 pg/ml IL-2, IL-7, IL-15 and SCF.</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
</tbody>
</table>

The table shows an overview of product release tests and product specifications for the ex-vivo generated NK cells using a closed cell culture process. The table summarized the facts needed to provide a certificate of analysis to release an UCB-NK cell therapy product for a patient. n.a. = not analyzed in validation runs but these parameters will be scored for the clinical production and the certificate of analysis. n.d.: not detected; the test do not show any positive events, yes = the results of the test fulfill the specification relevant for the certificate of analysis. doi:10.1371/journal.pone.0020740.t004
bags and up-scaling of the NK cell expansion step into the bioreactor systems resulted in the generation of products containing more than 3.5\times10^9 NK cells with a purity of up to 95%. Furthermore, the UCB-NK-cell products could be finally processed for infusion using a closed system and be stored until all product control tests will be available in order to release the UCB-NK-cell therapy product. The safety and toxicity of infusing these allogeneic ex vivo-expanded UCB-NK cell therapy products will be investigated in phase I dose escalation trial in elderly AML patients not eligible for allogeneic stem cell transplantation.

Materials and Methods

Cell lines

Cell line K562 (LGC Standards, Wesel, Germany) was cultured in Iscove's modified Dulbecco's medium (IMDM; Invitrogen, Carlsbad CA, USA) containing 50 U/ml penicillin, 50 µg/ml streptomycin and 10% fetal calf serum (FCS; Integro, Zaandam, the Netherlands).

Isolation of CD34+ stem and progenitor cells

UCB units have been obtained at birth after normal full-term delivery after written informed consent with regard of scientific use from the cord blood bank of the Radboud University Nijmegen Medical Center (RUNMC, Nijmegen, The Netherlands). The use of these UCB units for our study was approved by the RUNMC Institutional Review board. UCB samples were stored at room temperature and processed within 24 h after collection. Before storage, the red blood cell content has been reduced using standard EloHAES separation and the mononuclear cells have been washed, cryopreserved and stored in liquid nitrogen [29]. Stored UCB units were thawed at 37°C and resuspended in thawing buffer consisting of CliniMACS PBS/EDTA buffer (Miltenyi Biotech, Bergisch Gladbach, Germany), 5% HSA (Sanquin blood bank, Amsterdam, The Netherlands), 3.5 mM MgCl₂ (Pharmacy Department, RUNMC, Nijmegen, The Netherlands) and 100 U/ml Pulmozyme (Roche, Almere, The Netherlands). Thawed UCB cells were incubated for 30 minutes at room temperature (RT) and subsequently centrifuged. After two washing steps, thawed UCB cells were resuspended in 8 ml washing buffer consisting of CliniMACS PBS/EDTA buffer, 0.5% HSA, 3.5 mM MgCl₂ and 100 U/ml Pulmozyme and labeled for 30 minutes at RT with 0.75 ml CliniMACS CD34 reagent (Miltenyi Biotech) and 1 ml Nanogam (Sanquin blood bank, Amsterdam, The Netherlands). After incubation, the CD34-labeled UCB sample was washed and

Figure 4. Stability tests of ex vivo generated and processed NK cell products. (A) The NK cell content of the processed final product was followed over time, while the products were either stored at 4°C or room temperature (RT) for a maximum of 3 days. The percentage of the CD45+/CD56+ cells is displayed from 3 different stability tests. (B) Viability of the final NK cell product was followed over time, while the products were either stored at 4°C or room temperature (RT). The percentage of the CD45+/CD56+/7-AAD− cells is displayed from 3 different stability tests. doi:10.1371/journal.pone.0020740.g004
resuspended in 100 ml washing buffer. The automated Clin- 
imACS cell separator was equipped with a closed disposable 
CliniMACS tubing set type 161-01 (Miltenyi Biotech). The CD34+ 
cell selection was performed using an automated program and 
after the enrichment procedure, the CD34+ cell fraction was 
collected, and the cell number and purity were analyzed by flow 
cytometry. Finally, the obtained CD34+ UCB cells were used 
directly for the NK cell generation bioprocess.

Ex vivo expansion and differentiation of CD34-positive 
progenitor cells

CD34+ UCB cells were transferred into Vuelife™ bags 290AC 
or 750AC (Cellgenix, Freiburg, Germany), and expanded and 
differentiated according to culture method III as described 
previously with some minor modifications [18]. In brief, for day 
0–9 Expansion Medium I was used consisting of Glycostem 
Basal Growth Medium (GBGM®) for cord blood (CB) (Clear Cell 
Technologies, Beernem, Belgium) supplemented with 10% human 
serum (HS; Sanquin Bloodbank, Nijmegen, The Netherlands), a 
high-dose cytokine cocktail containing SCF, Flt3L, TPO and IL-7 
(all CellGenix, Freiburg, Germany) and a low-dose cytokine 
cocktail consisting of GM-CSF (Neupogen) (Amgen, Breda, 
The Netherlands), G-CSF and IL-6 (both CellGenix, Freiburg, 
Germany). Between day 10 and 14, Expansion II medium was 
used in which TPO was replaced by IL-15 (CellGenix, Freiburg, 
Germany). During the first 14 days of culture, low molecular 
weight heparin (LMWH) (Clivarin®, Abbott, Wiesbaden, Ger-
many) was added to the expansion medium. Cell cultures were 
refreshed with new medium every 2–3 days, and adjusted to a cell 
density of >0.5 x 10^9/ml. Cultures were maintained in a 37°C, 
95% humidity, 5% CO² incubator. Expanded cultures in 
Vuelife™ bags were either maintained in Vuelife™ bags or 
transferred to a bioreactor at around day 14. We have expanded 
both the single use WAVE Bioreactor™ System 2/10 (GE Health, 
Uppsala, Sweden) and BIOSTAT® CultiBag RM system (Sarto-
rius Stedim Biotech, Göttingen, Germany). From day 14 onward, 
expanded CD34+ UCB cells were differentiated and further 
expanded using NK cell differentiation medium. This medium 
consisted of Glycostem Basal Growth Medium (GBGM®) for 
cord blood (CB) as used for the CD34 expansion step supplemented 
with 10% HS, the low-dose cytokine cocktail (as previously 
mentioned) and a new high-dose cytokine cocktail consisting of IL-
7, SCF, IL-15 and IL-2 (Proleukin®, Chiron, Munchen, 
Germany). The cell density was checked two times a week and 
adjusted to ~1.0 x 10^9 cells/ml by the addition of GBGM® NK 
cell differentiation medium. The conditions of the bioreactor were 
as follows: temperature 37°C, CO² 5%, air-flow 0.1–0.2 l/min, 
racking rate 10/min, rocking angle of 6°.

Flow cytometry

Cell numbers and expression of cell-surface markers were 
determined by flow cytometry. For immunophenotypical staining, 
cells were incubated with the appropriate concentration of antibodies 
for 30 min at 4°C. After washing, cells were resuspended in Coulter® 
Isotof® II DIHuent (Beckman Coulter) and analyzed using the Coulter 
FC500 flow cytometer (Beckman Coulter). For determining the 
content of CD34+ cells in the UCB and the purity of the CD34+ 
selected cells the following monoclonal antibodies were used: CD34-
FITC (J33) and CD34-PE (581) (both from Beckman Coulter, 
Woerden, The Netherlands). The population of living CD34+ cells 
was determined by exclusion of 7AAD (Sigma) positive cells. Ten color 
analysis was used to determine the phenotype of the cultured NK 
cells. The following monoclonal antibodies were used in the 
appropriate concentration: CD16-FTC (NKp15), CD336(NKp46)- 
PE (Z31), CD3-FTC (UCHT1), CD337(NKp30)-PC5.5 (Z25), 
CD335(NKp46)-PE-Cy7 (BAB281), CD341(NKG2D)-APC (ON72), 
CD244(2B4)-APC-alexas70 (C1.7.1), CD56-APC-Alexa750 (N901), 
CD161-PB (191B6), CD45-PO (J33) (all provided by Beckman 
Coulter, Marseille, France). The acquisition was performed on the 
Navios™ flowcytometer and the data were further analyzed using the 
Kahazu™ software (all from Beckman Coulter, Miami, Florida, 
USA).

Flow cytometry-based cytotoxicity and degranulation 
studies

Flow cytometry-based cytotoxicity assays were performed as 
described previously [18,30]. Briefly, after incubation for 4 h or 
overnight at 37°C, 50 μl supernatant was collected and stored at 
−20°C for later use to measure cytokine production. Cells in the 
remaining volume were harvested and the number of viable target 
cells was quantified by flow cytometry. Target cell survival 
was calculated as follows: % survival = [(absolute no. viable CFSF* 
target cells co-cultured with NK cells)/[absolute no. viable CFSF* 
target cells cultured in medium]]*100%. The percentage specific 
lysis was calculated as follows: % lysis = [(100−% survival)]. 
Degranulation of NK cells during co-culture was measured by cell 
surface expression of CD107a [31]. After 18 hrs of incubation at 
37°C, the percentage of CD107a+ cells was determined by flow 
cytometry..

Preparation of the final NK cell product

At the end of culture, NK cells were harvested, and the number 
and viability of CD56+ cells was determined by flow cytometry 
and ACT counter (Beckman Coulter). The UCB-NK-cell product 
was transferred into 600 ml transfer bags (Baxter, Deerfield, USA), 
centrifugated 200 g for 15 min without break and the supernatant 
was collected for testing of bacterial, fungal and mycoplasm 
contamination. NK cells were resuspended and washed twice with 
500 ml CliniMACS PBS/EDTA buffer supplemented with 0.5% 
HSA (Sanquin Blood Bank, Amsterdam, The Netherlands). After 
washing, NK cells were resuspended in infusion buffer consisting of 
NaCl 0.9% and viability of CD56+CD3- 
NK cells in the end-product was determined by flow cytometry 
and the concentration of residual SCF, IL-7, IL-15 and IL-2 was 
measured by ELISA (R&D Systems, Abingdon, Oxon, UK).

Karyotyping of the NK cell product

Cytogenetic analysis was performed on the final NK cell 
products according to standard methods. In total 20 metaphases 
were G-banded using trypsin and Giemsa (GTG) and were 
examined per case. Karyotypes were described according to ISCN 
2009 [32].

Sterility testing of the NK cell product

Before and after washing in bags, samples were taken and 
processed to check for bacterial and fungal contaminations. These 
samples were transferred to Bacter Ped plus flasks (BD). Bacterial 
growth till day 6 should be reported as positive. The testing was
done by the Department of Microbiology, RUNMC, Nijmegen, The Netherlands.

Mycoplasma testing
Mycoplasma detection was performed on final products using the MycoAlert® Mycoplasma detection kit (Lonza, Rockland, USA) following the manufacturer’s instructions. The signals were measured with the Fluostar Optima (BMG Labtech, IJsselstein, The Netherlands).

Endotoxin test
Endotoxin level in the final products was determined using the chromogenic Limulus Amebocyte Lysate (LAL) assay (Charles River Endosafe, Charleston, SC, USA) following the manufacturer’s guidelines by the Pharmacy Department, RUNMC, Nijmegen, The Netherlands. A level of <0.25 EU/ml was set as negative endotoxin limit.

Cytokine detection
Cytokine levels in the final products were determined using ELISA. Briefly, Maxisorp 96-well plates (NUNC) were coated overnight with 1 μg/ml of monoclonal coating antibody for IL-2, IL-7, IL-15 and SCF (all from R&D systems, Minneapolis, USA). For sample detection, biotinylated antibodies were added for IL-2 (0.02 μg/ml polyclonal Ab), IL-7 (0.2 μg/ml polyclonal Ab) and SCF (0.05 μg/ml polyclonal Ab), respectively. The extinction was measured by the TiterTek Multiscan MCC/340 plate reader (TiterTek, Huntsville, AL). Concentrations of triplicate measurements were determined using a standard curve ranging from 1 to 2000 pg/ml of the specific cytokine.

Statistics
Results from different experiments are described as mean ± standard deviation of the mean (SD), range and median. Statistical analysis was performed using students t-test. A p-value of <0.05 was considered statistically significant.

References