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- (71) **Applicant (for all designated States except US):**
AVARIS AB [SE/SE]; Hälsovägen 7, S-141 52 Hud-
dinge (SE).
- (72) **Inventor; and**
- (75) **Inventor/Applicant (for US only):** ALICI, Evren
[TR/SE]; Skebokvarnsvägen 317, S-124 53 Bandhagen
(SE).
- (74) **Agent:** BERGENSTRÅHLE & LINDVALL AB; Box
17704, S-118 93 Stockholm (SE).
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(54) **Title:** EXPANSION OF NK CELLS

(57) **Abstract:** A method of large scale expansion and simultaneous activation of NK cells and NK- like T cells in a closed cell culture system is presented, wherein the expanded cells exhibit increased cytotoxicity.

EXPANSION OF NK CELLS

Technical field

[0001] The present invention relates to cell culture and immunotherapy, in particular large scale expansion and simultaneous activation of NK cells and NK-like T cells for therapeutic uses. The cells are expanded in a closed automated culture system, for example using a bioreactor.

Background of the invention

[0002] The use of cellular immunotherapy against cancer has been thoroughly investigated since the introduction of lymphokine-activated killer (LAK) cells in the mid-1980s (Grimm EA. *et al.*, 1982; Rosenberg S., 1985).

[0003] One of the most experimented approaches has been adoptive transfer of autologous or allogeneic cytotoxic effectors with tumor cell killing potential to trigger a graft-vs-tumor (GvT) effect. Among the various effector populations that have a potential anti-tumor effect, natural killer (NK) and NK-like T cells stand out with their high cytotoxic capacity (Sutlu T and Alici E., 2009).

[0004] NK and NK-like T cells are normally present only in low numbers in peripheral blood mononuclear cells (PBMCs) and effector cell preparations such as LAK cells. Therefore methods involving current good manufacturing practice (cGMP)-compliant components that allow expansion of polyclonal NK cells and NK-like T cells in cell culture flasks using PBMCs from healthy donors (Carlens S. *et al.*, 2001 and US 10/242,788), as well as patients with B-cell chronic lymphocytic leukemia (Guyen H. *et al.*, 2003), and multiple myeloma (MM) (Alici E. *et al.*, 2008) have been developed. These cells have been shown to exert specific cytotoxic activity against fresh human tumor cells in vitro and in experimental models of human tumors (Guimaraes F. *et al.*, 2006) which opens up the possibility to be evaluated in clinical settings. However, the conventional flask-based culture is labor-intensive and cumbersome, thus limiting the cell number that can be handled practically.

Previously disclosed protocols (e.g. Miller JS. *et al.*, 1994; Pierson BA. *et al.*, 1996; Luhm J. *et al.*, 2002; Klingermann HG and Martinson J., 2004) directed to effector cell preparation also include steps such as NK precursor or CD56 separation prior to culture and the use of feeder cells or cGMP-incompatible components. These disadvantages render previous protocols suboptimal and unfeasible to support large clinical studies.

[0005] Furthermore, expansion of NK cells 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 cells.

[0006] Since hematopoietic cells are relatively sensitive to shear it is reasonable to assume that high shear processes are unsuitable for *ex vivo* expansion (Nielsen, 1999). Thus, stirred-tank bioreactors (Pierson. *et al.*, 1996) or perfusion culture systems relying on external filters and high flow rate are unlikely to provide a high efficiency.

[0007] There are many promising approaches for the treatment of cancer with NK cell and NK-like T cell based immunotherapy. However, *ex vivo* expansion and activation of these effector cells under GMP compatible closed systems are crucial factors for facilitating frequent clinical use.

[0008] There have also been attempts by other investigators to expand and/or activate NK cells *ex vivo* and treatment options using purified/resting, short term or highly purified and long term activated NK cells are being investigated. These studies report NK cell infusions to be well tolerated and partially effective. Yet, 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. These disadvantages render such protocols suboptimal for GMP production and unfeasible for supporting large clinical studies.

[0009] A thorough evaluation of the above-mentioned reports shows a need for an automated method for optimized *ex vivo* expansion of NK cells. One problem with conventional flask-based cultures is related to scale, i.e. the cell number is limited due to the cumbersome handling of the flasks. Further, the risk of infections is quite high since the system is exposed to the environment each time the media are changed or the cells split. Further problems to be solved involve developing a method for the expansion of effector cells which is cost-effective, easy to handle and which includes well-defined cGMP quality components. Preferably the culture system is also free of animal products and feeder cells.

Summary of invention

[0010] The present invention relates to a closed system for large scale expansion and simultaneous activation of natural killer (NK) cells and NK-like T-cells, to be used as cell therapy products. In particular the invention discloses a method for large scale expansion and simultaneous activation of NK cells with the phenotype CD3⁻CD56⁺ and NK-like T cells with the phenotype CD3⁺CD56⁺, wherein the expanded cells exhibit increased cytotoxicity compared to cells cultured using conventional methods, such as flasks, under the same or similar conditions. The inventive method will be further disclosed in the description, non-limiting examples, and claims.

[0011] The inventors have investigated the feasibility of large-scale NK cell expansion using closed systems. Two different closed systems (cell culture bags and an automated bioreactor) have been evaluated in comparison to conventional cell culture flasks using PBMCs from healthy donors and patients with MM, with the aim of developing an automated GMP-compatible protocol that allows large-scale production of NK cells to be used for immunotherapy.

[0012] Concurrently, the inventors have successfully completed a safety evaluation of this cell product in an allogeneic setting in a phase I clinical trial with cancer patients (Barkholt L. *et al.*, 2009).

[0013] According to a first embodiment of the present invention, a method for large scale expansion and simultaneous activation of certain cell types is provided, wherein a closed cell culture system is used, and wherein the expanded cells obtained by this method exhibit an increased cytotoxicity as determined by *in vitro* cytotoxicity tests. Preferably the cell types are natural killer (NK) cells with the phenotype CD3⁻CD56⁺ and/or NK-like T cells with the phenotype CD3⁺CD56⁺.

[0014] According to a preferred embodiment the method comprises the steps of adding said cells to said closed system comprising a growth medium supplemented with serum, interleukin-2 (IL-2) and anti-CD3 antibodies; expanding said cells within said system with agitation and heating until: at least 35% of the expanded cell population comprises activated NK cells and NK-like T cells, and said expanded cells exhibit an increased cytotoxicity as determined by *in vitro* cytotoxicity tests.

[0015] According to one embodiment of the described method, the serum is selected from the group consisting of human serum and autologous serum. The medium is supplemented with about 50 to about 1500 U/ml IL-2, about 1 to about 50 ng/ml anti-CD3-antibodies and about 1 to about 40% serum.

[0016] The agitation and heating is, according to another embodiment, performed under the following conditions: a temperature of about 36 – 40 °C; a CO₂ concentration of about 4.7 – 5.1%; and gentle rocking at a rate and angle permitting the cells to adhere to the surface of the closed cell system. The rocking is performed at a rocking rate of about 4-8/min and at a rocking angle of about 4-8°.

[0017] The expansion is preferably performed until the total number of cells has expanded at least about 10-fold or until at least about 50 % of the expanded cell population comprises activated NK cells and NK-like T cells, respectively.

[0018] The cell sample used to expand said cells is preferably a sample of peripheral blood, cell lines, or cytokine stimulated peripheral blood. Most

preferably the cell sample is a sample of peripheral blood mononuclear cells (PBMC's).

[0019] According to one embodiment of the method, the cell sample is collected from a healthy subject.

[0020] According to another embodiment, the cell sample is collected from a tumour bearing subject, preferably having a tumour selected from the group consisting of haematological tumours and solid tumours.

[0021] According to another embodiment, the cells predominantly consist of natural killer (NK) cells with the phenotype CD3⁻CD56⁺

[0022] The concentration of cells initially added to the closed cell system is preferably about 0.5×10^6 to about 2×10^6 /ml growth medium.

[0023] In one embodiment of the invention, the method further comprises the step of adding per day a volume of the medium supplemented with serum and IL-2 corresponding to about 50% of the total culture volume and discarding about the same volume of growth medium/day from the closed cell system, wherein said step is performed when the total cell density has increased with at least about 50% of the initial cell density.

[0024] Preferably said step is performed when the total cell density has increased with at least about 300 % from the initial cell density, but wherein about 75 % of the total culture volume is exchanged.

[0025] Alternatively, said step is performed when the total cell density has increased with at least about 500% from the initial cell density, but wherein about 100 % of the total culture volume is exchanged.

[0026] According to yet another embodiment, freely combinable with one or more of the above disclosed embodiments, the cells are incubated for at least about 10 days.

[0027] According to one embodiment the closed cell system is a pre-sterile bag.

[0028] According to another embodiment, the closed cell system is a bioreactor.

[0029] Another embodiment of the present invention is a suspension of natural killer (NK) cells with the phenotype CD3⁻CD56⁺ and NK-like T cells with the phenotype CD3⁺CD56⁺ exhibiting increased cytotoxicity.

[0030] Preferably these cells exhibit an increased cytotoxicity, as determined by *in vitro* cytotoxicity tests, compared to cells expanded in flasks.

[0031] In a suspension of natural killer (NK) cells with the phenotype CD3⁻CD56⁺ and NK-like T cells with the phenotype CD3⁺CD56⁺ according to the invention, at least 35 %, and preferably at least 50 % of the expanded cell population, comprises activated NK cells.

[0032] In the context of the present invention a bioreactor is a re-usable automatic chamber-system that may refer to any device or system that supports a biologically active environment. Within the scope of the described invention the term bioreactor refers to a device or system meant to grow cells or tissues in the context of cell culture. A non-limiting example of a bioreactor system is the Wave bioreactor system 2/10 from GE-Healthcare, but a skilled person will appreciate that there are alternative bioreactors available from other sources, or will be able to construct a bioreactor in which the inventive method can be performed.

[0033] In the context of the present invention a closed system is a cell-growth chamber system consisting of a central cell culture bag wherein cells can be efficiently and quickly proliferated *ex vivo*, without any further steps of passage.

[0034] The term "cytotoxicity" means the quality of being toxic to cells. Examples of toxic agents include chemical substances, or immune cells such as cytotoxic lymphocytes such as cytotoxic T cells, NK cells and NK like T-cells. NK cells and NK like T-cells stand out with their high cytotoxic capacity.

[0035] A skilled person can determine the cytotoxicity using available methods. One way of determining if cells exhibit an increased cytotoxicity is to use the *in vitro* analysis of cell mediated cytotoxicity against K562 cells using the standard 4 hour ⁵¹Cr-release assay. Alternatively, the degranulation assay can be used. Both these methods are disclosed in the attached examples.

[0036] In the content of the present invention the terms "activation" and "activated NK cells" refer to NK cells that have received an activating signal. Activated NK cells are capable of killing cells with deficiencies in MHC class I expression.

[0037] The term "simultaneous activation" of NK cells and NK-like T cells means the cells are activated substantially simultaneously, preferably in the same cell culture.

[0038] Given their strong cytolytic activity and the potential for auto-reactivity, natural killer (NK) cell activity is tightly regulated. In order to kill cells with a missing or abnormal MHC class I expression the NK cells need to be activated. NK cells must receive an activating signal which can come in a variety of forms, the most important of which are cytokines, Fc-receptors, activating and inhibitory receptors.

[0039] In the content of the present invention the term "cell expansion" relates to the culturing of cells that go through a series of cell division steps and thus expand the number of cells present in the culture. Thus, the term "NK cell expansion" relates to the culturing of NK cells that go through a series of cell division steps and thus expand the number of cells present in the culture. The term "expanded NK cells" relates to NK cells obtained through NK cell expansion. More specifically, in one embodiment the term "expanded NK cells" relates to a polyclonal group of chronically activated CD3⁻ CD56⁺ cells as well as NK-like T cells with the phenotype CD3⁺CD56⁺, expanded in a specific cGMP grade environment and cGMP grade medium.

[0040] In the context of the present invention the term “effector cell” relates to a cell that performs a specific function in response to a stimulus such as cells in the immune system. In one embodiment effector cells are a type of lymphocytes that have cytotoxic capacity (i.e. they induce death of other cells). *Another embodiment are lymphocytes actively engaged in secreting antibodies.* Non-limiting examples of effector cells are NK cells, T cells and NK-like T cells.

Brief description of drawings

[0041] Figures 1 A-D are diagrams showing a comparison of fold expansion in flask and bags (1A) and in flask and bioreactor (1B) and final product purity in flask and bags (1C) and in flask and bioreactor (1D).

[0042] Figures 2 A-C are diagrams showing a comparison of phenotype (A and B) and cytotoxic activity (C) of the expanded cells following different expansion protocols.

[0043] Figures 3 A-D are diagrams showing the expansion of cell cultures initiated in the bioreactor in comparison to flasks.

[0044] Figures 4 A-B are diagrams showing the functional comparison of cells expanded directly in bioreactors and flasks, respectively.

[0045] Figure 5 shows the phenotypic comparison of NK cells expanded directly in bioreactor and flasks, respectively.

Detailed description of the invention

[0046] Before the present invention is described, it is to be understood that the terminology employed herein is used for the purpose of describing particular embodiments only and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

[0047] If nothing else is defined, any terms and scientific terminology used herein are intended to have the meanings commonly understood by those skilled in the art to which this invention pertains.

[0048] It must be noted that, as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise.

[0049] Also, the term "about" is used to indicate a deviation of +/- 2 % of the given value, preferably +/- 5 %, and most preferably +/- 10 % of the numeric values, where applicable.

[0050] Ex vivo expansion and reinfusion of natural killer (NK) cells to patients afflicted with a malignant disease offers a new and potentially interesting therapeutic approach to combat such a disease. A prerequisite for this is the possibility to expand and use NK cells that meet the demands of clinical use. The present inventors have surprisingly been able to expand NK cells in a large scale for clinical applications. Thus, a GMP compliant automated closed culture system for preparation of large scale NK and NK-like T cell enriched effector populations that is applicable in clinical settings is provided.

[0051] As demonstrated in the examples below, the inventive method expands and activates NK cells having a satisfactory cytotoxic capacity (i.e. they induce death of other cells). The cytotoxicity of NK cells towards other cells can easily be measured, for example, by traditional cell counting before and after exposure to active NK cells. Such methods are well known to a person skilled in the art.

Examples

1. Ex vivo expansion of NK cells and NK-like T cells from peripheral blood

Materials & Methods

Sampling and isolation of blood mononuclear cells (PBMCs)

[0052] Buffy coats, peripheral blood and apheresis products were obtained from healthy donors or MM patients via the blood bank at the Karolinska University Hospital, Huddinge. The experimental protocol was approved by the local research ethics committee.

[0053] The peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation, using Lymphoprep (Nyegaard, Oslo, Norway). PBMCs were washed twice with phosphate-buffered saline (PBS) (Gibco, Grand Island, NY, USA), and cell viability was assessed by trypan blue exclusion.

Growth medium and cell counts

[0054] 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 rhIL-2 (Proleukin^R, Novartis Pharmaceuticals, East Hanover, NJ, USA) was used as growth medium. At the beginning of the culture, the medium was further supplemented with monoclonal anti-CD3 antibody (Orthoclone OKT-3, Ortho Biotech, Raritan, NJ, USA) at a final concentration of 10 ng/ml. Total cell numbers were assessed by staining cells with Trypan blue dye on days 0, 5–6, 9–10, 14–15, and 21 of culture. The final products were evaluated for safety, purity, and identity (cell viability and phenotype). Absolute cell counts were calculated by multiplying the total number of cells with the percentage of these subsets determined by flow cytometry (BD FACS Calibur; BD Biosciences, San Jose, CA, USA).

Expansion in cell culture flasks

[0055] The culture conditions for the expansion of cytotoxic cells in cell culture flasks have previously been optimized on PBMCs from healthy individuals (Carlens S. *et al.*, 2001). In short, PBMCs were initially cultured in T25 flasks (TPP, Trasadingen, Switzerland) at a concentration of 0.5×10^6 cells/ml. After five 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 (Heiskala M. *et al.*, 1987), the cells were transferred to bigger flasks (T75 or T150: TPP, Trasadingen, Switzerland) or passaged into multiple flasks when necessary. During medium replenishment, the cell concentration was adjusted to 0.5×10^5 cells/ml until day 10 and to 1×10^6

cells/ml after day 10. Occasionally, certain portions of the cells were frozen in order to keep the number of flasks manageable.

Expansion in the Wave Bioreactor System

[0056] The Wave Bioreactor is a cell culture system where the cells are grown inside a temperature and CO₂ controlled disposable, sterile bag that is placed on a rocking heated platform. The inventors have used a Wave Bioreactor System2/10 (GE Healthcare, Somerset, NJ, USA). Our previous experience with this system has shown suboptimal efficiency when started in low volumes and low cell numbers. Yet, the amount of cells in peripheral blood samples of donors does not allow directly starting in the bioreactor. Therefore, in initial optimization experiments the inventors started the cultures in flasks and transferred the cells into the bioreactor around Day 5 when sufficient number of cells was reached. The bioreactor cultures at this day were started with 2×10^6 cells/ml in 800 ml.

[0057] In the final validation experiments, a whole unit of peripheral blood, or apheresis product from the donors was obtained and the cultures were initiated directly in bioreactors from day 0. The settings for the bioreactor were as follows at all times: Temperature 37°C, CO₂: 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 500 ml of medium per day (50ml/shot). When the cells reached a density of 6×10^6 cells/ml, the feeding was increased to 750 ml/day and after 1×10^7 cells/ml, to 1000 ml/day.

Expansion in Vuelife™ bags

[0058] Vuelife™ (American Fluoroseal Corporation, MD, USA) is a sterile cell culture bag made of fluorinated ethylene-propylene which is 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% CO₂. Fresh medium was added every 2-3 days to adjust the concentration to 1x10⁶ cells/ml until day 10 and of 2x10⁶ cells/ml after day 10. Cells were split to bigger bags when necessary.

2. Analysis of lymphocyte subsets and phenotyping by flow cytometry

Materials & methods

[0059] The cell phenotype and percentage of subpopulations was analyzed by flow cytometry on days 0, 5-6, 9-10, 14-15 and 20 of culture using standard procedures with fluorochrome conjugated mAbs against CD3, CD14, CD19, CD45 and CD56.

[0060] In initial optimization experiments, Day 0 cells and day 20 cells from each expansion condition were subjected to a more detailed immunophenotypic analysis. To avoid inter-acquisition variability, all frozen samples were thawed simultaneously for a detailed phenotypic characterization of the NK cell subset by flow cytometry. This 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), NKp46 (BAB281), from Beckman Coulter Inc., Fullerton, CA, USA; NKG2A (131411), NKG2C (134591), KIR2DL1 (143211), KIR2DL3 (180701) from R&D Systems, Minneapolis, MN, USA.

[0061] 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% PFA prior to data acquisition. Data acquisition was done on a FACSCalibur (BD) or CyFlow ML (Partec GmbH, Munster, Germany)

and data were analysed with CellQuest or FloMax software. For the analysis, appropriate SSC/FSC gates around the CD14⁻CD19⁻ lymphocyte population were used. 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.

[0062] 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, the inventors calculated MFI ratios (MFI_{day20}/MFI_{day0}) for each receptor. When the MFI for day 20 samples was higher than for day 0, the MFI ratio was higher than 1, which indicated the relative extent of up regulation in that receptor. Likewise, an MFI ratio below 1 was interpreted as down regulation in the expression of that receptor.

3. Evaluation of cell mediated cytotoxicity

Materials & methods

[0063] The cytotoxic capacity of the final product was evaluated *in vitro* with a standard 4 hour ⁵¹Cr-release assay against K562 cells (Aktas E. *et al.*, 2009; Alter, G. *et al.*, 2004). In short, K562 target cells were labeled with 100 μ Ci of ⁵¹Cr for 1 hour at 37°C, washed twice with PBS, and resuspended in RPMI medium. A total of 3×10^4 target cells in 100 μ l RPMI medium was placed in triplicates into V-bottomed 96-well plates and incubated for 4 hours with 100 μ l of effector cells at appropriate concentrations to obtain effector:target ratios from 1:3 to 10:1. Aliquots of supernatants were counted using a Packard Cobra Auto-Gamma 5000 Series Counting System (Meridien, CT, USA). The percentage specific ⁵¹Cr release was calculated according to the formula: percent specific release = $[(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})] \times 100$.

Analysis of NK cell degranulation

[0064] The expansion products were co-incubated with K562 target cells at a ratio of 1:1 in a final volume of 200 μ l in round-bottomed 96-well plates at 37°C

and 5% CO₂ 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 coincubation, Monensin (GolgiStop, Becton Dickinson) was added at a 1:100 dilution. Surface staining was done by incubating cells with anti-CD3 and anti-CD56 mAbs for 30 min on ice. The cells were then washed, resuspended in PBS and immediately analyzed by flow cytometry.

4. Statistical analysis

[0065] Data analysis, preparation of graphs and statistical comparisons were done with the GraphPad Prism Software (GraphPad Software Inc. CA, USA).

RESULTS

Evaluation of cell culture bags and bioreactor for NK cell expansion

[0066] In an attempt to use a closed culture system for the expansion of NK cells, the inventors have initially compared cell culture bags with flasks using PBMCs from five healthy donors. 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 flask yielded a mean of 1100-fold expansion. The NK expansion in bags appeared impressive when compared to flask especially in three out of five donors. However, when the percentage of NK cells in the final product was taken into account (Figure 1C), it was seen that expansion in bags does not correlate with expansion in flasks and might 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%.

[0067] In search for a closed expansion system that results in comparable and correlating yield with flasks, the inventors have evaluated the use of an automated bioreactor system in comparison with expansion in flasks, using PBMCs from five healthy donors. The inventors have observed 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 correlating with flask expansion (Figure 1D). The end product of the expansion protocols had a median of 38% NK cells in the bioreactor while there was 44% NK cells and 16% in flasks.

[0068] When compared with the previously mentioned results from bags, the percentage of NK cells in the bioreactor was higher than that in bags, even despite the fact that this set of donors had yielded a worse NK cell purity in flask expansion. Taken together, the above results suggest that the fold expansion of NK cells is better in bags, whereas the purity is slightly higher in the bioreactor.

[0069] Due to the use of different donor sets and the high inter-individual variability in NK cell expansion, it was not possible to directly see the relative efficiencies of the bag and bioreactor systems. Owing to the fact that two of the five donors in these different groups were actually the same individuals (Donors 1 and 2), the inventors had a chance to directly compare 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%) when compared to flask (74%) which was higher than bags (47%). In the case of NK-like T cells, the percentages were very close to 5% in all three systems.

[0070] 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 an inter-individual variability as usual, the changes in receptor expression levels in the different final products were very similar (Figure 2A and 2B). It can be clearly seen that the extent of up- or down-regulation in a given receptor is the same no matter which culture protocol is used.

[0071] In order to clarify if the NK cells in the final products of different systems retain the same activation status and show comparable cytotoxicity, the inventors have evaluated the cytotoxic activity of the final products against the NK-sensitive cell line K562 (Figure 2C). The inventors have observed no significant difference between the cytotoxic activities of cell preparations expanded under different conditions. These results ensure that the methods in question are all sufficient to produce cell preparations with increased cytotoxic capacity.

Validation of NK cell expansion process in the bioreactor

[0072] After demonstrating the feasibility of using the bioreactor for the expansion process, the inventors have continued with validation of the expansion process in 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. For comparison purposes, the starting material, PBMCs, were expanded in parallel using flasks. Figure 3 presents the expansion curves of bulk cells and lymphocyte subpopulations for all donors in bioreactor and flasks. The total number of cells reached was much higher in the bioreactor expansions, with an average purity of 37.5% while the purity was 43% in flasks. Although the NK cell purity was slightly lower in bioreactors, the final number of NK cells reached is impressive enough to facilitate clinical use of expanded NK cells in cancer immunotherapy settings.

NK cells expanded in the bioreactor display higher cytotoxic capacity

In previous experiments, where the expansion was initiated in flasks and subsequently transferred to bioreactors, the inventors had detected no difference in cytotoxic capacity or the phenotype of final products when compared to flask expansions. Interestingly, the inventors have observed that when expansion was initiated directly in bioreactors, the cytotoxic activity of the final product against K562 cells was remarkably higher when compared to the final product of flask expansions in 3 out of 4 donors (Figure 4A). In order to better look into this

phenomenon, the inventors have carried out degranulation assays against K562 cells, and measured the percentage of degranulating cells in each lymphocyte subpopulation (Figure 4B). Surprisingly, the inventors have observed that the extent of degranulation observed in the NK cell fraction from bioreactor expansions was significantly higher than NK cells from flask expansions in all 4 donors. Likewise, the degranulation of the NK-like T cell fraction was significantly higher in 3 out of 4 donors. Taken together, these results suggest that the expansion process carried out in the bioreactor performs better in terms of elevating the cytotoxic capacity of NK cells.

In an attempt to explain the difference in cytotoxic capacity of the final products from bioreactor and flask expansions, the inventors have performed detailed phenotypic characterization of the NK cells using multi-color flow cytometry. Figure 5A shows the expression of various surface receptors on NK cells from bioreactor and flask expansions and summarizes the results from all donors and gives an overall picture of the phenotypic comparison between bioreactor and flask expansions. Overall, the NK cells in the final product look similar regardless of which expansion system is used, with slight but noticeable differences in the expression levels of CD11b, NKG2D and NKp44. Up regulation of NKp44 is one of the key factors in elevated cytotoxic capacity.

[0073] This study comparatively evaluates the use of cell culture flasks, bags and a bioreactor for the *ex vivo* expansion of NK and NK-like T cells originating from bulk PBMCs of human donors in an attempt to investigate the feasibility of producing GMP-quality effector cells in closed automated systems.

[0074] The inventors have previously reported a GMP-compliant culture medium which promotes the selective enrichment of activated NK cells in cell culture flasks (Carlens S. *et al.*, 2001). Here, the inventors present the last step of optimizing a GMP quality automated closed culture system for preparation of large scale NK cell enriched effector populations that is applicable in clinical settings (Miller JS. *et al.*,

1994; Luhm J. *et al.*, 2002; Klingemann HG and Martinson J., 2004; Koehl U. *et al.*, 2005) and/or use of feeder cell lines (Ishikawa E. *et al.*, 2004; Torelli GF, *et al.*, 2005) have been widely used for NK cell expansion in previous reports. In this study, the inventors do not utilize any separation steps but rather use bulk PBMCs for culture which results in a cell population enriched in NK cells that is distinct from LAK (Ramsdell FJ and Golub SH., 1987) and cytokine-induced killer (CIK) cells (Chan JK. *et al.*, 2006; Lu PH and Negrin RS., 1994) both in terms of NK cell content and anti-tumor activity (Bordignon C. *et al.*, 1999).

[0075] The inventors have demonstrated that both bags and the bioreactor systems provide 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 amount of NK cells with higher purity and moreover has much less of T cells in the final product when compared to bags.

[0076] Surprisingly, the inventors have observed that NK cell activity is significantly higher in expansion products from bioreactors, as compared to those from flasks. Correlation of receptor expression levels with the response of NK cells against K562 has revealed that CD132, CD25, CD57 and NKG2C expression levels were inversely correlated with the response while expression levels of NKp30, NKp44 and NKp46 were directly correlated. 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 the expression of its ligand HLA-E (Khalil-Daher I. *et al.*, 1999). Statistical analysis of receptors correlating with NK cell response has revealed that NKp44 both correlates positively with response, and is expressed at a significantly higher level in bioreactor products when compared to flask expansions. This can, at least in part, explain the observation of high cytotoxic capacity of the bioreactor products. Unlike the other NCRs, NKp44 (Vitale M. *et al.*, 1998) is expressed exclusively on

activated NK cells and is upregulated after *in vitro* IL-2 stimulation (Biassoni R. *et al.*, 2002). Therefore, in this case, it might be presenting as a surrogate marker of how well the IL-2 in the culture is being used and what the extent of activation in the NK cell population is. 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.

[0077] Regarding practicalities, the inventors have observed that all systems in question have 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 cells. Initiation of culture in flasks does not demand a high number of cells but as the cells have to be kept within a certain concentration (Heiskala M. *et al.*, 1987), splitting into new flasks during expansion ends up in an impossible number of flasks to handle. Culturing can also be initiated with few cells in small bags and results in good expansion but the purity of NK cells are lower than the other systems and the cells still need to be split into more than one bag. Yet, expansion in bags can be easily 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 the bioreactor needs extra investment for purchase of the machine and can be used for one expansion at a time.

[0078] The bioreactor is the most practical method, as it requires minimum hands-on time. However, the initiation of this system requires many cells and expansion rate is lower. The continuous rocking motion of the bioreactor ensures a dynamic and homogenous culture environment that provides many advantages such as uniform culture conditions, ease of sampling and better quality of control processes like the 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 cells more concentrated in the bioreactor. This avoids any waste of media or additional components, which dramatically decreases the overall cost of the process. Using the average expansion curves for each protocol, the inventors have estimated that, for acquiring similar number of NK cells, the bioreactor system uses approximately 1/10 times the media components used for bags and 1/25 times the media components used for flasks. The consumption of media and cytokines is even more (around 2.5 times of bags) in flasks.

[0079] 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 (Nielsen LK., 1999). Thus, stirred-tank bioreactors (Pierson BA. *et al.*, 1996) 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 (Nielsen LK., 1999) and the bioreactor system used in this study can meet these expectations.

[0080] Another factor to consider carefully is the material used in the culture environment. Only few materials can support the growth of hematopoietic cells efficiently and factors such as cleaning, sterilization and reuse significantly affect their performance (Laluppa JA. *et al.*, 1997). 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.

[0081] One difference observed was a slightly lower viability of cells in the final products of the bioreactor and bags cultures when compared to flasks. This is most probably due to the fact that the dead cells are being continuously washed away in the flask culture every 2-3 days when the medium is being changed while no such

procedure is involved in the closed systems. Yet, it is always possible to utilize a GMP quality washing step at the end of the culture, prior to administration.

[0082] All systems in question have certain practical advantages and disadvantages. Expansion of NK cells 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 cells. Culturing in flasks can be initiated with very low number of cells but as the cells have to be kept within a certain concentration, splitting into new flasks during expansion ends up in an impossible number of flasks to handle.

[0083] Culturing can also be initiated with few cells in small bags and results in very good expansion but the purity of NK and NK-like T cells are lower than the other systems and the cells still need to be split into more than one bag. Expansion in bags can be easily 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 the bioreactor can be used for one expansion at a time.

[0084] In conclusion, the results presented here clearly demonstrate that large amounts of highly activated effector cells for possible use in adaptive immunotherapy settings can be produced in a closed culture system under GMP conditions.

[0085] Although particular embodiments have been disclosed herein in detail, this has been done by way of example for purposes of illustration only, and is not intended to be limiting with respect to the scope of the appended claims that follow. In particular, it is contemplated by the inventors that various substitutions, alterations, and modifications may be made to the invention without departing from the spirit and scope of the invention as defined by the claims.

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US 10/242,7881

CLAIMS

1. A method for large scale expansion and simultaneous activation of natural killer (NK) cells with the phenotype CD3⁻CD56⁺ and NK-like T cells with the phenotype CD3⁺CD56⁺ wherein said expansion and activation is performed in a closed system, and the expanded cells obtained exhibit an increased cytotoxicity as determined by an *in vitro* cytotoxicity test.
2. The method of claim 1, wherein said method comprises the steps:
 - adding said cells to said closed system comprising a growth medium supplemented with serum, interleukin-2 (IL-2) and anti-CD3 antibodies;
 - expanding said cells within said system with agitation and heating until at least 35% of the expanded cell population comprises activated NK cells and NK-like T cells, and said expanded cells exhibit an increased cytotoxicity as determined by *in vitro* cytotoxicity tests.
3. The method according to claim 2, wherein the agitation and heating is performed under the following conditions: a temperature of about 36 to about 40°C; a CO₂ concentration of about 4.7 to about 5.1%; and gentle rocking at a rate and angle permitting the cells to adhere to the surface of the closed cell system.
4. The method according to claim 2, wherein the rocking is performed at a rocking rate of about 4 - 8/min.
5. The method according to claim 2, wherein the rocking is performed at a rocking angle of about 4 - 8 °.
6. The method according to claim 2, wherein the expansion is performed until the total number of cells has expanded at least about 10-fold.
7. The method according to claim 2, wherein the expansion is performed until at least about 50 % of the expanded cell population comprises activated NK cells and NK-like T cells, respectively.

8. The method according to any one of claim 1 – 7, wherein the cell sample is a sample of peripheral blood, cell lines, or cytokine stimulated peripheral blood.
9. The method according to claim 8, wherein the cell sample is a sample of peripheral blood mononuclear cells (PBMC's).
10. The method according to claim 2, wherein the concentration of cells initially added to the closed cell system is about 0.5×10^6 to about 2×10^6 /ml growth medium.
11. The method according to any one of claims 1 – 10, further comprising the step of adding per day a volume of the medium supplemented with serum and IL-2 corresponding to about 50% of the total culture volume and discarding about the same volume of growth medium/day from the closed cell system and said step is performed when the total cell density has increased with at least about 50% of the initial cell density.
12. The method according to claim 11 comprising repeating the step disclosed in claim 11 when the cell density has increased with at least about 300 % from the initial cell density but adding and discarding about 75 % of the total culture volume.
13. The method according to claim 11, comprising repeating the step disclosed in claim 11 when the cell density has increased with a least about 500% from the initial cell density but adding and discarding about 100 % of the total culture volume.
14. The method according to any one of the preceding claims, wherein the cells are incubated for at least about 10 days.
15. The method according to any one of the preceding claims, wherein the closed cell system is a pre-sterile bag.
16. The method according to any one of the preceding claims, wherein the closed cell system is a bioreactor.

17. The method according to claim 2, wherein the serum is selected from the group consisting of human serum and autologous serum.
18. The method according to claim 2, wherein the medium is supplemented with about 50 to about 1500 U/ml IL-2, about 1 to about 50 ng/ml anti-CD3-antibodies and about 1 to about 40% serum.
19. The method according to any one of the preceding claims, wherein the cell sample is collected from a healthy subject.
20. The method according to any one of the preceding claims, wherein the cell sample is collected from a tumour bearing subject.
21. The method according to claim 20, wherein the tumour is selected from the group consisting of haematological tumours and solid tumours.
22. The method according to any one of the preceding claims, wherein the cells predominantly consist of natural killer (NK) cells with the phenotype CD3⁻CD56⁺
23. A suspension of natural killer (NK) cells with the phenotype CD3⁻CD56⁺ and NK-like T cells with the phenotype CD3⁺CD56⁺ obtainable by a method according to any one of claims 1 – 21.
24. A suspension of natural killer (NK) cells with the phenotype CD3⁻CD56⁺ and NK cell like T cells with the phenotype CD3⁺CD56⁺ wherein said cells exhibit an increased cytotoxicity as determined by an *in vitro* cytotoxicity test, when compared to cells expanded in flasks.
25. A suspension of natural killer (NK) cells with the phenotype CD3⁻CD56⁺ and NK-like T cells with the phenotype CD3⁺CD56⁺ wherein at least 35 %, preferably at least 50 % of the expanded cell population comprises activated NK cells.

Figure 1A

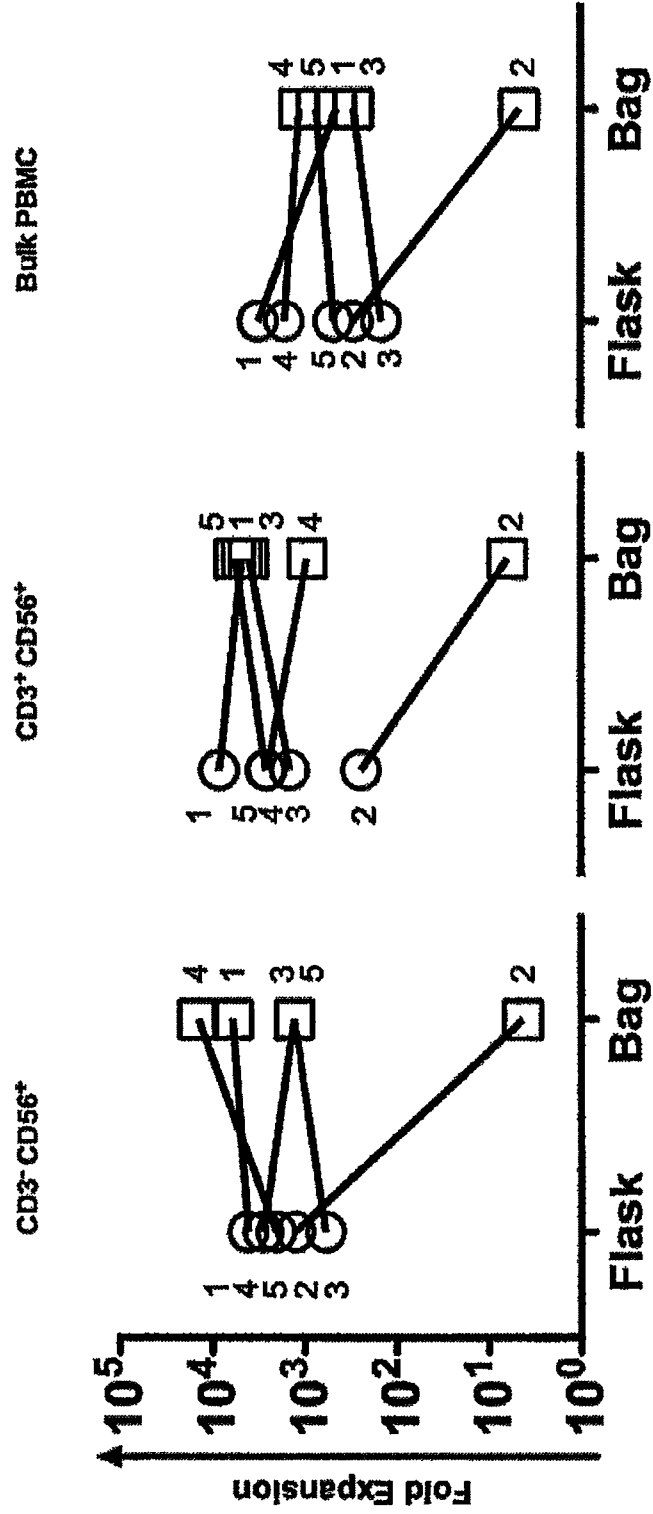


Figure 1B

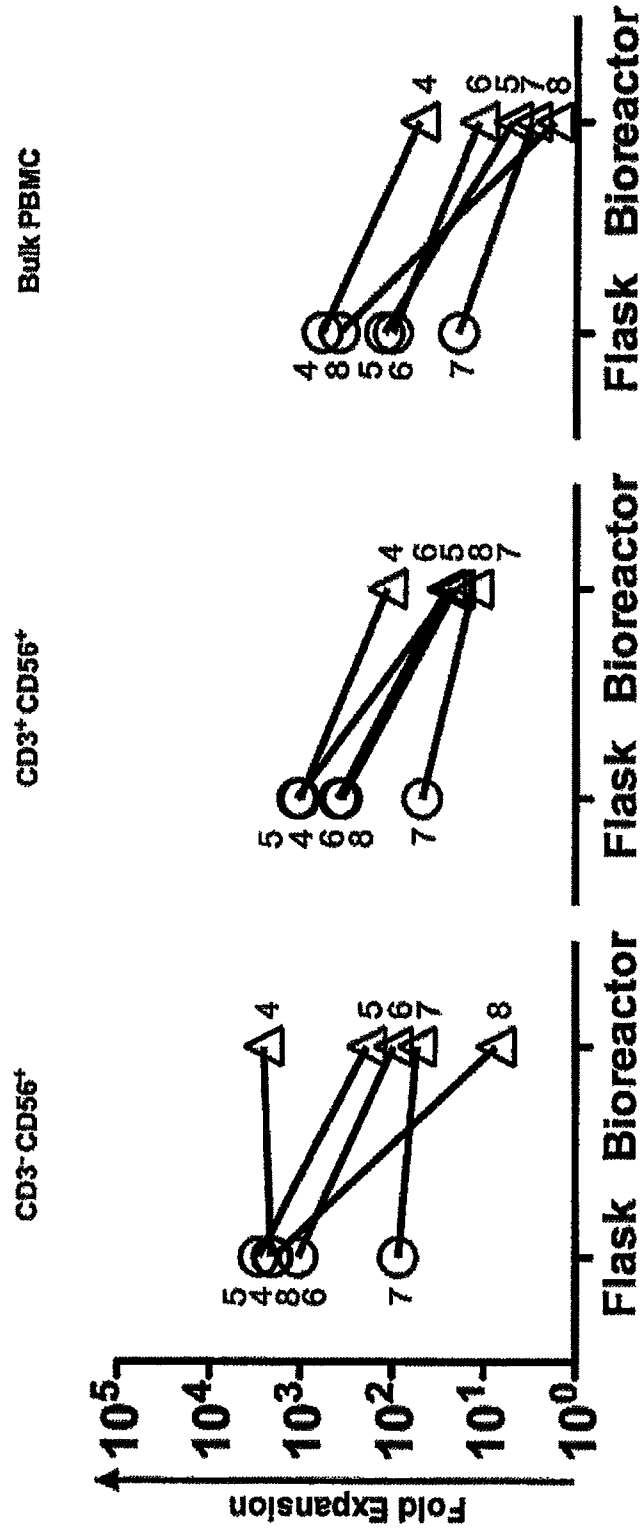


Figure 1C

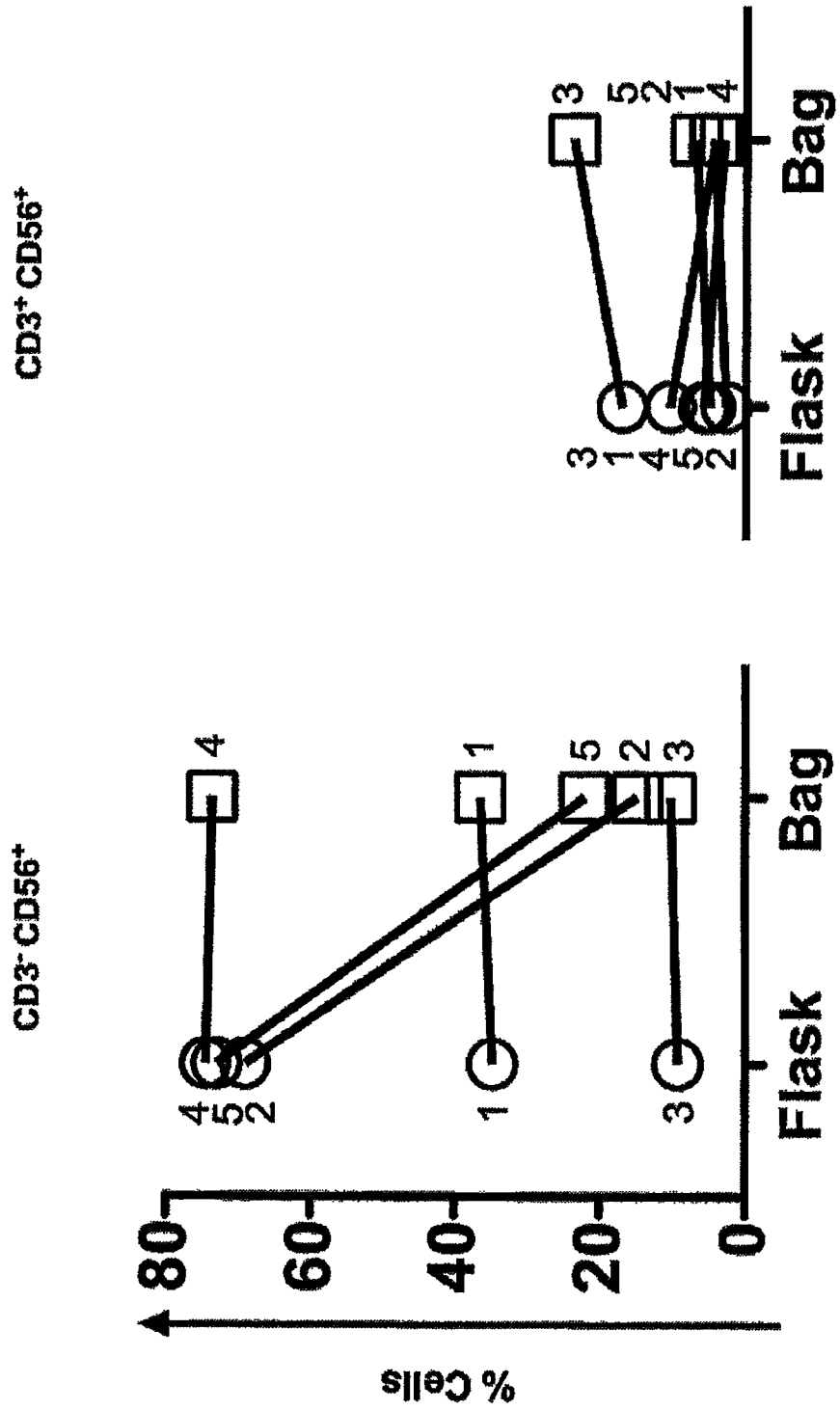


Figure 1D

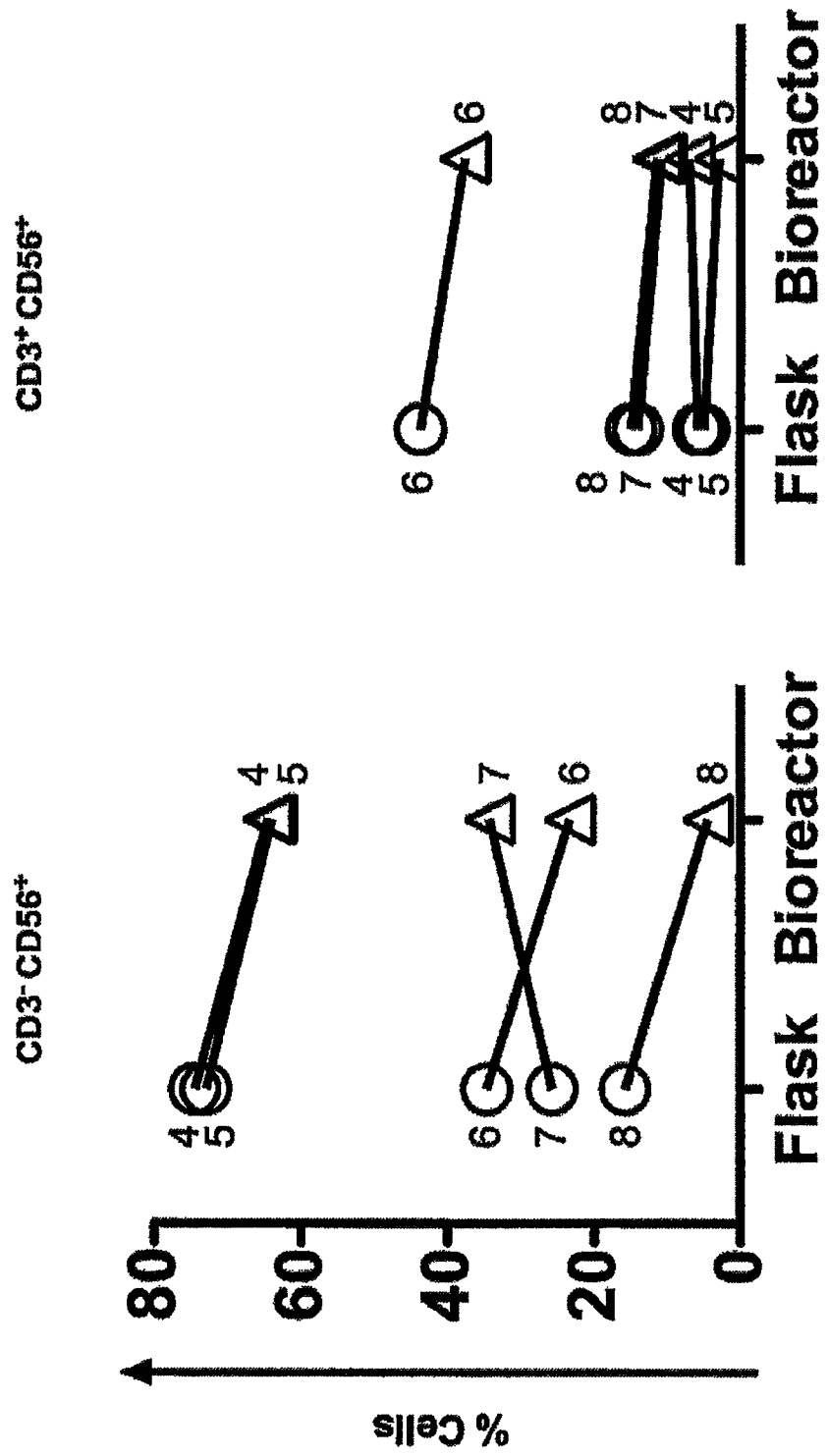


Figure 2A

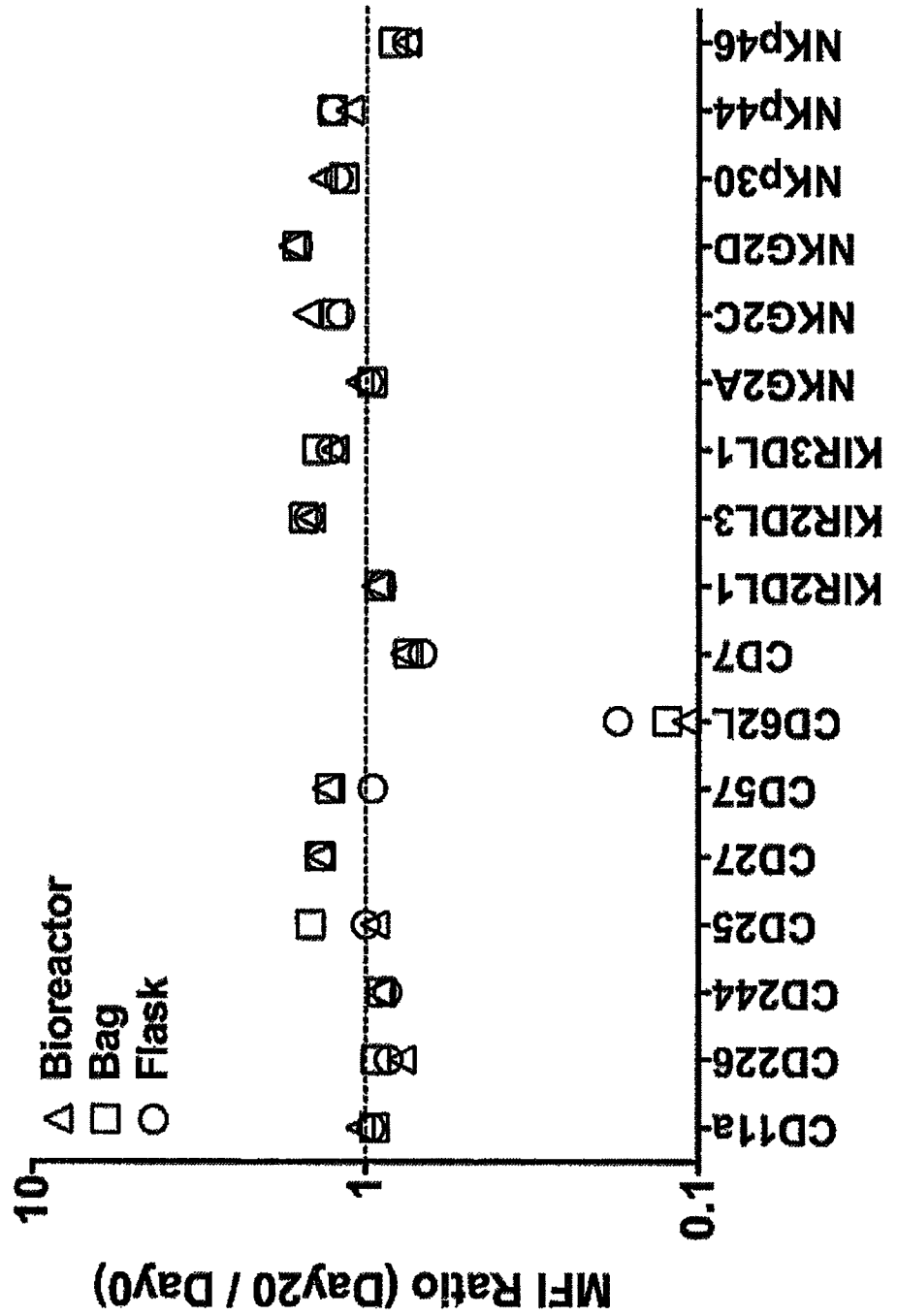


Figure 2B

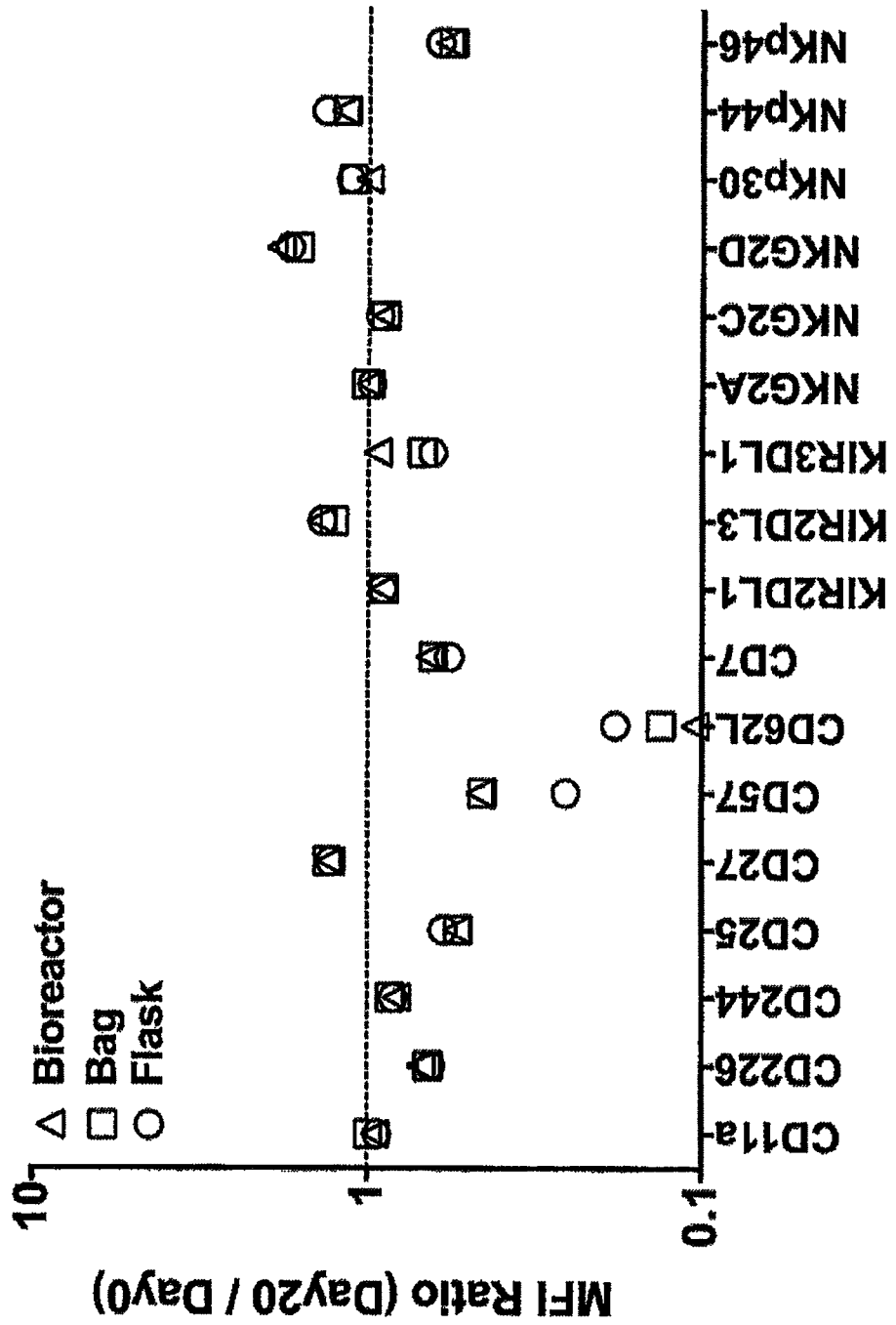


Figure 2C

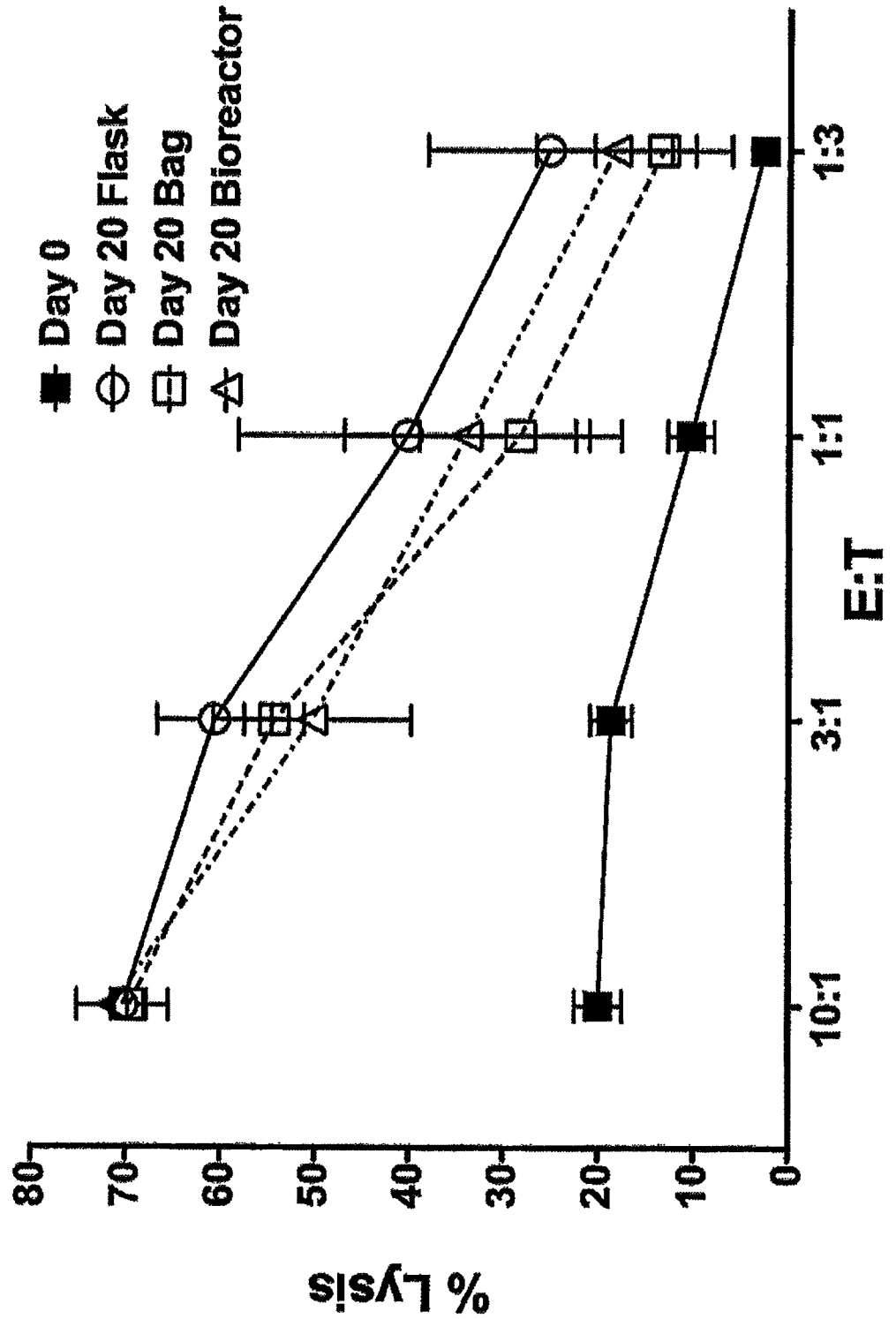


Figure 3A

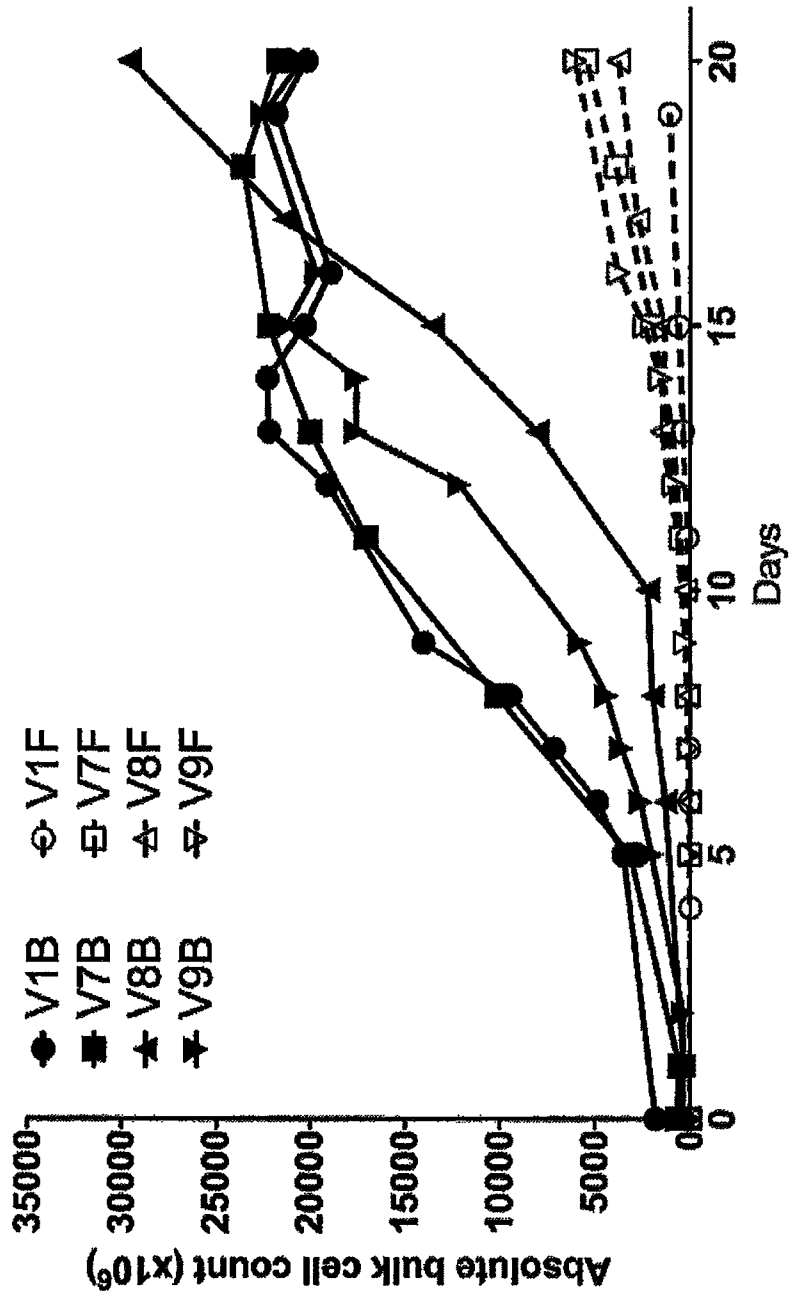


Figure 3B

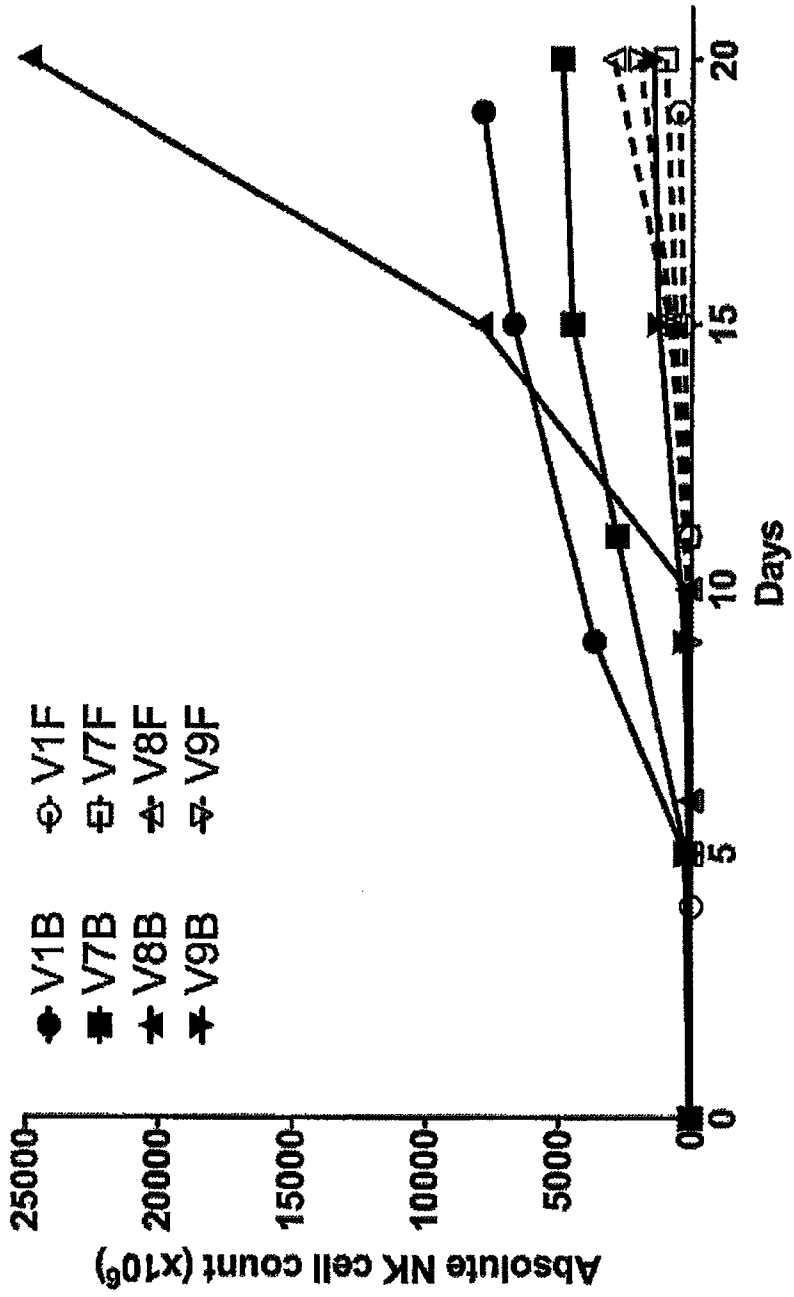


Figure 3C

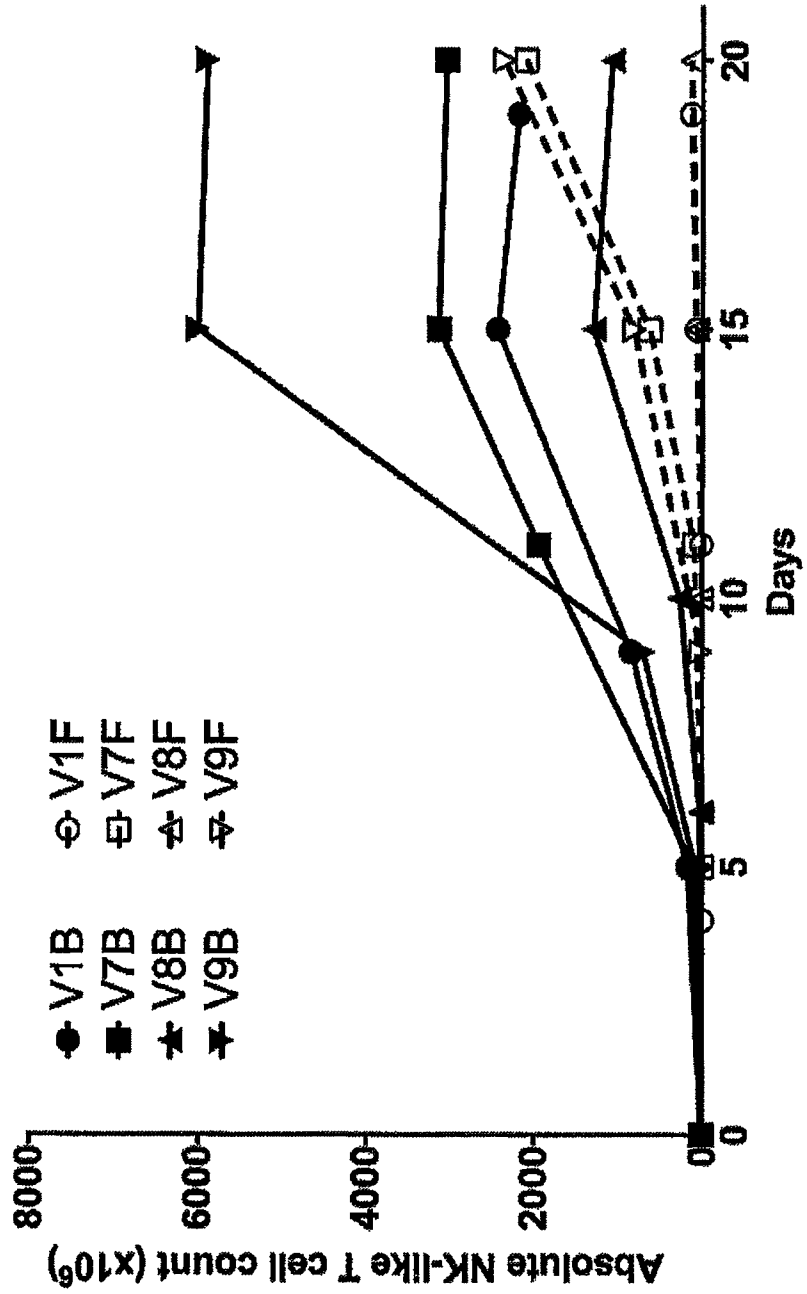


Figure 3D

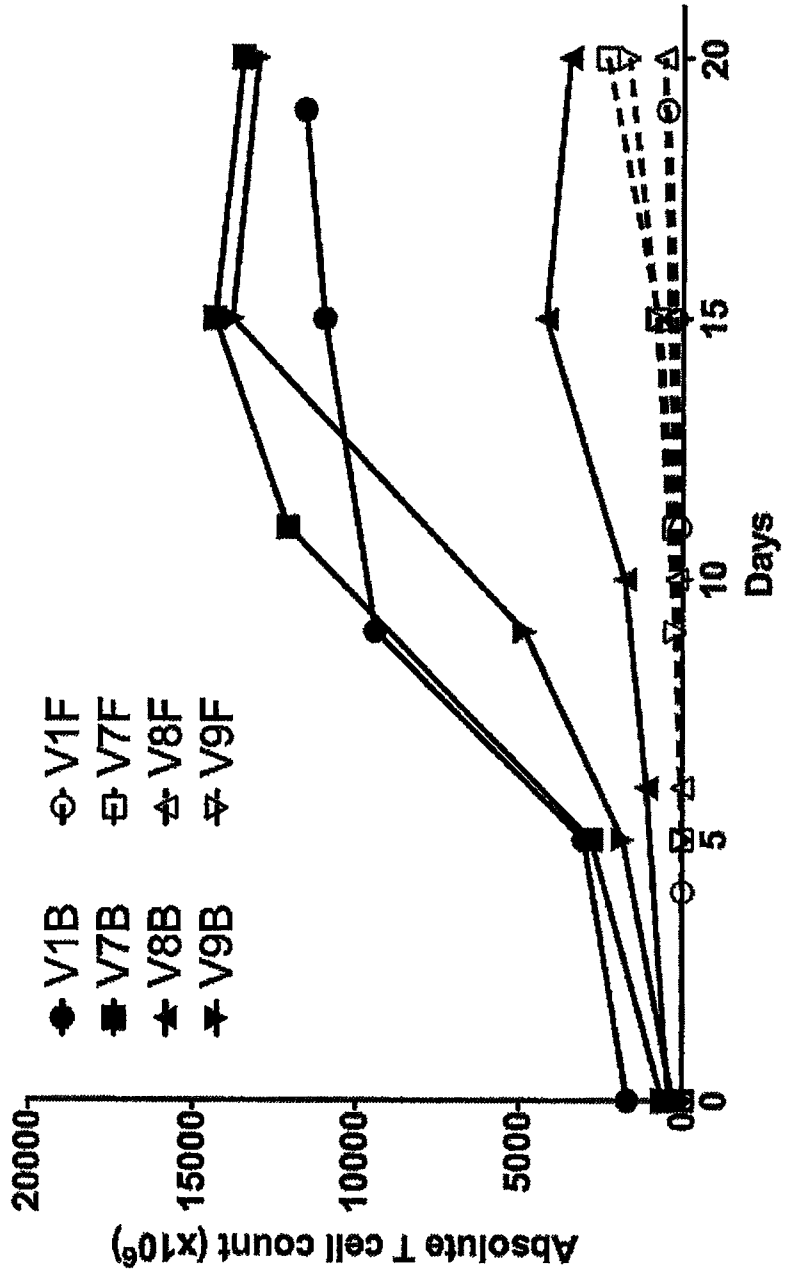


Figure 4A

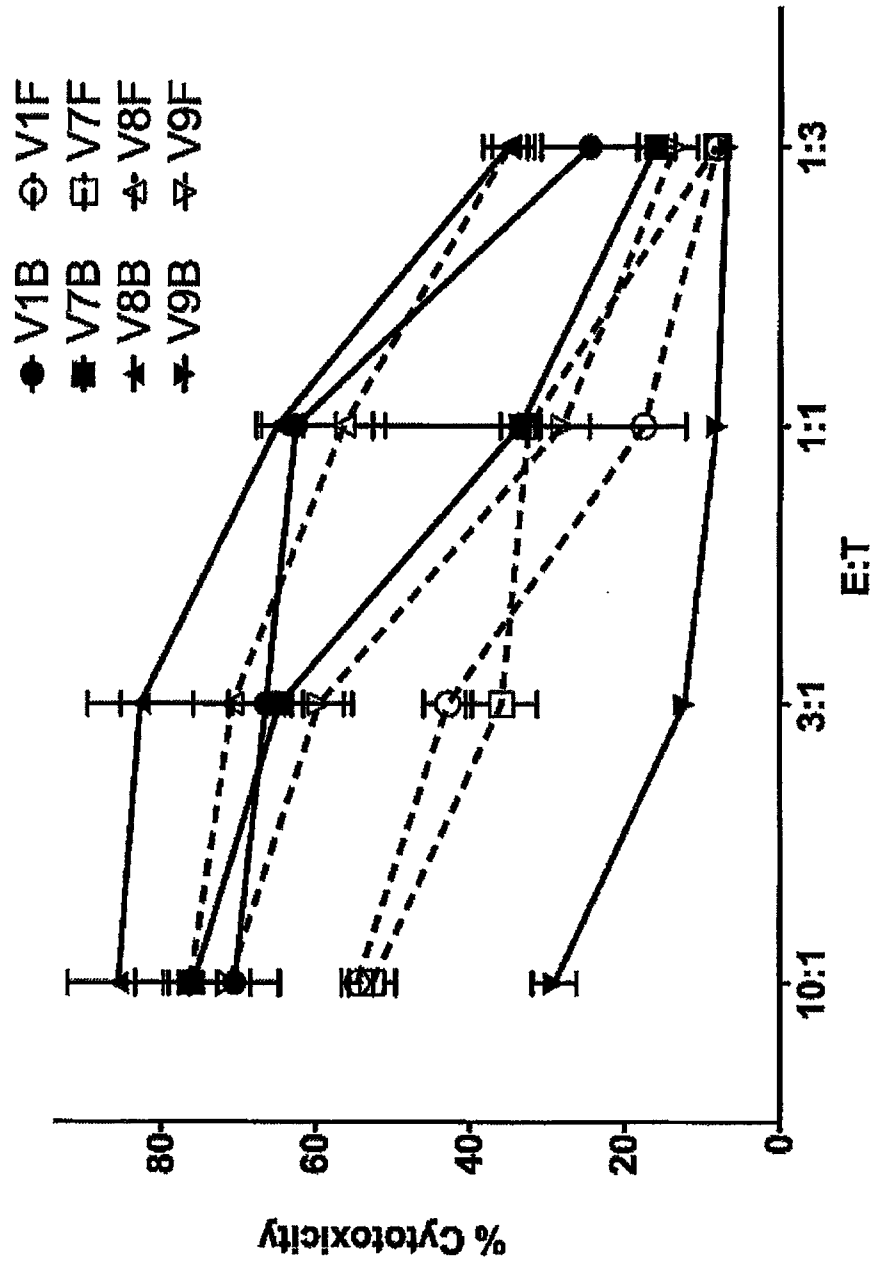


Figure 4B

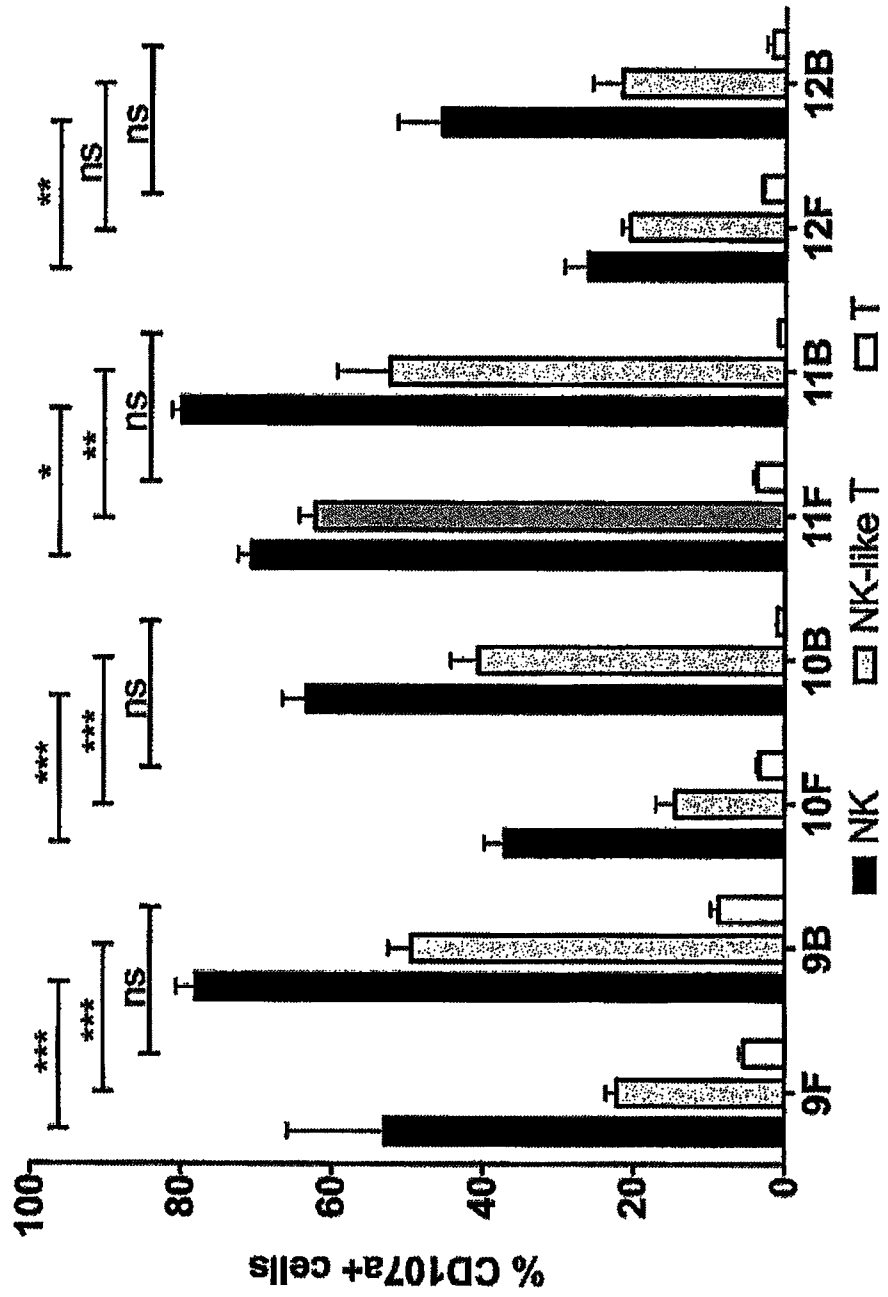
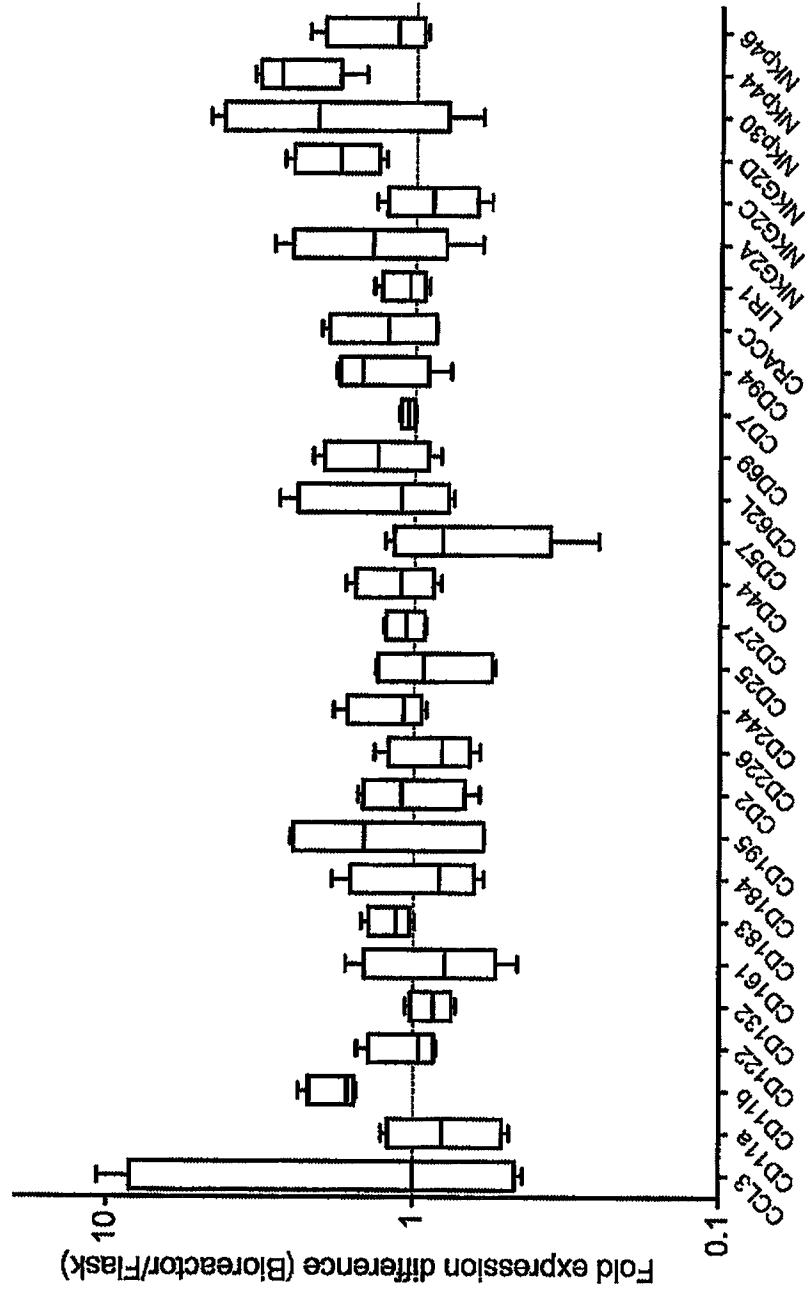


Figure 5



INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2010/050333

A. CLASSIFICATION OF SUBJECT MATTER

IPC: see extra sheet

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC:C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE, DK, FI, NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, PAJ, WPI data, BIOSIS, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Carlens et al. "A new method for in vitro expansion of cytotoxic human CD3-CD56+ natural killer cells" Human immunology, 2001, vol 62, no 10, pages 1092-1098, ISSN 0198-8859.; abstract; pages 1094-1097	25
Y	--	1-24
Y	Meehan et al. "Development of a clinical model for ex vivo expansion of multiple populations of effector cells for adoptive cellular therapy", Cytotherapy, 2008, vol 10, no 1, pages 30-37, ISSN 1465-3249.; abstract; pages 31-34	1-24
Y	Guyen et al. "Expansion of natural killer (NK) and natural killer-like T (NKT)-cell populations derived from patients with B-chronic lymphocytic leukemia (B-CLL): A potential source for cellular immunotherapy." Leukemia, 2003, vol 17, no 10, pages 1973-1980, ISSN 0887-6924.; abstract; pages 1974-1975	1-24

 Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

30-06-2010

Name and mailing address of the ISA/SE
Patent- och registreringsverket
Box 5055
S-102 42 STOCKHOLM
Facsimile No. + 46 8 666 02 86

Authorized officer

Magnus Hallberg

Telephone No. + 46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2010/050333

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Alici et al. "Autologous antitumor activity by NK cells expanded from myeloma patients using GMP-compliant components", Blood, 2008, vol 111, no 6, pages 3155-3162, ISSN 0006-4971.; whole document	1-24
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Y	US 20030068306 A1 (DILBER MEHMET SIRAC), 10 April 2003 (2003-04-10); abstract; paragraphs [0006], [0049], [0072]; claims	1-24
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A	Arai et al. "Infusion of the allogeneic cell line NK-92 in patients with advanced renal cell cancer or melanoma: a phase I trial", Cytotherapy, 2008, vol 10, no 6, pages 625-632, ISSN 1465-3249.; abstract; pages 626-627	1-24
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A	Tam et al. "Ex vivo expansion of the highly cytotoxic human natural killer-92 cell-line under current good manufacturing practice conditions for clinical adoptive cellular immunotherapy", Cytotherapy, 2003, vol 5, no 3, pages 259-272, ISSN 1465-3249.; abstract; pages 266, 269-270; figure 5	1-24
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A	Luhm et al. "Large-scale generation of natural killer lymphocytes for clinical application" Journal of hematotherapy & stem cell research, 2002, vol 11, no 4. Pages 651-657, ISSN 1525-8165.; abstract	1-24
	--	
A	Pierson et al. "Production of human natural killer cells for adoptive immunotherapy using a computer-controlled stirred-tank bioreactor", Journal of hematotherapy, 1996, vol 5, no 5, pages 475-483, ISSN 1061-6128.; whole document	1-24
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P, Y	WO 2009151183 A1 (NKBIO CO LTD ET AL), 17 December 2009 (2009-12-17); abstract; paragraphs [0001], [0042]	1-24
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2010/050333

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: **24**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claim 24 relates to a suspension of NK-cells and NK-like T cells defined by that the

.../...

3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

- Remark on Protest**
- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
 - The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
 - No protest accompanied the payment of additional search fees.

Continuation of: Box No. II

cells have an increased cytotoxicity compared to cells expanded in flasks. Thereby the cells are defined by reference to a desirable characteristic. Since it is impossible to know what cytotoxic activity a cell culture expanded in a closed system would have if it instead would have been expanded in a flask, it is impossible to determine if previously described cell cultures would fall within the scope of the claim. Furthermore, the description only provide support for that a culture of NK-cell/NK-like T cells have increased cytotoxicity, compared to cells expanded in flasks, when expanding the culture in a particular bioreactor under specific conditions. Apparently, expanding the culture in another closed system, i.e. a culture bag, did not increase the cytotoxicity. Moreover, only three out of four cell cultures expanded in the bioreactor has an increased cytotoxicity compared to expansion in flasks. Still, the scope of the claim covers all NK-cells/NK-like T cells having the desired characteristic. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely to a cell culture obtainable by the method described in the description.

Continuation of: second sheet

International Patent Classification (IPC)

C12N 5/0783 (2010.01)

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Cited literature, if any, will be enclosed in paper form.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/SE2010/050333

US	20030068306 A1	10/04/2003	NONE		
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