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Clinical-grade, large-scale, feeder-free expansion of highly active human natural killer cells for adoptive immunotherapy using an automated bioreactor

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Abstract

Background aims. Natural killer (NK) cell-based adoptive immunotherapy is a promising approach for the treatment of cancer. *Ex vivo* expansion and activation of NK cells under good manufacturing practice (GMP) conditions are crucial for facilitating large clinical trials. The goal of this study was to optimize a large-scale, feeder-free, closed system for efficient NK cell expansion. *Methods*. Peripheral blood mononuclear cells (PBMCs) from healthy donors and myeloma patients were cultured for 21 days using flasks, cell culture bags and bioreactors. Final products from different expansions were evaluated comparatively for phenotype and functionality. *Results.* Significant NK cell expansions were obtained in all systems. The bioreactor yielded a final product rich in NK cells (mean 38%) ensuring that a clinically relevant cell dose was reached (mean 9.8×10^9 NK cells). Moreover, we observed that NK cells expanded in the bioreactor displayed significantly higher cytotoxic capacity. It was possible to attribute this partially to a higher expression level of NKp44 compared with NK cells expanded in flasks. *Conclusions*. These results demonstrate that large amounts of highly active NK cells for adoptive immunotherapy can be produced in a closed, automated, large-scale bioreactor under feeder-free current GMP conditions, facilitating clinical trials for the use of these cells.

Key Words: *adoptive immunotherapy,* ex vivo *expansion, good manufacturing practice, natural killer cells, NKp44*

Introduction

The use of cellular immunotherapy against cancer has been investigated thoroughly since the introduction of lymphokine-activated killer (LAK) cells in mid-1980s (1,2). One of the most common approaches has been adoptive transfer of autologous or allogeneic cytotoxic effectors with tumor cell killing potential to trigger a graft-versus-tumor (GvT) effect. Among the various effector populations that have a potential anti-tumor effect, natural killer (NK) cells have drawn considerable attention in recent years (3).

The main obstacle in clinical studies with these cells originates from the fact that they are normally present only in low numbers in peripheral blood mononuclear cells (PBMCs) and effector cell preparations such as LAK cells. We have previously described a novel method comprising current good manufacturing practice (cGMP)-compliant components that facilitates expansion of high numbers of polyclonal NK cells in cell-culture flasks using PBMCs from healthy donors (4) as well as patients with B-cell chronic lymphocytic leukemia (5) and multiple myeloma (MM) (6). These cells were shown to exert specific cytotoxic activity against fresh human tumor cells *in vitro* (6) and in experimental models of human tumors (7), which opens up the possibility of evaluation in clinical settings. Concurrently, we have successfully completed a safety evaluation of this cell product in an allogeneic setting in a phase I clinical trial with cancer patients (8).

There have also been attempts by other investigators to expand and/or activate NK cells *ex vivo* (9–16), and treatment options using purified/resting (17,18), short term (13,19,20) or highly purified and long-term activated (14) NK cells have been investigated. These studies report NK cell infusions to be well tolerated and partially effective. However, the protocols used for effector cell preparation commonly include additional steps, such as NK precursor or CD56 separation prior to culture, and the use of feeder cells and/or cGMP-incompatible components.

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These disadvantages render such protocols either expensive or suboptimal for GMP production and unfeasible for large-scale clinical studies.

A thorough evaluation of the above-mentioned reports highlights a need for an automated method for optimized *ex vivo* expansion of NK cells. The conventional flask-based culture is labor-intensive and cumbersome, thus limiting the cell number that can be handled practically. An optimal procedure for the expansion of effector cells should be cost-effective, easy to handle and must include well-defined cGMP quality components, as well as a preferably closed and disposable culture system free of animal products and feeder cells.

Therefore we investigated the feasibility of large-scale NK cell expansion using closed systems. We evaluated two different closed systems, cell culture bags and an automated bioreactor, compared with conventional cell-culture flasks, using PBMCs from healthy donors as well as patients with MM, with the aim of optimizing an automated GMP-compatible protocol that will allow large-scale production of NK cells to be used in a phase I/II clinical trial for the immunotherapy of MM in an autologous setting.

Methods

Ex vivo expansion of NK cells

Buffy coats, peripheral blood and apheresis products were obtained from healthy donors ($n = 10$) and MM patients ($n = 2$) via the blood bank at the Karolinska University Hospital Huddinge (Stockholm, Sweden). The experimental protocol was approved by the local research ethics committee.

The PBMCs were isolated by gradient centrifugation using Lymphoprep (Nyegaard, Oslo, Norway) and washed twice with phosphate-buffered saline (PBS; Gibco, Grand Island, NY, USA). Cell count and viability were assessed by Türk and trypan blue dye exclusion or Nucleocounter NC-100 (Chemo-Metec A/S, Allerød, Denmark).

For all systems, CellGro SCGM serum-free medium (CellGenix, Freiburg, Germany), with the addition of 5% human serum (Biowhittaker-Cambrex, Walkersville, MD, USA) and 500 U/mL recombinant human interleukin-2 (IL-2) (Proleukin®, Novartis Pharmaceuticals, East Hanover, NJ, USA), was used as growth medium. At the beginning of the culture, the medium was further supplemented with GMP-grade monoclonal anti-CD3 antibody (Orthoclone OKT-3; Ortho Biotech, Raritan, NJ, USA) at a final concentration of 10 ng/mL. Total cell numbers and phenotype were assessed on days 0, 5, 10, 15 and 20 of culture. Absolute cell counts were calculated by multiplying the total number of cells with the percentage of these subsets determined by flow cytometry.

Expansion in cell-culture flasks

The culture conditions for the expansion of cytotoxic cells in cell-culture flasks have been optimized previously with PBMCs from healthy individuals (4). In brief, PBMCs were initially cultured in T25 flasks (TPP, Trasadingen, Switzerland) at a concentration of 0.5×10^6 cells/mL. After 5 days, the cultures were replenished with fresh medium with 5% human serum and IL-2 (500 U/mL) but without OKT-3, every 2–3 days until the end of the culture. To prevent contact inhibition of cell growth (21), the cells were transferred to bigger flasks (T75 or T150; TPP) or split into multiple flasks when necessary. During medium replenishment, the cell concentration was adjusted to 0.5×10^6 cells/mL until day 10 and to 1×10^6 cells/mL thereafter. Occasionally, certain portions of the cells were frozen in order to keep the number of flasks manageable.

Expansion in the Wave Bioreactor system

The Wave Bioreactor is a cell-culture system where the cells are grown inside a temperature- and CO_2-controlled disposable, sterile bag that is placed on a rocking, heated platform. We used a Wave Bioreactor System 2/10 (GE Healthcare, Somerset, NJ, USA). Previous experience with this system has shown suboptimal efficiency when the expansion is initiated with low volumes and/or low cell doses. Thus, the amount of cells in regular peripheral blood samples from healthy donors did not allow starting the expansions directly in the bioreactor. Therefore, in initial optimization experiments, we initiated the cultures in flasks and transferred the cells into the bioreactor at around day 5, when sufficient numbers of cells were reached. The bioreactor cultures on this day were started with 2×10^6 cells/mL in 800 mL. In final validation experiments, a whole unit of peripheral blood, or apheresis product from donors and MM patients, was obtained and the cultures were initiated directly in bioreactors from day 0. The conditions for the bioreactor were as follows, at all times: temperature 37°C, CO_2 5%, airflow 0.1, rocking rate 6/min, rocking angle 6°. The cells were sampled and counted every other day and no further feeding was done until the cell density reached 3×10^6 cells/mL. From then on, the culture was fed with 300 mL medium/day. When the cells reached a density of 7×10^6 cells/mL, the feeding was increased to 500 mL/day; after 1×10^7 cells/mL, to 750 mL/day; and after 2.5×10^7, to 1 L/day.

Expansion in Vuelife bags

Vuelife™ (American Fluoroseal Corporation, Gaithersburg, MD, USA) is a sterile cell-culture bag made of fluorinated ethylene propylene that is claimed to be biologically, immunologically and chemically inert. It is highly permeable to gases and optically clear. The cultures in Vuelife bags were initiated with 5×10^5 cells/mL in 60 mL medium using 72-mL Vuelife bags. The bags were incubated in a humidified incubator at 37°C and 5% CO_2. Fresh medium was added every other day to adjust the concentration to 1×10^6 cells/mL until day 10, and 2×10^6 cells/mL thereafter. Cells were split to larger bags when necessary.

Analysis of lymphocyte subsets and phenotyping by flow cytometry

The cell phenotype and percentage of subpopulations were analyzed by flow cytometry on days 0, 5, 10, 15 and 20 of culture using standard procedures with fluorochrome-conjugated monoclonal antibodies (mAbs) against CD3, CD14, CD19, CD45 and CD56.

For detailed phenotypic characterization of the NK cell subset by flow cytometry, all frozen samples were thawed simultaneously. This additional panel included fluorochrome-conjugated mAbs against the following surface antigens: CD11a (HI111), CD3 (UCHT-1), CD7 (M-T701), CD14 (MOP9), CD16 (3G8), CD19 (HIB19), CD25 (M-A251), CD27 (M-T271), CD56 (B159), CD57 (NK-1), CD226 (DX11), NKB1 (DX9) and CD62L (DREG56), purchased from BD Biosciences (San Jose, CA, USA); CD244(2B4) (C1.7), NKG2D (ON71), NKp30 (Z25), NKp44 (Z231) and NKp46 (BAB281), from Beckman Coulter Inc. (Fullerton, CA, USA); and NKG2A (131411), NKG2C (134591), KIR2DL1 (143211) and KIR2DL3 (180701) from R&D Systems (Minneapolis, MN, USA). Other antibodies used for further characterization of the final cell product were CD38 (HIT2), CD138 (MI15) and FoxP3 (250D/C7), from BD Biosciences (San Jose, CA, USA).

All antibody stainings for flow cytometry were done according to the following protocol. The cells were washed once with PBS and incubated with appropriate amounts of antibody at 4°C for 30 min. The labeled cells were then washed with PBS and fixed in 4% paraformaldehyde prior to data acquisition. Data acquisition was done on a FACSCalibur (BD Biosciences, San Jose, CA, USA) and CyFlow ML (Partec GmbH, Munster, Germany). Data were analyzed with CellQuest Pro (Biosciences, San Jose, CA, USA), FloMax (Partec GmbH, Munster, Germany) and FlowJo (Treestar Inc., Ashland, OR, USA) software. For the analysis, appropriate Side Scatter (SSC)/Forward Scatter (FSC) gates around the $CD45^+$ $CD14^-$ $CD19^-$ lymphocyte population were used. LIVE/DEAD Fixable Aqua stain (Invitrogen, Carlsbad, CA, USA) was used for gating out dead cells. NK cells were gated as the $CD3^-$ $CD56^+$ population. NK-like T cells and T cells were gated as $CD3^+$ $CD56^+$ and $CD3^+$ $CD56^-$ populations, respectively.

For each cell-surface receptor analyzed, mean fluorescence intensity (MFI) values were calculated for day 0 and day 20 samples. To estimate the change in receptor expression between different samples, we calculated MFI ratios (MFI_{day20}/MFI_{day0} or $MFI_{bioreactor}/MFI_{flask}$) for each receptor. When the MFI for a sample was higher than another, the MFI ratio was higher than 1, which indicated the relative extent of over-expression in that receptor. Likewise, an MFI ratio below 1 was interpreted as down-regulation in the expression of that receptor.

Evaluation of cell-mediated cytotoxicity

The cytotoxic capacities of the final products were evaluated *in vitro* with a standard 4-hour ^{51}Cr-release assay against K562 cells. In brief, K562 target cells were labeled with 100 μCi ^{51}Cr for 1 h at 37°C, washed twice with PBS, and resuspended in RPMI medium. A total of 3×10^4 target cells in 100 μL RPMI medium was placed in triplicate into V-bottomed 96-well plates and incubated for 4 h with 100 μL effector cells at appropriate concentrations to obtain effector:target (E:T) ratios from 1:3 to 10:1. Aliquots of supernatants were counted using a Cobra Auto-Gamma 5000 Series counting system (Packard, Meridien, CT, USA). The percentage-specific ^{51}Cr release was calculated according to the formula: percentage specific release = [(experimental release – spontaneous release)/(maximum release – spontaneous release)] × 100.

Analysis of NK cell degranulation

The expansion products were co-incubated with K562 target cells at a ratio of 1:1 in a final volume of 200 μL in round-bottomed 96-well plates at 37°C and 5% CO_2 for 6 h. Fluorochrome-conjugated anti-CD107a MAb or the corresponding IgG1 isotype control was added at the initiation of the assay. After 1 h of co-incubation, Monensin (GolgiStop; BD Biosciences, San Jose, CA, USA) was added at a 1:100 dilution. Surface staining was done by incubating cells with anti-CD3 and anti-CD56 MAb for 30 min at +4°C. The cells were then washed, resuspended in PBS and immediately analyzed by flow cytometry. The degranulation of NK cells, T cells and NK-like T cells was acquired within the same assay by gating on the appropriate populations.

Statistical analysis

Data analysis, preparation of graphs and statistical comparisons were done with GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA).

Results

Evaluation of cell culture bags and bioreactor for NK cell expansion

In an attempt to use a closed culture system for the expansion of NK cells, we primarily compared cell-culture bags with flasks using PBMCs from five donors. As the initiation of cultures in bags or bioreactor required a high number of cells, for these first sets of experiments we initiated cultures in flasks and transferred to a bag or bioreactor when a sufficient amount of cells was reached. Figure 1A shows the fold expansion of bulk cells as well as NK and NK-like T cells from each donor, at the end of the expansion period. The mean bulk cell expansion was 530-fold in bags, while the flasks yielded a mean of 1100-fold expansion. The NK expansion in bags appeared impressive compared with the flasks, especially for three of the five donors. However, when the percentage of

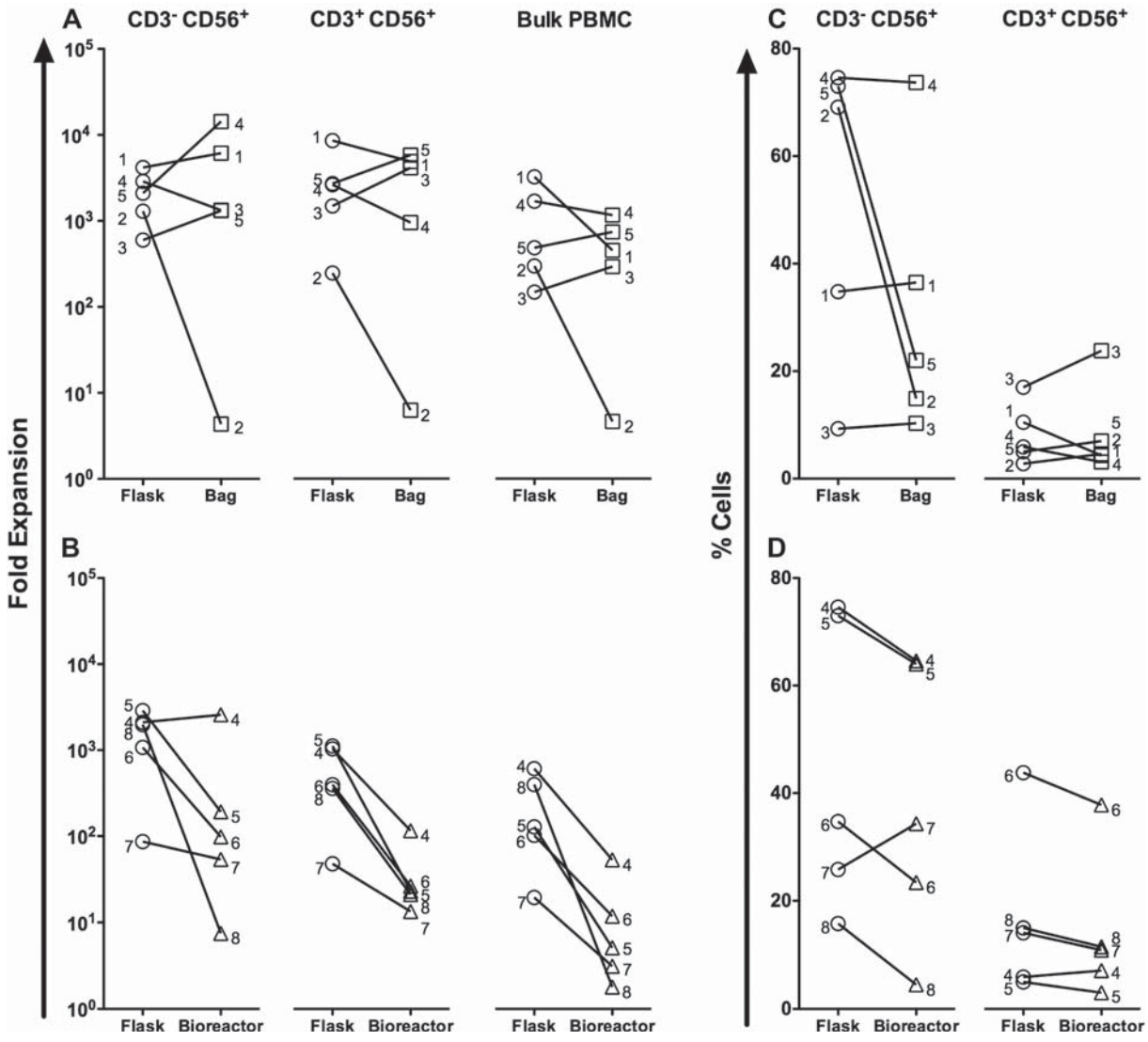

Figure 1. Comparison of fold expansion and final product purity in flasks, bags and the bioreactor. (A) Fold expansion of bulk, NK and NK-like T cells at day 20 using flasks and bags with a set of five donors. (B) Fold expansion of bulk, NK and NK-like T cells at day 20 using flasks and the bioreactor with a different set of five donors. (C) Percentages of NK and NK-like T cells at the end of *ex vivo* expansion in flasks and bags with a set of five donors. (D) Percentages of NK and NK-like T cells at the end of *ex vivo* expansion in flasks and the bioreactor with a different set of five donors. The remaining cells were mainly conventional $CD3^+$ $CD56^-$ T cells. The values for each donor are shown along with a line connecting the values for the same donor in different expansion protocols. Each data point is labeled with a number indicating the donor code.

NK cells in the final product was taken into account (Figure 1C) the expansion in bags did not correlate with the expansion in flasks and could result in a lower NK cell purity at the end. The end product in bags had a mean of 31% NK cells, while the mean NK percentage in the final product of flask was 53.

In the search for a closed expansion system that results in comparable and correlating yield with flasks, we evaluated the use of an automated bioreactor system compared with expansion in flasks, using PBMCs from five donors. We observed the expansion of bulk cells (mean expansions, flask 770-fold, bioreactor 77-fold) while NK cells expanded preferentially and increased their share of the population in both conditions. Although fold expansions (Figure 1B) of NK cells were lower than flasks in four out of five donors, percentages of these subpopulations in the final product were more comparable and correlated with flask expansion (Figure 1D). The end product of the expansion protocols had a median of 38% NK cells and 14% NK-like T cells in the bioreactor, while there were 44% NK cells and 16% NK-like T cells in flasks.

Compared with the previously mentioned results from bags, the percentage of NK cells in the bioreactor was higher than that in bags, despite the fact that this set of donors had yielded a worse NK cell purity in flask expansion. Taken together, the results suggested that NK cell fold expansions were better in bags, whereas expansion in the bioreactor yielded a higher NK cell purity with a feasible expansion.

Due to the use of different donor sets and the high inter-individual variations in NK cell expansions, it was not possible to see directly the relative efficiencies of the bag and bioreactor systems. Because two of the five donors in these different groups were the same donors (donors 4 and 5), we had a chance to compare directly the efficiency of expansion. Although it had a lower fold expansion rate, the bioreactor had a comparable percentage of NK cells in the final product (64%) compared with flasks (74%), which were higher than bags (47%). In the case of NK-like T cells, the percentages were very close to 5% in all three systems.

Expansion products from these two donors were subjected to further phenotypic analysis in order to see the pattern of change in receptor expression. Although there was inter-individual variability as usual, the changes in receptor expression levels in the different final products were very similar (Figure 2A, B).

In order to clarify whether the NK cells in the final products of different systems retain the same activation status and show comparable cytotoxicity, we evaluated the cytotoxic activity of the final products against the NK-sensitive cell line K562 (Figure 2C). We observed no significant differences between the cytotoxic activities of NK cells expanded under different conditions.

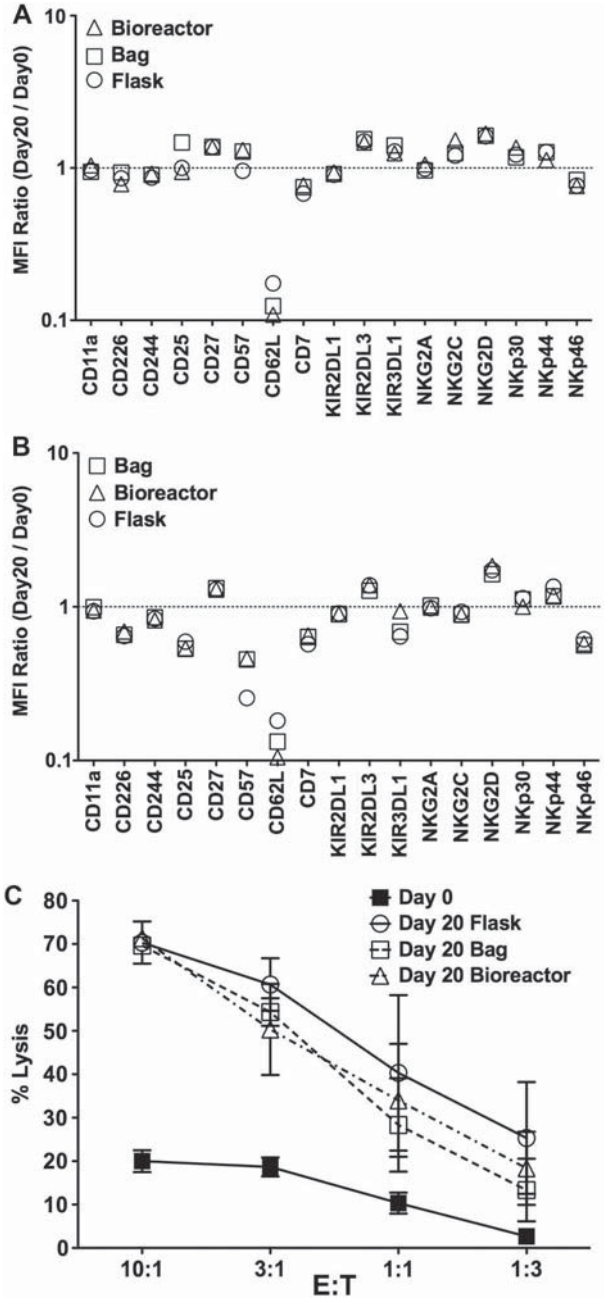

Figure 2. Comparison of phenotype and cytotoxic activity in different expansion protocols. (A and B) NK cells from flask, bag and bioreactor cultures of two donors were analyzed by multicolor flow cytometry for expression of various cell-surface receptors at days 20 and 0. To estimate the change in receptor expression, MFI ratios (MFI_{day20}/MFI_{day0}) for each receptor were depicted. The dashed line shows the MFI ratio = 1, which means unaltered expression. (C) Cytotoxic activity of end products from flask, bag and the bioreactor cultures against K562 cells, as measured by a standard ^{51}Cr-release assay.

Validation of the NK cell expansion process in the bioreactor

After demonstrating the feasibility of using the bioreactor for the expansion process, we continued with validation of the expansion process in a bioreactor under cGMP conditions using apheresis products or whole-unit peripheral blood to initiate the culture directly in the bioreactor from day 0. PBMCs from two healthy donors and two MM patients were used for this validation process. Table I presents the donor and sample characteristics. For comparison purposes, PBMCs from the same four donors were also expanded using flasks. Figure 3 presents the expansion curves of bulk cells and lymphocyte subpopulations for all donors in the bioreactor and flasks. The viability of the cells in the bioreactor, as assessed by trypan blue staining as well as Nucleocounter NC-100, was above 93% at all times during the culture and above 98% at the time of culture harvest.

The bioreactor expansions yielded an average of 9.8×10^9 NK cells (Figure 3) with an average purity of 37.5%, while the purity was 43% in flasks (Figure 4). Although the NK cell purity was slightly lower in the bioreactor, the final number of NK cells reached was sufficient to facilitate clinical trials for the use of expanded NK cells in cancer immunotherapy settings.

NK cells expanded in the bioreactor display a higher cytotoxic capacity

In initial experiments, where the expansion was initiated in flasks and subsequently transferred to bioreactors, we had detected no difference in cytotoxic capacity or the phenotype of final products compared with flask expansions. Interestingly, we observed that when expansions were initiated in a bioreactor from the beginning, the cytotoxic activity of the final product against K562 cells was remarkably higher compared with the final product of flask expansions, in three out of four donors (Figure 5A). Subsequently, we carried out degranulation assays against K562 cells and measured the percentage of degranulating lymphocytes (Figure 5B). Surprisingly, we observed that the extent of degranulation observed in the NK cell fractions from bioreactor expansions was significantly higher than NK cells from flask expansions, in all four donors. Likewise, degranulation of the NK-like T-cell fractions was significantly higher in three out of four donors. Taken together, these results suggested that the expansion process carried out in the bioreactor performed better in terms of elevating the cytotoxic capacity of NK cells.

Phenotypic characterization of cells expanded in the bioreactor

In an attempt to explain the difference in cytotoxic capacity of the final products from bioreactor and flask expansions, we performed detailed phenotypic characterization of the final product using multicolor flow cytometry. Figure 6A presents the dynamics of lymphocyte subpopulations throughout the expansion period from one representative donor (donor 11). It was clearly seen that NK cells dominated the culture with time, while contaminating T cells decreased in percentage, especially after day 5. Likewise, other impurities in cultures, such as $CD14^+$ monocytes and $CD19^+$ B cells, decreased to insignificant levels over time. Overall, the percentage of monocytes at the end of the culture was below 3% in all cases, and the percentage of B cells was below 1% in all cases. The NK cells in the final product almost uniformly expressed high levels of CD16 regardless of whether the culture was carried out in flasks or bags (Figure 6B). The final products were also analyzed for evidence of regulatory T-cell (T_{reg}) expansion, and we observed that in all cases the percentage of $CD4^+$ $CD25^{high}$ T cells was below 1%, indicating negligible, if any, levels of T_{reg} presence in the final product (Figure 6C). Moreover, these cells had no detectable FoxP3 expression, as analyzed by flow cytometry.

We also investigated whether there was any tumor cell contamination in the expansions where PBMCs from MM patients were used. No detect-

Table I. Donor and sample characteristics for PBMC expanded directly in bioreactors.

Donor code	Donor status	Sex	Age	Sample	Number of PBMCs used for culture ($\times 10^6$)	Total volume at initiation of bioreactor culture (mL)	Cell concentration at initiation of bioreactor culture ($\times 10^6$ cells/mL)
9	Healthy	F	24	Apheresis	970	1000	0.97
10	Healthy	F	25	PB	700	800	0.87
11	MM patient	M	55	PB	546	800	0.68
12	MM patient	F	59	PB	460	800	0.57

F, female; M, male.

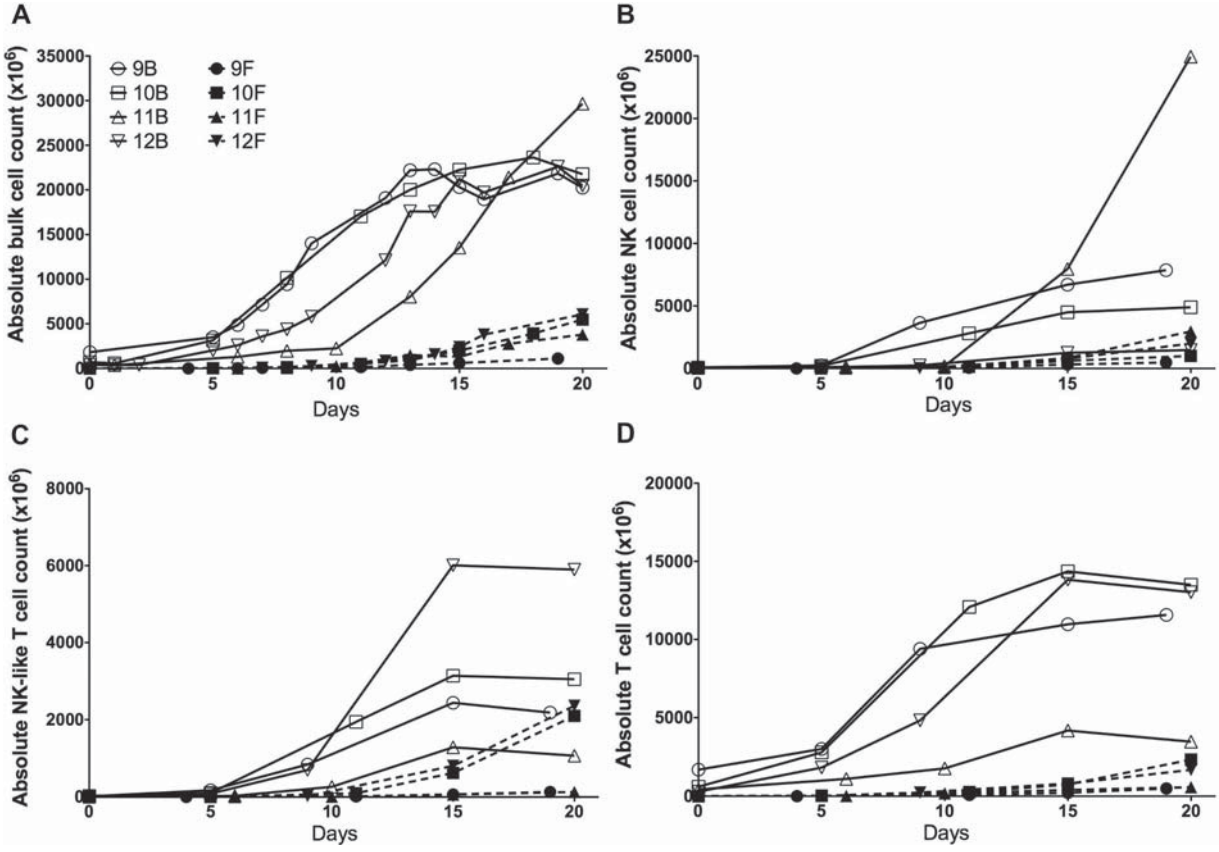

Figure 3. Expansion of cultures initiated in the bioreactor in comparison with flasks. PBMCs from four donors were expanded directly in bioreactors and compared with the expansion dynamics in flasks. Figures present the expansion of (A) bulk cells, (B) NK cells, (C) NK-like T cells and (D) T cells. The numbers indicate donor codes, and the letters B and F indicate the expansion system; for example, 9B is donor 9 bioreactor expansion, 9F is donor 9 flask expansion.

able $CD38^+$ $CD138^+$ cells were present in the final products for either of the donors, as assessed by flow cytometry.

Up-regulation of NKp44 is a key factor in elevated cytotoxic capacity

The expression of various surface receptors analyzed on the NK cells from bioreactor and flask expansions, as well as the distribution of killer cell immunoglobulin-like receptor (KIR) subpopulations within the NK cells from donor 11, is presented Figure 6D. Figure 7A and B summarizes the results from all donors and gives a general picture of the phenotypic comparison between bioreactor and flask expansions. Overall, the NK cells in the final product looked similar regardless of which expansion system was used, with slight but noticeable differences in the expression levels of CD11b, NKG2D and NKp44.

To analyze the effect of receptor expression levels on the response of NK cells, we analyzed the correlation between receptor expression level (MFI) and degranulation percentage using linear regression analysis in all samples of all four donors. Receptors giving a statistically significant correlation with degranulation percentage are presented in Figure 7C. We observed a negative correlation of CD132, CD25, CD57 and NKG2C expression levels with degranulation, while the expression levels of all three natural cytotoxicity receptors (NCR) correlated positively with the NK cell response against K562. When the expression data of these seven receptors from bioreactor and flask expansions as presented in Figure 7A were compared, the only significant difference in receptor expression was presented for NKp44 ($P < 0.05$). Taken together, these results suggested that the significantly higher expression of NKp44 in NK cells expanded in the bioreactor could be a key factor in explaining the high cytotoxic potential of these cells.

Discussion

We aimed to evaluate comparatively the use of cell-culture flasks, bags and a bioreactor for the *ex vivo* expansion of NK cells originating from bulk PBMCs of healthy donors and MM patients. Our results

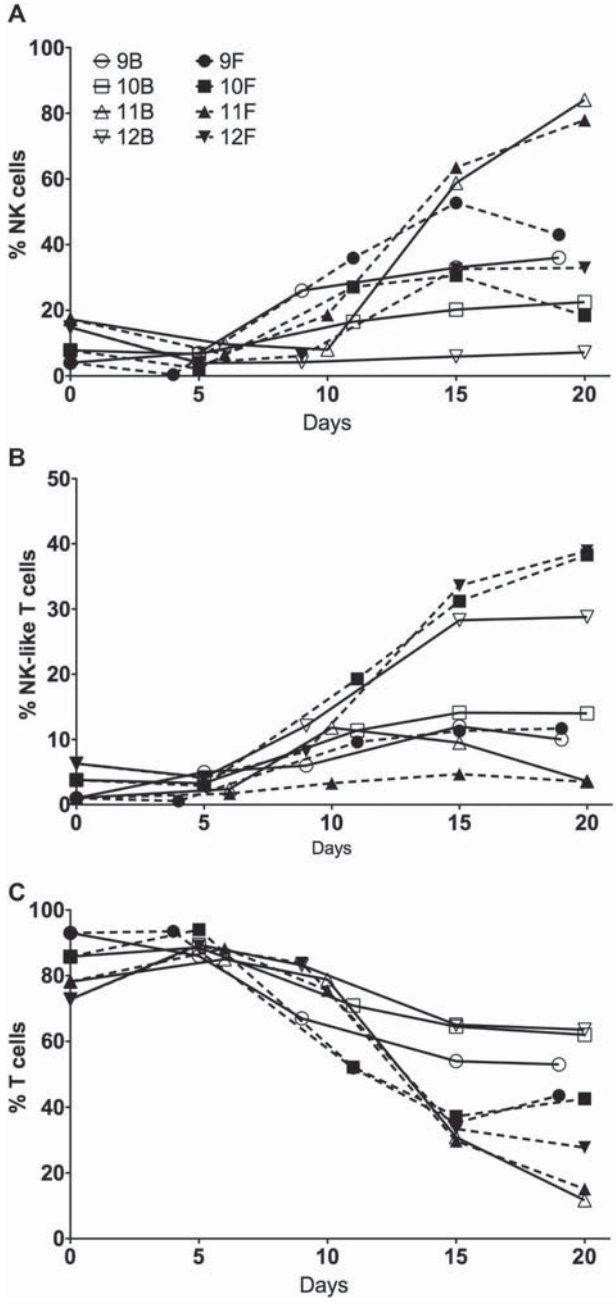

Figure 4. Dynamics of lymphocyte subpopulations during culture in bioreactors and flasks. Figures present the percentage of (A) NK cells, (B) NK-like T cells and (C) T cells in culture during the expansion process. The numbers indicate donor codes, and the letters B and F indicate the expansion system; for example, 9B is donor 9 bioreactor expansion, 9F is donor 9 flask expansion.

demonstrate the feasibility of producing clinical-grade effector cells in closed automated systems under GMP conditions.

We have previously reported a GMP-compliant culture medium that promotes the selective enrichment of activated NK cells in cell-culture flasks (4). Here, we present the last step in optimizing a GMP-quality automated closed culture system for the preparation of large-scale NK cell-enriched effector populations that is applicable in clinical settings.

The separation of NK cells or NK precursors prior to *ex vivo* culture (9,11,12,14) and/or use of feeder cell lines (13,15) has been used widely for NK cell expansion in previous reports. In this study, we did not utilize any feeder cells or separation steps but rather used bulk PBMCs for culture, which results in a cell population enriched in NK cells that is distinct from LAK (22) and cytokine-induced killer (CIK) cells (23,24), both in terms of NK cell content and anti-tumor activity (25).

We have demonstrated that both bags and the bioreactor system can sustain expansion of NK cells. An overall comparison of the expansion rates and end-product purity between the two closed systems utilized in this study reveals that the bioreactor system provides sufficient amounts of NK cells with higher purity and moreover has much less T cells in the final product compared with bags.

Surprisingly, we observed that NK cell activity is significantly higher in expansion products from a bioreactor compared with those from flasks. Observing a reverse correlation with the expression of the activating NK cell receptor NKG2C is unusual but has very little meaning in this case, as the target K562 cells are known to lack expression of its ligand human leukocyte antigen (HLA)-E (26). Statistical analyzes of receptors correlating with the NK cell response revealed that NKp44 expression both correlated positively with the response and was at a significantly higher level in bioreactor products compared with flask expansions. This may partially explain the observation of a high cytotoxic capacity of the bioreactor products. Unlike other NCRs, NKp44 (27) is expressed exclusively on activated NK cells and is up-regulated after IL-2 stimulation (28). Therefore, in this case, it might be acting as a surrogate marker for the efficiency of IL-2 usage in culture and the extent of NK cell activation. Thus the elevated expression of NKp44 provides a functional significance to the expansion procedure being carried out in the bioreactor rather than conventional cell-culture flasks.

Regarding practicalities, we observed that all systems in question had certain advantages and disadvantages. Expansion in cell-culture flasks has the inherent risk of exposure to external agents and contamination. Although this risk is minimized in GMP laboratory environments, the use of closed automated systems is definitely preferred as long as it supplies sufficient amounts of active cells. Initiation of cul-

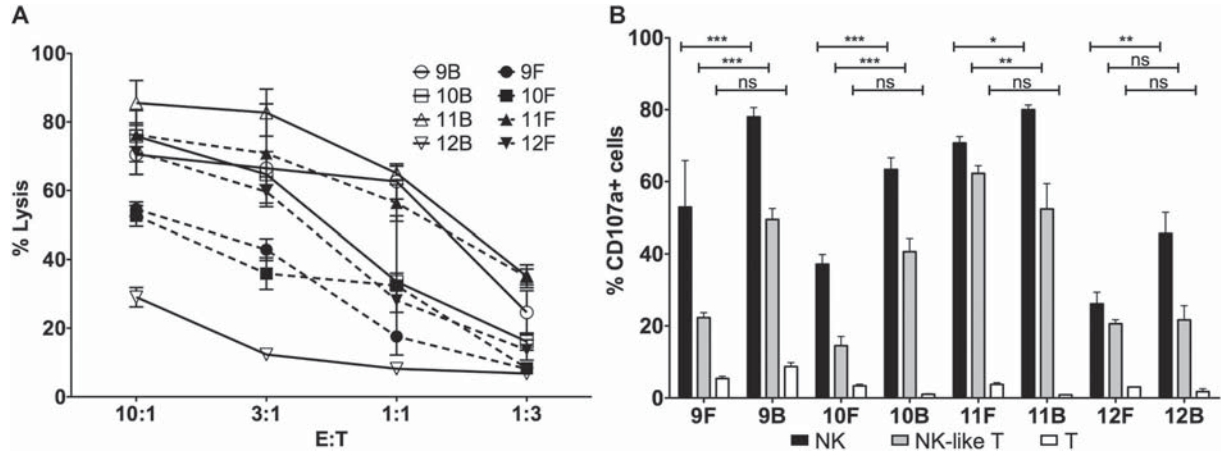

Figure 5. Functional comparison of cells expanded directly in bioreactors or flasks. (A) Cytotoxic activity of end products from flask and bioreactor cultures against K562 cells as measured by a standard ^{51}Cr-release assay. (B) Percentage of degranulating cells against K562 targets as measured by CD107a expression after co-culture with K562 cells at an E:T ratio of 1:1. The numbers indicate donor codes, and the letters B and F indicate the expansion system; for example, 9B is donor 9 bioreactor expansion, 9F is donor 9 flask expansion. ***, $P < 0.001$; **, $P < 0.01$, *, $P < 0.05$.

ture in flasks does not demand a high number of cells but, as the cells have to be kept within a certain concentration (21), splitting into new flasks during expansion results in an impossible number of flasks to handle. Culturing can also be initiated with a few cells in small bags and results in very good expansion, but the purity of the NK cells is lower than in the other systems and the cells still need to be split into more than one bag. However, expansion in bags can easily be optimized in a standard cell culture laboratory without the need to invest in additional equipment. More than one expansion can be carried out simultaneously using bags with the only limitation being incubator space, whereas a bioreactor needs extra investment for the purchase of the machine and can be used for only one expansion at a time.

The bioreactor is the most practical method, as it requires minimum hands-on time. However, initiation of the system requires many cells and the expansion rate is lower. The continuous rocking motion of the bioreactor ensures a dynamic and homogeneous culture environment that provides many advantages, such as uniform culture conditions, ease of sampling and better quality of control processes such as measurement of pH and dissolved oxygen. The use of such dynamic culture conditions is most probably a major factor contributing to the feasibility of growing more concentrated cells in a bioreactor. This saves any waste of media or additional components, which dramatically decreases the overall cost of the process.

Another factor to consider carefully is the material used in the culture environment. Only a few materials can support the growth of hematopoietic cells efficiently, and factors such as cleaning, sterilization and reuse significantly affect their performance (29). Thus the use of disposable and pre-sterilized suitable materials is preferred. Both the bags and the bioreactor system used in this study are suitable for production in this respect.

Naturally, the use of a dynamic bioreactor system for *ex vivo* culture demands a number of factors to be evaluated carefully. Hematopoietic cells are relatively sensitive to shear and it is reasonable to assume that high shear processes are unsuitable for *ex vivo* expansion (30). Thus stirred-tank bioreactors (10) or perfusion culture systems relying on external filters and high flow rate are unlikely to provide a high efficiency. In order to achieve the full benefit of a bioreactor, it would be desirable to use a low shear stress-producing system with an internal perfusion filter for removing media (30), and the bioreactor system used in this study can meet these expectations.

Nevertheless, the choice of which method to use depends on the availability of infrastructure and the demands of different applications in terms of cell number and purity, as well as many other practical issues. Bearing in mind that cGMP-compliant protocols for the enrichment of NK cells exist, it could be possible to purify the final product further before infusion to a patient.

In conclusion, we have optimized the expansion of clinical-grade NK cells from PBMCs of healthy individuals and MM patients using an automated bioreactor. The results presented here clearly demonstrate that large amounts of highly activated effector cells for possible use in adoptive immunotherapy settings can be produced in a closed, feeder-free culture system under GMP conditions. These cells may be used for the treatment of patients with malignancies and clinical trials will help to shape the future role of NK cells in cancer therapy.

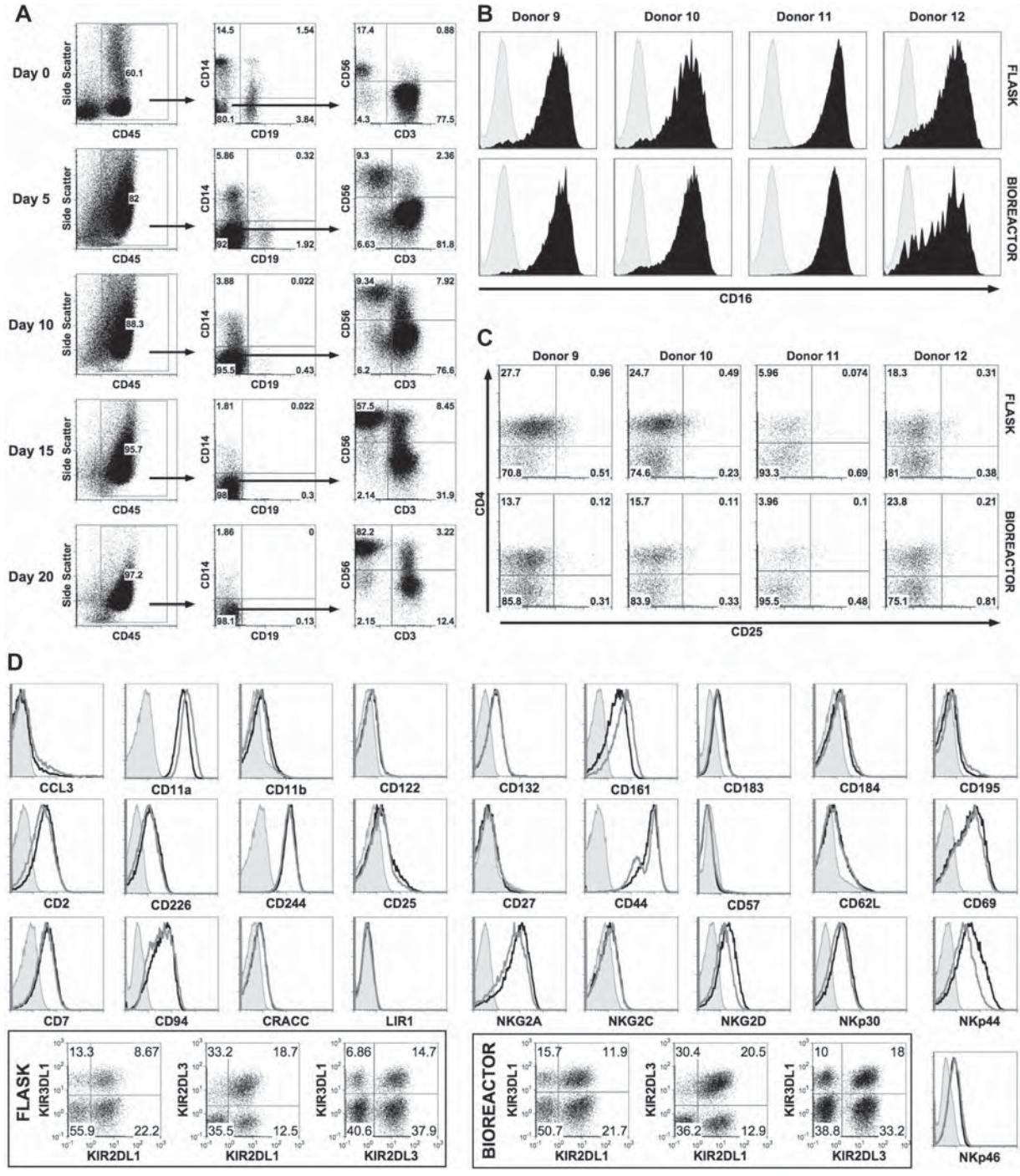

Figure 6. Phenotypic characterization of expansion products. (A) Measurement of contaminating $CD14^+$ monocytes, $CD19^+$ B cells and distribution of lymphocyte subpopulations throughout the culture period for bioreactor expansion of donor 11. It can be seen that the monocytes, B cells and T cells gradually fade away while the culture is dominated by NK cells. (B) Expression of CD16 on NK cells in the final cell product. Gray, isotype controls; black, CD16 staining gated on $CD3^-$ $CD56^+$ NK cells. (C) Analysis of possible T_{reg} presence in the final expansion products from flask and bioreactor expansions of four donors. Analysis was carried out on the $CD3^+$ $CD56^-$ T cells and the presence of $CD4^+$ $CD25^{high}$ cells within this gate was below 1% at all times. (D) Expression of various cell-surface receptors on NK cells and distribution of KIR-expressing populations as analyzed by multicolor flow cytometry on flask and bioreactor expansions of donor 11 at day 20. Gray lines represent flask, black lines represent bioreactor samples. Filled gray histograms represent isotype controls for the bioreactor samples. Isotype controls for flask samples looked identical but were left out of the picture to keep the images less complicated.

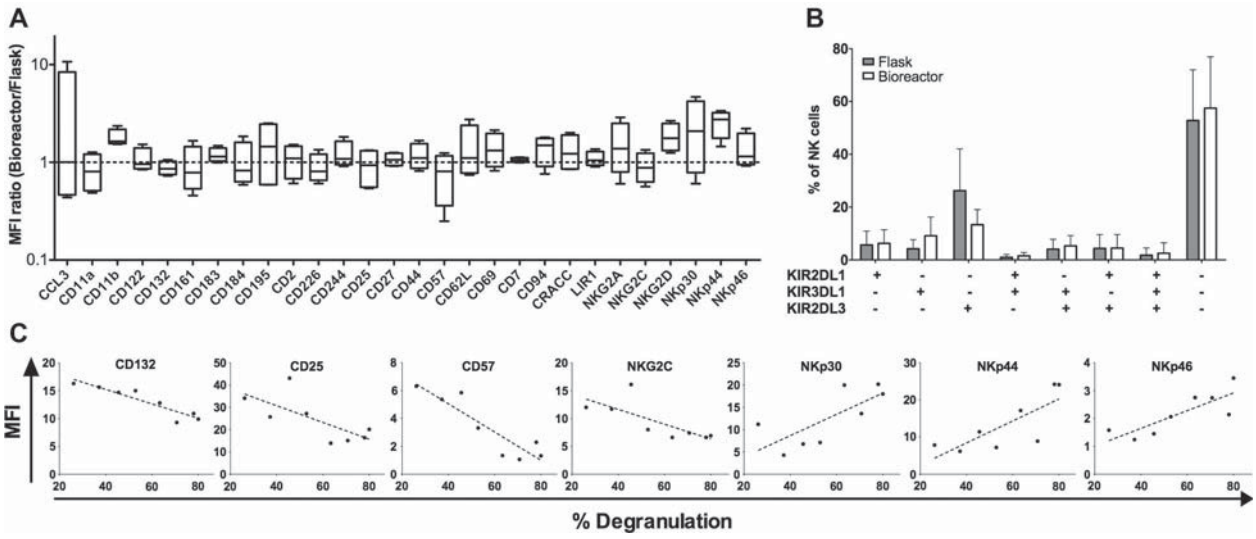

Figure 7. Phenotypic comparison of NK cells expanded in flasks or a bioreactor. (A) Comparison of bioreactor and flask expansion products in terms of expression levels of various cell-surface receptors on NK cells from four different donors. Each sample was analyzed as shown in Figure 6. The bars depict the MFI ratios of receptor expression after normalization with isotype controls. Values above 1 indicate higher levels of expression in bioreactor products, and values below 1 indicate lower levels of expression in bioreactor products. (B) Distribution of KIR-expressing subpopulations as phenotyped by antibodies against three different KIR molecules. The existence of all three genes in the donors was confirmed by KIR genotyping. (C) Correlation between expression levels of various cell-surface receptors and functional response of NK cells as measured by degranulation assays. Correlation analysis was run on all receptors presented in (A) but only those receptors giving a statistically significant correlation are presented. CD132, $r^2 = 0.8635$, $P = 0.0008$; CD25, $r^2 = 0.5192$, $P = 0.0438$; CD57, $r^2 = 0.8241$, $P = 0.0018$; NKG2C, $r^2 = 0.5619$, $P = 0.0322$; NKp30, $r^2 = 0.5510$, $P = 0.0349$; NKp44, $r^2 = 0.6046$, $P = 0.0231$; NKp46, $r^2 = 0.6743$, $P = 0.0124$.

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