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**(54) METHOD FOR SEPARATING CELLS USING IMMUNOROSETTES AND MAGNETIC PARTICLES**

VERFAHREN ZUR ZELLSEPARATION UNTER VERWENDUNG VON IMMUNOROSETTEN UND MAGNETISCHEN TEILCHEN

PROCÉDÉ DE SÉPARATION DE CELLULES À L'AIDE DE ROSETTES IMMUNES ET DE PARTICULES MAGNÉTIQUES

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**Description****Field of the Disclosure**

5 [0001] The present disclosure relates to methods for separating cells using immunorosettes and magnetic particles.

**Background**

10 [0002] In many applications it is desirable to enrich, or alternatively deplete, certain cell populations in a biological sample. The fields of hematology, immunology and oncology rely on samples of peripheral blood and cell suspensions from related tissues such as bone marrow, spleen, thymus and fetal liver. The separation of specific cell types from these heterogeneous samples is key to research in these fields. Purified populations of immune cells such as T cells and B cells are necessary for the study of immune function and are used in immunotherapy. Investigation of the cellular, molecular and biochemical processes require analysis of certain cell types in isolation. Numerous techniques have been used to isolate lymphocyte subsets such as T cells, B cells and natural killer (NK) cells and granulocytes such as neutrophils, basophils and eosinophils.

15 [0003] Hematopoietic cells and immune cells have been separated on the basis of physical characteristics such as density and through direct targeting with magnetic particles. The advent of monoclonal antibodies against cell surface antigens has greatly expanded the potential to distinguish and separate distinct cell types. There are two basic approaches to separating cell populations from blood and related cell suspensions using monoclonal antibodies. They differ in whether it is the desired or undesired cells which are distinguished/labelled with the antibody(s). In positive selection techniques, the desired cells are labelled with antibodies and removed from the remaining unlabelled/unwanted cells. In negative selection, the unwanted cells are labelled and removed. Antibody and complement treatment and the use of immunotoxins is a negative selection technique, whereas fluorescence assisted cell sorting (FACS) and most bulk immunoadsorption techniques can be adapted to both positive and negative selection. In immunoadsorption techniques, cells are selected with monoclonal antibodies and preferentially bound to a surface which can be removed from the remainder of the cells e.g. column of beads, flasks, magnetic particles. Immunoadsorption techniques have won favour clinically and in research because they maintain the high specificity of targeting cells with monoclonal antibodies, but unlike FACS, they can be scaled up to deal directly with the large numbers of cells in a clinical harvest and they avoid the dangers of using cytotoxic reagents such as immunotoxins and complement. They do however, require the use of a "device" or cell separation surface such as a column of beads, panning flask or magnet.

20 [0004] Density separations are commonly used to isolate peripheral blood mononuclear cells from granulocytes and erythrocytes. Ficoll (GE Healthcare Life Sciences, Chalfont, United Kingdom) is the most popular density solution used for this application. In a Ficoll density separation whole blood is layered over Ficoll, and then centrifuged. The erythrocytes and granulocytes settle with the pellet and the mononuclear cells remain at the Ficoll:plasma interface. The success of this technique relies on the difference in density between mononuclear cells and granulocytes. If whole blood is stored for more than 24 hours the granulocytes change density and will not pellet with the erythrocytes. Suspensions of pure mononuclear cells cannot be obtained from stored blood or samples with altered cell density in a single density separation. RosetteSep is a commercially available immunodensity cell separation product from STEMCELL Technologies Inc. (Vancouver, Canada) that utilizes density gradient centrifugation to enrich cells from erythrocyte containing samples such as human peripheral whole blood by forming immunorosettes between undesired nucleated cells with erythrocytes. Although immunodensity cell separation can efficiently enrich nucleated cell populations from human peripheral whole blood, there can be residual erythrocyte contamination in the final enriched cell sample. For more information on the use of density gradient centrifugation for the separation of immunorosettes see U.S. Pat. No. 6,448,075 to Thomas.

25 [0005] Current cell separation methods using immunomagnetic negative selection methods for the isolation of immune cells, hematopoietic stem cells and circulating epithelial tumor cells typically involve an initial step to deplete erythrocytes followed by antibody mediated adherence to a device or artificial particle. Several commercial cell separation products are available that utilize a pre-processing step to reduce the erythrocyte content of peripheral whole blood such as hypotonic erythrocyte lysis, Ficoll density centrifugation or a gravity sedimentation approach prior immunomagnetic cell separation (Miltenyi Biotec Inc., Gladbach, Germany., Life Technologies Corp., Carlsbad, USA., STEMCELL Technologies Inc., Vancouver, Canada). Recently, a new method has been described (see WO2013/076070), that combines gravity sedimentation of erythrocytes with immunomagnetic cell separation in a single step to enrich for desired cells from peripheral human whole blood. This method however requires an additional immunomagnetic depletion step to further reduce erythrocyte contamination in the enriched sample.

30 [0006] In view of the foregoing, there is a need in the art to provide novel methods for improving erythrocyte removal and separating desired cells or removing unwanted cells from biological samples containing erythrocytes without the need for pre-processing or using time consuming gravity sedimentation approaches. There is currently no sole immunomagnetic cell separation method for the negative selection of cells directly from erythrocyte containing samples such

as, but not limited to human peripheral whole blood without reducing the erythrocyte content by density centrifugation, agglutination or hypotonic lysis of whole blood either prior to or in combination with immunomagnetic cell separation. Peters et al. (Methods in Molecular Biology, vol.302, 2005, page 95-115) describes the Immunorosette-technology ("RosetteSep™") used to isolate specific cell types from human whole blood.

## Summary

**[0007]** The present inventor has developed a method for separating target cells by immunorsetting the target cells with secondary targets such as erythrocytes already existing in, or added to the sample, and subsequently labeling the immunorsettes with particles that facilitate the removal of the immunorosette-particle complexes. The particles are magnetic particles.

**[0008]** The methods of the disclosure can efficiently separate target cells from a complex sample while improving erythrocyte depletion compared to existing methods. With the current disclosure there is no need for pre-processing a sample with a significant erythrocyte content (>1 erythrocyte per nucleated cell) prior to subjecting the sample to the immunomagnetic cell separation procedure. A further benefit of the magnetic particle approach is that since it is an immunomagnetic cell separation approach, it can be fully automated thereby further reducing sample handling and minimizing exposure to blood borne pathogens such as viruses or parasites.

**[0009]** Accordingly, the present invention provides a selection method for separating target cells from non-target cells in a sample comprising target cells, secondary targets and non-target cells, the method comprising:

a. contacting the sample with a first antibody composition comprising (a) at least one antibody that binds to the target cells wherein the at least one antibody is linked, either directly or indirectly, to (b) at least one antibody that binds to the secondary targets, under conditions to allow immunorsettes of the target cells and the secondary targets to form,

b. contacting the sample with a second antibody composition comprising: (a) at least one antibody that binds to the secondary targets either singly or within the immunorsettes, wherein the at least one antibody is linked, either directly or indirectly, to (b) at least one antibody that binds to magnetic particles, under conditions to allow binding of the magnetic particles to the immunorsettes and/or the secondary targets, and

c. separating the immunorosette - magnetic particle complexes and/or the secondary target - magnetic particle complexes from the sample to separate the target cells from the non-target cells, wherein the immunorosette-magnetic particle complexes and/or the secondary target-magnetic particle complexes are separated from the sample by placing the sample in a magnetic field gradient of sufficient strength to separate the immunorosette-magnetic particle complexes and/or the secondary target-magnetic particle complexes from the sample.

**[0010]** In one embodiment, the second antibody composition is bound to the magnetic particles prior to contacting with the sample. In one embodiment, the second antibody composition is bound to the magnetic particles and added to the sample prior to contacting the sample with the first antibody composition.

**[0011]** In one embodiment, the secondary targets are erythrocytes.

**[0012]** In another embodiment, the secondary targets are granulocytes.

**[0013]** In another embodiment, the secondary targets are beads, optionally polystyrene beads coated with a polymer such as polyethylene glycol (PEG).

**[0014]** As described herein, the secondary targets may be added to the sample prior to separation.

**[0015]** In accordance with the invention, the particles are magnetic particles.

**[0016]** In another embodiment, the secondary targets have a density similar to a cell and the magnetic particles have a density different from a cell.

**[0017]** In another embodiment, the selection method is a positive selection method to select for a desired cell and the target cell is the desired cell.

**[0018]** In another embodiment, the selection method is a negative selection method to remove a non-desired cell from the sample and the target cell is the non-desired cell.

**[0019]** Also described herein, the immunorosette-magnetic particle complexes may be separated from the sample in step c) by density separation.

**[0020]** Also described herein, the immunorosette-magnetic particle complexes may be separated from the sample in step c) by sedimentation.

**[0021]** In another embodiment, the method further comprises:

d. separating the target cells from the immunorosette-magnetic particle complexes.

**[0022]** In another embodiment, the target cells are separated from the immunorosette-magnetic particle complexes by physical, chemical, enzymatic or thermal dissociation.

**[0023]** In another embodiment, the method further comprises:

- d. lysis of the erythrocytes in the immunorosettes and
- e. separation of the target cells from the lysed erythrocytes and the magnetic particles.

[0024] In another embodiment, the sample is whole blood, bone marrow, fetal liver, cord blood, a buffy coat suspension, a leukapheresis sample, a pleural and peritoneal effusion or a sample of thymocytes and splenocytes.

[0025] In another embodiment, the target cells are stem cells, progenitor cells, monocytes, lymphocytes, or granulocytes.

[0026] In another embodiment, the first antibody composition is a bi-specific tetrameric complex and the second antibody composition is a mono-specific tetrameric complex.

[0027] In another embodiment, the lymphocytes are T cells, B cells or NK cells and the granulocytes are neutrophils, basophils or eosinophils.

### Brief Description of the Drawings

[0028] The disclosure will now be described in relation to the drawings in which:

Figure 1 shows a bright field image of A) peripheral human whole blood, B) peripheral human whole blood with immunorosettes, C) immunorosettes bound by magnetic particles and D) a magnified image of immunorosettes bound by magnetic particles as indicated by the arrow.

Figure 2 (A-C) shows enrichment of human CD3+ CD4+ T cells from erythrocyte depleted human peripheral blood mononuclear cells with pegylated polystyrene beads.

Figure 3 (A-C) shows enrichment of human CD3+ CD4+ T cells from human peripheral whole blood using immunomagnetic cell separation of immunorosettes according to example 4 and 5. Samples were stained with anti-human CD45 FITC, CD4 PerCP-Cy5.5 and CD3 APC and analyzed by flow cytometry. A) Following hypotonic lysis of erythrocytes in human peripheral blood, 99.9% of cells are CD45+ of which 12.7% are CD3+ CD4+ T cells. B) Following immunomagnetic cell separation according to example 4, 96.0% of cells are CD45+ of which are 94.6% CD3+ CD4+ T cells. C) Following immunomagnetic cell separation according to example 5, 99.8% of cells are CD45+ of which are 94.6% CD3+ CD4+ T cells.

Figure 4 shows enrichment of human CD3+ T cells, CD3+ CD4+ T cells, CD3- CD19+ B cells and CD3- CD56+ NK cells from human peripheral whole blood using immunomagnetic cell separation of immunorosettes according to example 3. Following immunomagnetic cell separation according to example 3, mean purities for each cell type indicated above was 97.2%, 92.0%, 73.9%, and 68.5%, with recovery values of 40.7%, 35.2%, 29.9%, and 36.6%, respectively.

Figure 5 shows enrichment of human CD3+ CD4+ T cells from human peripheral whole blood using a commercially available erythrocyte aggregation and immunomagnetic separation method MACSpress with and without the additional immunomagnetic erythrocyte depletion step.

Figure 6 shows enrichment of human CD3+ T cells from human peripheral whole blood using either A) ammonium chloride lysis, B) immunorosettes and density separation, C) erythrocyte aggregation and immunomagnetic separation, or D) immunorosettes and immunomagnetic separation according to example 3.

## DETAILED DESCRIPTION

### Methods of the Disclosure

[0029] The present disclosure relates to a method for separating target cells from non-target cells from a sample containing target cells, secondary targets and non-target cells by immunorosetting the target cells with the secondary targets and subsequently binding said immunorosettes and secondary targets to particles.

[0030] In one aspect, the present invention provides a selection method for separating target cells from non-target cells in a sample comprising target cells, secondary targets and non-target cells, the method comprising:

a) contacting the sample with a first antibody composition comprising (a) at least one antibody that binds to the target cells wherein the at least one antibody is linked, either directly or indirectly, to (b) at least one antibody that binds to the secondary targets, under conditions to allow immunorosettes of the target cells and the secondary targets to form,

b) contacting the sample with a second antibody composition comprising (a) at least one antibody that binds to the secondary targets either singly or within the immunorosettes, wherein at least one antibody is linked, either directly or indirectly, to (b) at least one antibody that binds to magnetic particles, under conditions to allow binding of the magnetic particles to the immunorosettes and/or the secondary targets, and

c) separating the immunorosette - magnetic particle complexes and/or the secondary target - magnetic particle complexes from the sample to separate the target cells from the non-target cells, wherein the immunorosette-magnetic particle complexes and/or the secondary target-magnetic particle complexes are separated from the sample by placing the sample in a magnetic field gradient of sufficient strength to separate the immunorosette-magnetic particle complexes and/or the secondary target-magnetic particle complexes from the sample.

**[0031]** The method can be used in both positive and negative selection protocols. In a positive selection protocol, desired cells are the target cells and are removed from a sample. In a negative selection protocol, desired cells are the non-target cells and remain in the sample following the selection protocol such that the remaining sample is enriched for the desired cells.

**[0032]** As used herein, the term "target cell" is a cell that is targeted by binding to the first antibody composition. The target cell is optionally a nucleated cell. In a positive selection protocol, the desired cell is the target cell. In a negative selection protocol, the desired cell is not the target cell. Rather, the desired cell is a non-target cell.

**[0033]** As used herein, the term "non-target cell" is a cell in the sample that does not bind to the first or second antibody composition. In a positive selection method, the non-target cell is not the desired cell. In a negative selection method the non-target cell is the desired cell.

As used herein, the term "secondary target" is a cell or other body such as a particle, bead or aggregate that binds to the first and second antibody composition and is to be removed from the sample. The secondary target is removed from the sample in both positive and negative selection protocols. In one embodiment, the secondary target is an erythrocyte.

**[0034]** In another embodiment, the secondary target is a granulocyte. In another embodiment, the secondary target is a bead such as a polystyrene bead. The bead may be coated with a polymer such as PEG. As described herein,, the secondary target may be added to the sample prior to performing the selection method. For example, beads may be added to the sample.

**[0035]** In accordance with the invention, the particles are magnetic particles. In other methods described herein, the particles may be non-magnetic particles. One example of non-magnetic particles useful in those methods described herein are buoyant particles. Immunorosettes which include buoyant particles will float when placed in an appropriate buffer, thereby allowing separation of the immunorosettes from a sample.

**[0036]** In a negative selection protocol, the desired cells are not immunorosetted and remain in the sample following the removal of the immunorosettes labeled with the particles. Accordingly, the non-desired cells are the "target cells" to be removed from the sample along with the secondary targets and the desired cells are "non-target cells". In a negative selection, the antibody composition will contain at least one antibody specific for the cells that one wishes to remove from the sample. Accordingly, the present disclosure provides a negative selection method for enriching and recovering desired cells in a sample containing desired cells, secondary targets, and non-desired cells comprising:

- a) contacting the sample with a first antibody composition comprising (a) at least one antibody that binds to the non-desired cells linked, either directly or indirectly, to (b) at least one antibody that binds to the secondary targets, under conditions to allow immunorosettes of the non-desired cells and the secondary targets to form,
- b) contacting the sample with a second antibody composition comprising (a) at least one antibody that binds to the secondary targets either singly or within the immunorosettes, linked, either directly or indirectly, to (b) at least one antibody that binds to particles, under conditions to allow binding of the particles to the immunorosettes and/or the secondary targets, and
- c) separating the immunorosette - particle complexes and/or the secondary target - particle complexes from the sample to obtain a sample enriched in the desired cells.

**[0037]** In a positive selection protocol, the desired cells are the target cells. The desired cells are immunorosetted to the secondary targets and subsequently bound to the particles. In such a situation, the method can further include the step of separating the desired cells from the immunorosettes. In a positive selection, the antibody composition will contain at least one antibody specific for the desired cells that one wishes to remove from the sample. Accordingly, the present disclosure provides a positive selection method for recovering desired cells from a sample containing the desired cells, secondary targets, and non-desired cells comprising:

- a) contacting the sample with a first antibody composition comprising (a) at least one antibody that binds to the desired cells linked, either directly or indirectly, to (b) at least one antibody that binds to the secondary targets, under conditions to allow immunorosettes of the desired cells and the secondary targets to form,
- b) contacting the sample with a second antibody composition comprising (a) at least one antibody that binds to the secondary targets either singly or within the immunorosettes, linked, either directly or indirectly, to (b) at least one antibody that binds to particles, under conditions to allow binding of the particles to the immunorosettes and/or the secondary targets, and

c) separating the immunorosette particle complexes and/or the secondary target - particle complexes from the sample to obtain a sample enriched in the desired cells within the immunorosette - particle complexes.

5 [0038] As described herein, the positive selection method includes the disaggregation of the immunorosette - particle complex to separate the desired cells from the immunorosette-particle complex through but not limited to, physical, chemical, enzymatic, or thermal dissociation. As described herein, if the secondary targets are erythrocytes, a further step includes the hypotonic lysis of the erythrocytes in the immunorosettes and separation of the desired cells from the lysed erythrocytes and the magnetic particles. The resulting positively selected desired cells will be free of bound magnetic particles.

10 [0039] The immunorosettes bound to particles formed in step (b) above for either negative or positive selection can be separated from the non-magnetic non-target cells using a variety of techniques.

[0040] In accordance with the invention, the particles are magnetic particles and the sample, containing the immunorosettes labeled with magnetic particles, is placed into a magnetic field. The immunorosettes labeled with magnetic particles migrate towards the magnetic field and are held in place allowing the non-magnetic non-target cells to be easily separated from the immunorosettes labeled with magnetic particles.

15 [0041] Alternatively, as described herein, the sample containing the immunorosettes labeled with particles may be layered over a buoyant density solution (such as Ficoll) and centrifuged. The immunorosettes labeled with the particles pellet due to their increased density and the target cells remain at the interface between the buoyant density solution and the sample. The target cells may then be removed from the interface for further use.

20 [0042] Alternatively, as described herein, the sample containing the immunorosettes obtained in step (a) or immunorosette - particles complexes obtained in step (b) may be mixed with a sedimentation reagent (such as hydroxyethyl starch, gelatin or methyl cellulose) and the immunorosettes or immunorosette - particle complexes are permitted to sediment. The desired cells remain in suspension and may then be removed for further use.

25 [0043] The methods of the disclosure may be used in the processing of biological samples that contain erythrocytes including blood (in particular, cord blood and whole blood) bone marrow, fetal liver, buffy coat suspensions, leukapheresis samples, pleural and peritoneal effusions and suspensions of thymocytes and splenocytes. The method can be used to separate desired cells or removing unwanted cells from biological samples containing erythrocytes such as whole blood or whole bone marrow without the need for pre-processing or using time consuming gravity sedimentation approaches prior to, or in combination with, immunomagnetic cell separation approaches. This offers a significant advantage of the method of the disclosure over the prior art methods. In particular, the present disclosure can reduce the final erythrocyte content to less than 5% of the final enriched population which is an improvement on existing methods. Furthermore, the entire procedure can be performed using only immunomagnetic cell separation without the aid of non-magnetic erythrocyte depletion methods such as density gradient centrifugation, density sedimentation or hypotonic lysis. Although the method uses a magnetic field to separate the magnetically labeled immunorosettes, the current procedure still retains its utility for use with density gradient centrifugation methodology or sedimentation reagents.

30 [0044] The method of the disclosure can be used to prepare enriched samples of any cell type including, but not limited to, T cells, B cells, NK cells, dendritic cells, monocytes, basophils, mast cells, progenitor cells, stem cells and tumor cells.

35 [0045] As described herein the method of the disclosure may be used to prepare a cell preparation from samples such as blood and bone marrow, which is enriched in a selected differentiated cell type such as T cells, B cells, NK cells, monocytes, dendritic cells, basophils and plasma cells. This will enable studies of specific cell to cell interactions including growth factor production and responses to growth factors. It will also allow molecular and biochemical analysis of specific cells types. Cell preparations enriched in NK cells, dendritic cells and T cells may also be used in immune therapy against certain malignancies.

#### 45 **Antibody and Particle Compositions**

[0046] The disclosure includes the antibody and particle compositions for use in the methods described herein. The first antibody composition will contain (a) at least one antibody that binds to an antigen on the target cell, linked either directly or indirectly, to (b) at least one antibody that binds to the secondary target.

50 [0047] As described herein, the second antibody composition will contain (c) at least one antibody that binds to the secondary target linked, either directly or indirectly, to (d) at least one antibody that binds to a particle.

[0048] As described herein, the second antibody composition may be directly bound to a particle using conventional techniques that are readily apparent to those skilled in the art such as physical adsorption or chemical conjugation. As described herein, the second antibody composition bound to a particle may contain (c) at least one antibody that binds to the secondary target linked, either directly or indirectly, to (d) at least one antibody that binds to the same secondary target. Alternatively as described herein, the second antibody composition may contain (c) at least one antibody that binds to the secondary target which is directly bound to the particle using conventional techniques that are readily apparent to those skilled in the art such as physical adsorption or chemical conjugation.

[0049] The term "at least one antibody" means that the antibody composition includes at least one type of antibody (as opposed to at least one antibody molecule). One type of antibody means an antibody that binds to a particular antigen. For example, antibodies that bind to the antigen CD3 are considered one type of antibody. Preferably, the antibody compositions of the disclosure contain (a) more than one antibody type that binds to target cells.

5 [0050] As described herein, the first antibody composition of the present disclosure comprises (a) at least one antibody specific for a target cell and (b) at least one antibody specific for the secondary target. By "indirectly linked" it is meant that antibody (a) and antibody (b) are not directly covalently linked to each other but are attached through a linking moiety such as an immunological complex. As described herein, the first antibody composition may contain at least one antibody to the target cell (a) that is indirectly linked to at least one antibody specific for the secondary target (b) by preparing a bi-specific tetrameric antibody complex. A bi-specific tetrameric antibody complex may be prepared by mixing (a) the first monoclonal antibody which is capable of binding to the target cells, and (b) second monoclonal antibody capable of binding the secondary target cells. The first and second monoclonal antibody are from a first animal species. The first and second antibodies are reacted with approximately an equimolar amount of monoclonal antibodies of a second animal species which are directed against the Fc-fragments of the antibodies of the first animal species. The first and second antibodies may also be reacted with an about equimolar amount of the full length or F(ab')<sub>2</sub> fragments of monoclonal antibodies of a second animal species which are directed against the Fc-fragments of the antibodies of the first animal species. (See U.S. Pat. No. 4,868,109 to Lansdorp, for a description of tetrameric antibody complexes and methods for preparing the same).

10 [0051] As described herein, the second antibody composition of the present disclosure comprises (c) at least one antibody specific for the secondary target cell and (d) at least one antibody specific for the particle. By "indirectly linked" it is meant that antibody (c) and antibody (d) are not directly covalently linked to each other but are attached through a linking moiety such as an immunological complex. As described herein, the second antibody composition may contain at least one antibody to the secondary target cell (c) that is indirectly linked to at least one antibody specific for the particle (d) by preparing a bi-specific tetrameric antibody complex as described for the first antibody composition.

15 [0052] As described herein, the second antibody composition of the present disclosure may comprise (c) at least one antibody specific for the secondary target cell and (d) at least one antibody specific for same secondary target indirectly linked by preparing a mono-specific tetrameric antibody complex. A mono-specific tetrameric antibody complex may be prepared by mixing the monoclonal antibody which is capable of binding to the secondary target cells from a first animal species with an equimolar amount of monoclonal antibodies of a second animal species which are directed against the Fc-fragments of the antibodies of the first animal species. The antibodies from the first animal species may also be reacted with an about equimolar amount of the full length or F(ab')<sub>2</sub> fragments of monoclonal antibodies of a second animal species which are directed against the Fc-fragments of the antibodies of the first animal species.

20 [0053] As described herein, the second antibody composition containing mono-specific tetrameric antibody complexes specific for the secondary targets may be directly bound to the particle using conventional techniques that are readily apparent to those skilled in the art such as physical adsorption or chemical conjugation.

25 [0054] As described herein, the antibody specific for the target cells may be linked directly to the antibody specific for the secondary target and the antibody specific for the secondary target may be linked directly to the antibody specific for the magnetic particle. As described herein, the antibody composition of the present disclosure may contain bifunctional antibodies comprising (a) at least one antibody specific for the target cells linked directly to (b) at least one antibody specific for the secondary targets and a second bifunctional antibody comprising (c) at least one antibody specific for the secondary targets linked directly to (d) at least one antibody specific for the particles. Bifunctional antibodies may be prepared by chemically coupling one antibody to the other using conventional techniques that are readily apparent to those of skill in the art as those disclosed in Ellerman and Scheer [1].

30 [0055] As described herein, the antibody composition may contain bispecific antibodies. The first bispecific antibodies contains a variable region of an antibody specific for the secondary targets and a variable region specific for at least one antigen on the surface of the target cells to be separated. The second bispecific antibody contains a variable region of an antibody specific for the secondary targets and the particles. The bispecific antibodies may be prepared by forming hybrid hybridomas. The hybrid hybridomas may be prepared using the procedures known in the art such as those disclosed in Moldenhauer [2]. Bispecific antibodies may also be constructed by expression of recombinant immunoglobulin gene constructs as described by Chang, Rossi, and Sharkey [3].

35 [0056] As described herein, the secondary targets may be erythrocytes and the antibody specific for the erythrocytes may be anti-glycophorin A. The anti-glycophorin A antibodies contained in the antibody composition of the disclosure are used to bind the erythrocytes. Examples of monoclonal antibodies specific for glycophorin A are 10F7MN (U.S. Pat. No. 4,752,582, Cell lines: ATCC accession numbers HB-8162), and D2.10 (Immunotech, Marseille, France). As described herein the antibody specific for the target cells may be a combination of antibodies. The combination of antibodies may be specific for a number of cell types so that many target cell types may be removed from the sample. When using a combination of antibodies, each antibody may be linked (either directly or indirectly) to an antibody specific for erythrocytes.

**[0057]** As described herein, the particles may be magnetic particles and the mono-specific tetrameric antibody complex specific for the secondary targets may be directly bound to the magnetic particle using conventional techniques such as physical adsorption or chemical conjugation that are readily apparent to those of skill in the art.

**[0058]** As described herein, the particles may be magnetic particles and the antibody specific for the secondary targets may be directly bound to the magnetic particle using conventional techniques such as physical adsorption or chemical conjugation that are readily apparent to those of skill in the art.

**[0059]** As described herein, the particles may be magnetic particles and the antibody specific for the magnetic particle may be anti-dextran. The anti-dextran antibodies contained in the antibody composition of the disclosure may be used to bind the magnetic particles which have been coated with a dextran polymer.

**[0060]** As described herein, the first antibody composition may be a tetrameric complex comprising (a) anti-glycophorin A antibodies to bind the erythrocytes, (b) an antibody that binds to a target cell type that one wishes to immunorosette and (c) antibodies that bind the Fc portion of both (a) and (b), optionally F(ab')<sub>2</sub> antibody fragments. The molar ratio of (a):(b):(c) may be approximately 1:3:4. When several types of cells are to be separated, complexes may be made with several anti-target cell antibodies (b). The complexes may then be mixed together to form an antibody composition for use in the method of the disclosure. The second antibody composition may be a mono-specific tetrameric complex comprising (a) anti-glycophorin A antibodies to bind the erythrocytes in immunorosettes, and (c) antibodies that bind the Fc portion of (a), optionally F(ab')<sub>2</sub> antibody fragments. The molar ratio of (a):(c) may be approximately 1:1. The mono-specific tetrameric complexes specific for the secondary targets may be directly bound to the magnetic particle using conventional techniques.

**[0061]** As described herein, the first antibody composition may be a tetrameric complex comprising (a) anti-glycophorin A antibodies to bind the erythrocytes, (b) an antibody that binds to a target cell type that one wishes to immunorosette and (c) antibodies that bind the Fc portion of both (a) and (b), optionally F(ab')<sub>2</sub> antibody fragments. The molar ratio of (a):(b):(c) may be approximately 1:3:4. When several types of cells are to be separated, complexes may be made with several anti-target cell antibodies (b). The complexes may then be mixed together to form an antibody composition for use in the method of the disclosure. The second antibody composition may be anti-glycophorin A antibodies directly bound to magnetic particles that can to bind individual erythrocytes and erythrocytes within immunorosettes.

**[0062]** As described herein, the first antibody composition may be a tetrameric complex comprising (a) anti-glycophorin A antibodies to bind the erythrocytes, (b) an antibody that binds to a target cell type that one wishes to immunorosette and (c) antibodies that bind the Fc portion of both (a) and (b), optionally F(ab')<sub>2</sub> antibody fragments. The molar ratio of (a):(b):(c) may be approximately 1:3:4. When several types of cells are to be separated, complexes may be made with several anti-target cell antibodies (b). The complexes may then be mixed together to form an antibody composition for use in the method of the disclosure. The second antibody composition may be a tetrameric complex comprising (a) anti-glycophorin A antibodies to bind the erythrocytes in immunorosettes, (d) an anti-dextran antibody that binds to a magnetic particle and (c) antibodies that bind the Fc portion of both (a) and (d), optionally F(ab')<sub>2</sub> antibody fragments. The molar ratio of (a):(d):(c) may be approximately 1:3:4.

**[0063]** As described herein, the second antibody composition may be a tetrameric antibody complex comprising (a) anti-glycophorin A antibodies to bind the erythrocytes in immunorosettes, (b) an anti-dextran antibody that binds to a magnetic particle and (c) antibodies that bind the Fc portion of both (a) and (d), optionally F(ab')<sub>2</sub> antibody fragments. The molar ratio of (a):(d):(c) may be approximately 1:3:4. The second antibody composition may be pre-loaded onto the magnetic particle prior to adding to the sample.

**[0064]** Within the context of the present disclosure, antibodies are understood to include monoclonal antibodies and polyclonal antibodies, antibody fragments (e.g., Fab, and F(ab')<sub>2</sub>), chimeric antibodies, bifunctional or bispecific antibodies and tetrameric antibody complexes. Antibodies are understood to be reactive against a selected antigen on the surface of a target cell or erythrocyte if they bind with an appropriate affinity (association constant), e.g. greater than or equal to 10<sup>7</sup> M<sup>-1</sup>.

**[0065]** Monoclonal antibodies are preferably used in the antibody compositions of the disclosure. Monoclonal antibodies specific for selected antigens on the surface of nucleated cells may be readily generated using conventional techniques that are readily apparent to those of skill in the art.

**[0066]** The disclosure also contemplates aptamers or chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. A variety of approaches for making chimeric antibodies have been described and can be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes selected antigens on the surface of differentiated cells or tumor cells. See for example, Kim and Hong [4].

**[0067]** The following examples are illustrative of the present disclosure:

## EXAMPLES

## Example 1

## 5 Preparation of tetrameric antibody complexes

[0068] In order to prepare a tetrameric antibody complex for use in the method of the present disclosure, the following protocol may be used: (a) take 1 mg of antibody specific for cells to be rosetted (e.g. anti-CD8, CD16, CD19, CD36, CD56, CD66b, etc.); (b) add 3 mg anti-glycophorin A antibody (against erythrocytes); mix well (c) then add 4.0 mg of P9 antibody or 2.72 mg of the P9 F(ab')<sub>2</sub> antibody fragment. Incubate overnight at 37° C. The P9 antibody binds the Fc portion of the antibodies added in steps (a) and (b) resulting in a tetrameric antibody complex. For the second antibody composition, the following protocol may be used: (a) take 1 mg of antibody specific to erythrocytes (anti-glycophorin A); (b) add 1mg of P9 antibody or 0.68 mg of P9 F(ab')<sub>2</sub> antibody fragment. Alternatively, (a) take 1 mg of antibody specific to erythrocytes (anti-glycophorin A); (b) add 3mg of anti-dextran antibody; mix well (c) then add 4.0 mg of P9 antibody or 2.72 mg of P9 F(ab')<sub>2</sub> antibody fragment. Incubate overnight at 37°C. For more information on the preparation of tetramers see U.S. Pat. No. 4,868,109 to Lansdorp. Tetrameric antibody complexes incorporating different antibodies to antigens expressed or nucleated cells are prepared separately and then mixed.

[0069] The antibody compositions are made by combining various tetrameric antibody complexes depending on which cells one wishes to deplete. The concentration of the various tetrameric antibody complexes varies: typically antibodies to antigens expressed on nucleated cells are at 10-40 ug/mL in tetrameric complexes. The composition is then diluted 1/20 into the cells so the final concentrations of each anti-nucleated cell antibody in the cell suspensions is 0.5-2.0 ug/mL.

[0070] In one embodiment, the second antibody composition comprised of anti-glycophorin A antibodies in a monospecific tetrameric antibody complex is at a concentration of 20-400ug/mL. The second composition is directly bound to a particle using conventional techniques such as physical adsorption or chemical conjugation that are readily apparent to those of skill in the art. The second antibody composition directly bound to the particle is then diluted 1/20 into the sample so the final concentration of anti-glycophorin A in the sample is 1-20ug/mL.

[0071] In another embodiment, the second antibody comprised of anti-glycophorin A antibodies is at a concentration of 20-400ug/mL. The second antibody is directly bound to a particle using conventional techniques such as physical adsorption or chemical conjugation that are readily apparent to those of skill in the art. The second antibody composition directly bound to the particle is then diluted 1/20 into the sample so the final concentration of anti-glycophorin A in the sample is 1-20ug/mL.

[0072] In another embodiment, the second antibody composition comprised of anti-glycophorin A and anti-dextran antibodies is at a concentration of 20-400ug/mL. The second composition is then diluted 1/20 into the immunorosetted cells so the final concentration of anti-glycophorin A in the cell suspension is 1-20ug/mL.

## Example 2

## 40 Preparation of particles with bound tetrameric antibody complexes or antibodies against the secondary target.

[0073] In order to prepare a particle with bound tetrameric antibody complexes for use in the method of the present disclosure, the following protocol may be used: (a) take 200ug of anti-glycophorin A antibody bound in a tetrameric antibody complex with 200ug of P9 antibody; (b) add 80mg of carboxydextran magnetic particles; and (c) incubate overnight at 15 - 37°C. The composition is then diluted 1/20 into the sample so the final concentration of anti-glycophorin A antibody is between 1-10ug/mL.

[0074] In another embodiment, physical adsorption or chemical crosslinking of anti-glycophorin A to magnetic particles is performed using conventional techniques that are readily apparent to those of skill in the art. A non-limiting example illustrative of the present disclosure would be the EDC-NHS crosslinking of anti-glycophorin A antibody to a carboxydextran magnetic particle. The composition is then diluted 1/20 into the sample so the final concentration of anti-glycophorin A antibody is 1-10ug/mL.

[0075] In another embodiment, (a) take 100ug of anti-glycophorin A antibody bound in a tetrameric antibody complex with 600ug of anti-dextran antibody and 600ug of P9 antibody or 408ug of P9 (F(ab')<sub>2</sub> antibody fragment; then add (b) 13.3mg of dextran-coated magnetic particles; and (c) incubate 5 minutes at room temperature. The composition is then diluted 1/20 into the cells so the final concentration of glycophorin A antibody is 1-10ug/mL.

**Example 3****Method of immunomagnetic cell separation of immunorosettes from human peripheral whole blood using mono-specific tetrameric antibody complexes specific for the secondary targets directly bound to magnetic particles**

**[0076]** A negative selection protocol for immunorosetting cells from human peripheral whole blood using magnetic cell separation is set out below.

1. Add 50uL of the first antibody composition per mL of human peripheral whole blood.
2. Add 50uL of the second antibody composition directly bound to magnetic particles per mL of human peripheral whole blood.
3. Incubate 5 minutes at room temperature.
4. Dilute sample with an equal volume of phosphate buffered saline (PBS) and mix gently.
5. Place the tube containing the sample into a magnet.
6. Incubate 5 minutes at room temperature.
7. Remove the enriched cells from the sample while the sample tube is retained within the magnet.
8. Add an equivalent volume of the second antibody composition directly bound to magnetic particles as in step 2 to the diluted enriched sample
9. Incubate 5 minutes at room temperature.
10. Place the tube containing the sample into a magnet.
11. Incubate 5 minutes at room temperature.
12. Remove the enriched cells from the sample while the sample tube is retained within the magnet.

**[0077]** A first antibody composition for the enrichment of a) CD3+ T cells, b) CD3+ CD4+ T cells, c) CD3- CD19+ B cells, and d) CD3- CD56+ NK cells from human peripheral whole blood each consisting of a cocktail of tetrameric antibody complexes containing antibodies against target cells and glycoprotein A (secondary targets) was prepared. A second antibody composition consisting of a mono-specific tetrameric antibody complex containing antibodies against glycoprotein A bound to carboxydextran magnetic particles was prepared. Following the method of the disclosure, mean purities for each cell type indicated above was a) 97.2%, b) 92.0%, c) 73.9% and d) 68.5%, with recovery values of a) 40.7%, b) 35.2%, c) 29.9%, and d) 36.6%, respectively as shown in Figure 4.

**Example 4****Method of immunomagnetic cell separation of immunorosettes from human peripheral whole blood using tetrameric antibody complexes specific for the secondary targets and magnetic particles preloaded onto magnetic particles**

**[0078]** A negative selection protocol for immunorosetting cells from human peripheral whole blood using magnetic cell separation is set out below.

1. Add 50uL of the first antibody composition per mL of human peripheral whole blood.
2. Add 50uL of the second antibody composition pre-loaded onto magnetic particles per mL of human peripheral whole blood.
3. Incubate 5 minutes at room temperature.
4. Dilute sample with an equal volume of phosphate buffered saline (PBS) and mix gently.
5. Place the tube containing the sample into a magnet.
6. Incubate 5 minutes at room temperature.
7. Remove the enriched cells from the sample while the sample tube is retained within the magnet.
8. Add an equivalent volume of the second antibody composition pre-loaded onto magnetic particles as in step 2 to the diluted enriched sample
9. Incubate 5 minutes at room temperature.
10. Place the tube containing the sample into a magnet.
11. Incubate 5 minutes at room temperature.
12. Remove the enriched cells from the sample while the sample tube is retained within the magnet.

**[0079]** A first antibody composition for the enrichment of CD4+ T cells from human peripheral whole blood consisting of a cocktail of tetrameric antibody complexes containing antibodies against CD8, CD16, CD19, CD36, CD56, CD66b, TCR $\gamma\delta$  and glycoprotein A was prepared. A second antibody composition consisting of a tetrameric antibody complex

containing antibodies against glycophorin A and dextran was prepared and preloaded onto carboxydextran magnetic particles. Following the method of the disclosure, 96.0% of cells were CD45+ of which, 94.6% were CD3+ CD4+ T cells as shown in figure 3B.

5 **Example 5**

**Method of immunomagnetic cell separation of immunorosettes from human peripheral whole blood using antibodies specific for the secondary targets chemically conjugated to magnetic particles**

10 **[0080]** A negative selection protocol for immunorosetting cells from human peripheral whole blood using magnetic cell separation is set out below.

1. Add 50uL of the first antibody composition per mL of human peripheral whole blood.
- 15 2. Add 50uL of the second antibody composition chemically conjugated to magnetic particles per mL of human peripheral whole blood.
3. Add 50uL of dextran coated magnetic particles per mL of human peripheral blood.
4. Incubate 10 minutes at room temperature.
5. Dilute sample with an equal volume of phosphate buffered saline (PBS) and mix gently.
6. Place the tube containing the sample into a magnet.
- 20 7. Incubate 5 minutes at room temperature.
8. Remove the enriched cells from the sample while the sample tube is retained within the magnet.
9. Add an equivalent volume of the dextran coated magnetic particles as in step 3 to the diluted enriched sample
10. Incubate 5 minutes at room temperature.
11. Place the tube containing the sample into a magnet.
- 25 12. Incubate 5 minutes at room temperature.
13. Remove the enriched cells from the sample while the sample tube is retained within the magnet.

30 **[0081]** A first antibody composition for the enrichment of CD4+ T cells from human peripheral whole blood consisting of a cocktail of tetrameric antibody complexes containing antibodies against CD8, CD16, CD19, CD36, CD56, CD66b, TCR $\gamma\delta$  and glycophorin A was prepared. A second antibody composition consisting of antibodies against glycophorin A chemically conjugated to carboxydextran magnetic particles was prepared. Following the method of the disclosure, 99.8% of cells were CD45+ of which, 94.6% were CD3+ CD4+ T cells as shown in figure 3C.

35 **Example 6**

**Method of immunomagnetic cell separation of immunorosettes from human peripheral whole blood using carboxydextran coated magnetic particles.**

40 **[0082]** A negative selection protocol for immunorosetting cells from human peripheral whole blood using magnetic cell separation is set out below.

1. Add 50uL of the first antibody composition per mL of human peripheral whole blood.
2. Add 50uL of the second antibody composition per mL of human peripheral whole blood.
3. Incubate 10 minutes at room temperature.
- 45 4. Add 50uL of carboxydextran coated magnetic particles per mL of human peripheral whole blood.
5. Incubate 5 minutes at room temperature.
6. Dilute sample with an equal volume of phosphate buffered saline (PBS) and mix gently.
7. Place the tube containing the sample into a magnet.
8. Incubate 5 minutes at room temperature.
- 50 9. Remove the enriched cells from the sample while the sample tube is retained within the magnet.
10. Add 5uL of carboxydextran coated magnetic particles per mL of diluted enriched sample.
11. Incubate 5 minutes at room temperature.
12. Place the tube containing the sample into a magnet.
13. Incubate 5 minutes at room temperature.
- 55 14. Remove the enriched cells from the sample while the sample tube is retained within the magnet.

**[0083]** A first antibody composition for the enrichment of CD4+ T cells from human peripheral whole blood consisting of a cocktail of tetrameric antibody complexes containing antibodies against CD8, CD16, CD19, CD36, CD56, CD66b,

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TCR $\gamma\delta$  and glycoporphin A was prepared. A second antibody composition consisting of a tetrameric antibody complex containing antibodies against glycoporphin A and dextran was prepared. Following the method of the disclosure, 92.6% purity of CD4+ T cells with a recovery of 24.7% was achieved as shown in Table 1.

### 5 Example 7

#### Method of immunomagnetic cell separation of immunorosettes from human peripheral blood mononuclear cells (PBMC)

10 [0084] A negative selection protocol for immunorosetting cells from erythrocyte depleted human PBMCs using magnetic cell separation is set out below.

1. Add an equivalent or greater number of non-magnetic particles to the erythrocyte depleted human PBMC sample.
2. Add 50uL of the first antibody composition per mL of sample.
- 15 3. Add 50uL of the second antibody composition per mL of sample.
4. Incubate 10 minutes at room temperature.
5. Add 50uL of magnetic particles per mL of sample.
6. Incubate 5 minutes at room temperature.
7. Dilute sample with an equal volume of PBS + 2% FBS and mix gently.
- 20 8. Place the tube containing the sample into a magnet.
9. Incubate 5 minutes at room temperature.
10. Remove the enriched cells from the sample while the sample tube is retained within the magnet.
11. Add 5uL of magnetic particles per mL of diluted enriched sample.
12. Incubate 5 minutes at room temperature.
- 25 13. Place the tube containing the sample into a magnet.
14. Incubate 5 minutes at room temperature.
15. Remove the enriched cells from the sample while the sample tube is retained within the magnet.

30 [0085] A first antibody composition for the enrichment of CD4+ T cells from human peripheral blood mononuclear cells consisting of a cocktail of tetrameric antibody complexes containing antibodies against CD8, CD16, CD19, CD36, CD56, CD66b, TCR $\gamma\delta$  and anti-PEG was prepared. A second antibody composition consisting of a tetrameric antibody complex containing antibodies against PEG and dextran was prepared. Following the method of the disclosure, 96.6% of cells were CD45+ of which, 96.3% were CD3+ CD4+ T cells as shown in figure 2C.

### 35 Example 8

#### Enrichment of human CD4+ T cells from peripheral whole blood using density gradient centrifugation or immunomagnetic separation of immunorosettes.

40 [0086] This example demonstrates the enrichment of CD4+ T cells from whole peripheral blood using the method described in Example 6 compared to CD4+ T cells enriched using immunorosettes isolated using density gradient centrifugation (Table 1). A CD4+ T cell enrichment cocktail of tetrameric antibody complexes containing antibodies against CD8, CD16, CD19, CD36, CD56, CD66b, TOR $\gamma\delta$  and glycoporphin A was prepared. A second tetrameric antibody complex containing antibodies against glycoporphin A and dextran was prepared. The results, shown in Table 1, demonstrate that the method of the disclosure results in 92.6% purity of CD4+ T cells with a recovery of 24.7%. In comparison, density gradient centrifugation of immunorosettes results in 80.6% purity of CD4+ T cells with 23.2% recovery primarily due to the presence of residual erythrocytes in the final enriched fraction (84.5% versus 97.5% CD45+ purity).

50 **Table 1. Enrichment of Human CD3+ CD4+ T cells from peripheral whole blood using density gradient centrifugation or immunomagnetic separation of immunorosettes**

Immunorosette depletion method of CD4+ T cell enrichment (n=4)	% CD45+	% CD3+ CD4+ (of CD45+)	% CD45+ CD3+ CD4+ recovery	% CD45+ CD3+ CD4+ (of total)
Density gradient centrifugation	84.5 +/- 7.8	96.6 +/- 0.5	23.2 +/- 5.5	80.6 +/- 7.9
Immunomagnetic separation	97.5 +/- 1.8	96.2 +/- 1.9	24.7 +/- 4.4	92.6 +/- 2.6

**Example 9****Enrichment of human CD4+ T cells from human PBMCs with PEGylated polystyrene beads.**

5 **[0087]** This example demonstrates the enrichment of CD4+ T cells from whole peripheral blood using the method described in Example 7 (Figure 2). Polyethylene glycol (PEG) functionalized 7um polystyrene beads were added to the erythrocyte depleted PBMC sample a final ratio of 10 beads to 1 nucleated cell. A CD4+ T cell enrichment cocktail of tetrameric antibody complexes containing antibodies against CD8, CD16, CD19, CD36, CD56, CD66b, TCR $\gamma\delta$  and PEG was prepared. A second tetrameric antibody complex containing antibodies against PEG and dextran was prepared. A) Ficolled PBMCs contain 71.5% CD45+ cells with 47.6% being CD3+ CD4+ T cells. B) The addition of 10 fold excess 7um PEG polystyrene beads reduces the CD45+ fraction to 7.2% with 83.6% being the beads. The proportion of CD3+ CD4+ T cells in the singlet fraction is similar to the PBMC sample at 44.4%. C) Following the method of the disclosure as outlined in Example 6, the final CD45+ purity is 96.6% and 96.3% of the singlets are CD3+ CD4+ T cells. The recovery of CD3+ CD4+ T cells was 23.6%.

**Example 10****Enrichment of human CD3+ CD4+ T cells using MACSxpress with or without additional erythrocyte depletion using immunomagnetic cell separation.**

20 **[0088]** This example demonstrates the negative enrichment of CD3+ CD4+ T cells from 2mL of human peripheral whole blood using a commercially available erythrocyte aggregation reagent and immunomagnetic separation system (MACSxpress®, Miltenyi Biotech) with and without the additional erythrocyte immunomagnetic depletion step recommended by the manufacturer (Figure 5). Samples were stained with anti-human CD4 FITC, CD3 PerCP-Cy5.5 and CD45 APC and analyzed by flow cytometry. A) Erythrocytes in human peripheral whole blood were lysed using ammonium chloride resulting in 85.0% CD45+ cells, of which 29.0% were CD3+ CD4+ T cells. A) Following the erythrocyte aggregation and immunomagnetic enrichment protocol as recommended by the manufacturer, the cell suspension is visibly red as shown in the image of the separation tube. Upon analysis of the sample, only 7.7% of recovered cells were CD45+, of which 72.1% were CD3+ CD4+ T cells. B) Following the additional immunomagnetic erythrocyte depletion protocol as per the manufacturers recommendation, the cell suspension is clear of residual erythrocytes. Upon analysis of the sample, 98.6% of the recovered cells were CD45+, of which 98.7% were CD3+ CD4+ T cells. This example demonstrates that the commercially available erythrocyte aggregation reagent and immunomagnetic separation platform MACSxpress requires both an erythrocyte aggregation reagent to sediment erythrocytes combined with a second step involving immunomagnetic erythrocyte depletion step to fully remove erythrocytes from human peripheral whole blood.

**Example 11****Enrichment of human CD3+ T cells from human whole peripheral blood using either density gradient or immunomagnetic separation of immunorosettes, or a method combining erythrocyte aggregation and immunomagnetic separation.**

40 **[0089]** This example demonstrates the negative enrichment of CD3+ T cells from 15mL of human peripheral whole blood (n=3) using either A) ammonium chloride lysis, B) immunorosettes and density separation (RosetteSep, STEMCELL Technologies), C) erythrocyte aggregation and immunomagnetic separation (MACSxpress, Miltenyi Biotech), or D) immunorosettes and immunomagnetic separation according to example 3 (Figure 6). A) Ammonium chloride lysis of human peripheral blood demonstrates that on average 26.6% of peripheral blood nucleated cells are CD3+ T cells present at  $1 \times 10^6$  cells per mL of whole blood. B) A first antibody composition for the enrichment of CD3+ T cells consisting of a cocktail of tetrameric antibody complexes containing antibodies against CD15, CD16, CD19, CD36, CD56, CD66b, and glycophorin A was prepared. Following immunodensity separation according the US patent US6448075B1, the mean purity of CD3+ T cells within the total cells including residual erythrocytes was 88.7% with  $3.6 \times 10^5$  CD3+ T cells recovered per mL of whole blood. C) Following the negative selection of CD3+ T cells using a commercially available erythrocyte aggregation reagent and immunomagnetic separation product (MACSxpress, Miltenyi Biotech) and an additional erythrocyte depletion step using immunomagnetic cell separation (MACSxpress), the mean purity of CD3+ T cells within the total cells including residual erythrocytes was 98.5% with  $4.5 \times 10^5$  CD3+ T cells recovered per mL of whole blood. D) Following the method of disclosure in example 3, a first antibody composition for the enrichment of CD3+ T cells consisting of a cocktail of tetrameric antibody complexes containing antibodies against CD15, CD16, CD19, CD36, CD56, CD66b, and glycophorin A was prepared. A second antibody composition consisting of a mono-specific tetrameric antibody complex containing antibodies against glycophorin A bound to carboxydextran magnetic particles

was prepared. Following the method of the disclosure, mean purities for CD3+ T cells within the total cells including residual erythrocytes was 96.8% with  $3.6 \times 10^5$  CD3+ T cells recovered per mL of whole blood. There was no significant difference between either the purity or CD3+ T cell recovery between methods C) and D). The method of disclosure in example 3, was solely a immunomagnetic negative selection method for the enrichment of CD3+ T cells from whole human peripheral blood.

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## Claims

1. A selection method for separating target cells from non-target cells in a sample comprising target cells, secondary targets and non-target cells, the method comprising:
  - a) contacting the sample with a first antibody composition comprising (a) at least one antibody that binds to the target cells wherein the at least one antibody is linked, either directly or indirectly, to (b) at least one antibody that binds to the secondary targets, under conditions to allow immunorosettes of the target cells and the secondary targets to form;
  - b) contacting the sample with a second antibody composition comprising:
    - (a) at least one antibody that binds to the secondary targets either singly or within the immunorosettes, wherein the at least one antibody is linked, either directly or indirectly, to (b) at least one antibody that binds to magnetic particles under conditions to allow binding of magnetic particles to the immunorosettes and/or the secondary targets;
    - and
    - c) separating the immunorosette - magnetic particle complexes and/or the secondary target - magnetic particle complexes from the sample to separate the target cells from the non-target cells, wherein the immunorosette-magnetic particle complexes and/or the secondary target-magnetic particle complexes are separated from the sample by placing the sample in a magnetic field gradient of sufficient strength to separate the immunorosette-magnetic particle complexes and/or the secondary target-magnetic particle complexes from the sample.
2. The method of claim 1 wherein the second antibody composition is bound to the magnetic particles prior to contacting with the sample.
3. The method of claim 1 or 2 wherein the second antibody composition is bound to the magnetic particles and added to the sample prior to contacting the sample with the first antibody composition.
4. The method of claim 1, wherein the secondary targets are erythrocytes, granulocytes, or beads, optionally polystyrene beads, optionally polystyrene beads coated with a polymer.
5. The method of claim 1, wherein the selection method is a positive selection method to select for a desired cell and the target cell is the desired cell or a negative selection method to remove a non-desired cell from the sample and the target cell is the non-desired cell.
6. The method of claim 1, wherein the secondary targets have a density similar to a cell and the magnetic particles have a density different from a cell.

7. The method of claim 1, wherein the method further comprises:  
d) separating the target cells from the immunorosette-magnetic particle complexes.
8. The method of claim 7, wherein the target cells are separated from the immunorosette-magnetic particle complexes by physical, chemical, enzymatic or thermal dissociation.
9. The method of claim 1, wherein the method further comprises:  
d) lysis of the erythrocytes in the immunorosettes and  
e) separation of the target cells from the lysed erythrocytes and the magnetic particles.
10. The method of claim 1, wherein the sample is blood, whole blood, bone marrow, fetal liver, cord blood, a buffy coat suspension, a leukapheresis sample, a pleural and peritoneal effusion or a sample of thymocytes and splenocytes.
11. The method of claim 1, wherein the target cells are stem cells, progenitor cells, monocytes, lymphocytes or granulocytes.
12. The method according to any one of claims 1 to 11 wherein the first antibody composition is a bi-specific tetrameric complex and the second antibody composition is a mono-specific tetrameric complex.
13. The method of claim 11 wherein the lymphocytes are T cells, B cells or NK cells and the granulocytes are neutrophils, basophils or eosinophils.

#### Patentansprüche

1. Auswahlverfahren zum Trennen von Zielzellen von Nicht-Zielzellen in einer Probe, die Zielzellen, Sekundärziele und Nicht-Zielzellen umfasst, wobei das Verfahren Folgendes umfasst:  
a) Inkontaktbringen der Probe mit einer ersten Antikörperzusammensetzung, umfassend (a) mindestens einen Antikörper, der an die Zielzellen bindet, wobei der mindestens eine Antikörper entweder direkt oder indirekt mit (b) mindestens einem Antikörper verbunden ist, der an die Sekundärziele bindet, unter Bedingungen, die es ermöglichen, Immunrosetten der Zielzellen und der Sekundärziele zu bilden;  
b) Inkontaktbringen der Probe mit einer zweiten Antikörperzusammensetzung, umfassend:  
(a) mindestens einen Antikörper, der entweder einzeln oder innerhalb der Immunrosetten an die sekundären Ziele bindet, wobei der mindestens eine Antikörper entweder direkt oder indirekt mit (b) mindestens einem Antikörper verbunden ist, der an magnetische Partikel bindet, unter Bedingungen, die eine Bindung von Magnetpartikeln an die Immunrosetten und/oder die Sekundärziele ermöglichen; und  
c) Trennen der Immunrosette-Magnetpartikel-Komplexe und/oder der Sekundärziel-Magnetpartikel-Komplexe von der Probe, um die Zielzellen von den Nicht-Zielzellen zu trennen, wobei die Immunrosette-Magnetpartikel-Komplexe und/oder die Sekundärziel-Magnetpartikel-Komplexe von der Probe getrennt werden, indem die Probe in einen Magnetfeldgradienten mit ausreichender Stärke platziert wird, um die Immunrosette-Magnetpartikel-Komplexe und/oder die Sekundärziel-Magnetpartikel-Komplexe von der Probe zu trennen.
2. Verfahren nach Anspruch 1, wobei die zweite Antikörperzusammensetzung an die Magnetpartikel gebunden ist, bevor sie mit der Probe in Kontakt kommt.
3. Verfahren nach Anspruch 1 oder 2, wobei die zweite Antikörperzusammensetzung an die Magnetpartikel gebunden ist und zu der Probe hinzugefügt wird, bevor die Probe mit der ersten Antikörperzusammensetzung in Kontakt gebracht wird.
4. Verfahren nach Anspruch 1, wobei die Sekundärziele Erythrozyten, Granulozyten oder Kügelchen, optional Polystyrolkügelchen, optional mit einem Polymer beschichtete Polystyrolkügelchen sind.
5. Verfahren nach Anspruch 1, wobei das Auswahlverfahren ein positives Auswahlverfahren zum Auswählen für eine gewünschte Zelle ist und die Zielzelle die gewünschte Zelle ist, oder ein negatives Auswahlverfahren zum Entfernen einer nicht gewünschten Zelle aus der Probe und die Zielzelle die nicht gewünschte Zelle ist.

6. Verfahren nach Anspruch 1, wobei die Sekundärziele eine Dichte ähnliche wie eine Zelle aufweisen und die Magnetpartikel eine von einer Zelle verschiedene Dichte aufweisen.
- 5 7. Verfahren nach Anspruch 1, wobei das Verfahren ferner Folgendes umfasst:  
d) Trennen der Zielzellen von den Immunrosette-Magnetpartikel-Komplexen.
8. Verfahren nach Anspruch 7, wobei die Zielzellen durch physikalische, chemische, enzymatische oder thermische Dissoziation von den Immunrosette-Magnetpartikel-Komplexen getrennt werden.
- 10 9. Verfahren nach Anspruch 1, wobei das Verfahren ferner Folgendes umfasst:  
d) Lyse der Erythrozyten in den Immunrosetten, und  
e) Trennung der Zielzellen von den lysierten Erythrozyten und den Magnetpartikeln.
- 15 10. Verfahren nach Anspruch 1, wobei die Probe Blut, Vollblut, Knochenmark, fetale Leber, Nabelschnurblut, eine Leukozytenfilm-Suspension, eine Leukapherese-Sonde, ein Pleura- und Peritonealerguss oder eine Probe von Thymozyten und Splenozyten ist.
- 20 11. Verfahren nach Anspruch 1, wobei die Zielzellen Stammzellen, Vorläuferzellen, Monozyten, Lymphozyten oder Granulozyten sind.
12. Verfahren nach einem der Ansprüche 1 bis 11, wobei die erste Antikörperzusammensetzung ein bi-spezifischer tetramerer Komplex ist und die zweite Antikörperzusammensetzung ein monospezifischer tetramerer Komplex ist.
- 25 13. Verfahren nach Anspruch 11, wobei die Lymphozyten T-Zellen, B-Zellen oder NK-Zellen sind, und die Granulozyten Neutrophile, Basophile oder Eosinophile sind.

### Revendications

- 30 1. Procédé de sélection destiné à séparer des cellules cibles de cellules non cibles dans un échantillon comprenant des cellules cibles, des cibles secondaires et des cellules non cibles, le procédé comprenant :
- 35 a) la mise en contact de l'échantillon avec une première composition d'anticorps comprenant (a) au moins un anticorps qui se lie aux cellules cibles dans lequel l'au moins un anticorps est lié, soit directement ou indirectement, à (b) au moins un anticorps qui se lie aux cibles secondaires, dans des conditions qui permettent à des rosettes immunes des cellules cibles et des cibles secondaires de se former ;  
b) la mise en contact de l'échantillon avec une deuxième composition d'anticorps comprenant :  
40 (a) au moins un anticorps qui se lie aux cibles secondaires soit individuellement ou au sein des rosettes immunes, dans lequel l'au moins un anticorps est lié, soit directement ou indirectement, à (b) au moins un anticorps qui se lie à des particules magnétiques dans des conditions qui permettent la liaison de particules magnétiques aux rosettes immunes et/ou aux cibles secondaires ; et  
c) la séparation des complexes rosette-immune-particule magnétique et/ou des complexes cible secondaire-particule magnétique de l'échantillon pour séparer les cellules cibles des cellules non cibles, dans lequel les  
45 complexes rosette-immune-particule magnétique et/ou les complexes cible secondaire-particule magnétique sont séparés de l'échantillon par le positionnement de l'échantillon dans un gradient de champ magnétique de force suffisante pour séparer les complexes rosette-immune-particule magnétique et/les complexes cible secondaire-particule magnétique de l'échantillon.
- 50 2. Procédé selon la revendication 1 dans lequel la deuxième composition d'anticorps est liée aux particules magnétiques avant la mise en contact avec l'échantillon.
3. Procédé selon la revendication 1 ou 2 dans lequel la deuxième composition d'anticorps est liée aux particules magnétiques et ajoutée à l'échantillon avant la mise en contact de l'échantillon avec la première composition d'anticorps.
- 55 4. Procédé selon la revendication 1, dans lequel les cibles secondaires sont des érythrocytes, des granulocytes, ou des billes, facultativement des billes de polystyrène, facultativement des billes de polystyrène revêtues d'un poly-

mère.

- 5
5. Procédé selon la revendication 1, dans lequel le procédé de sélection est un procédé de sélection positive pour sélectionner une cellule désirée et la cellule cible est la cellule désirée ou un procédé de sélection négative pour éliminer une cellule non désirée de l'échantillon et la cellule cible est la cellule non désirée.
- 10
6. Procédé selon la revendication 1, dans lequel les cibles secondaires ont une densité semblable à une cellule et les particules magnétiques ont une densité différente d'une cellule.
- 15
7. Procédé selon la revendication 1, dans lequel le procédé comprend en outre :
- d) la séparation des cellules cibles des complexes rosette immune-particule magnétique.
8. Procédé selon la revendication 7, dans lequel les cellules cibles sont séparées des complexes rosette immune-particule magnétique par une dissociation physique, chimique, enzymatique ou thermique.
- 20
9. Procédé selon la revendication 1, dans lequel le procédé comprend en outre :
- d) la lyse des érythrocytes dans les rosettes immunes et
- e) la séparation des cellules cibles des érythrocytes lysés et des particules magnétiques.
- 25
10. Procédé selon la revendication 1, dans lequel l'échantillon est du sang, du sang entier, de la moelle osseuse, du foie foetal, du sang de cordon, une suspension leuco-plaquettaire, un échantillon de leucaphérèse, une effusion pleurale et péritonéale ou un échantillon de thymocytes et de splénocytes.
- 30
11. Procédé selon la revendication 1, dans lequel les cellules cibles sont des cellules souches, des cellules progénitrices, des monocytes, des lymphocytes ou des granulocytes.
- 35
12. Procédé selon l'une quelconque des revendications 1 à 11 dans lequel la première composition d'anticorps est un complexe tétramérique bispécifique et la deuxième composition d'anticorps est un complexe tétramérique monospécifique.
- 40
- 45
- 50
- 55
13. Procédé selon la revendication 11 dans lequel les lymphocytes sont des cellules T, des cellules B ou des cellules NK et les granulocytes sont des neutrophiles, des basophiles ou des éosinophiles.

FIGURE 1

Cell separation using a combination of non-magnetic and magnetic particles

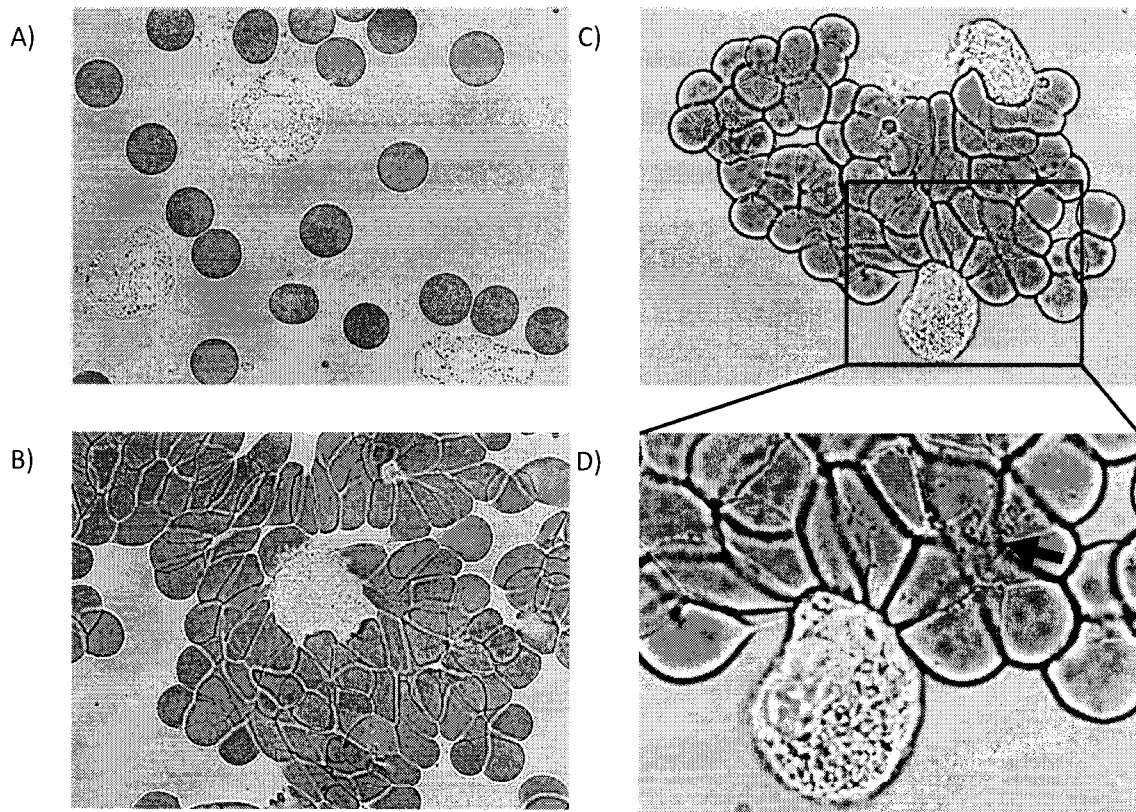
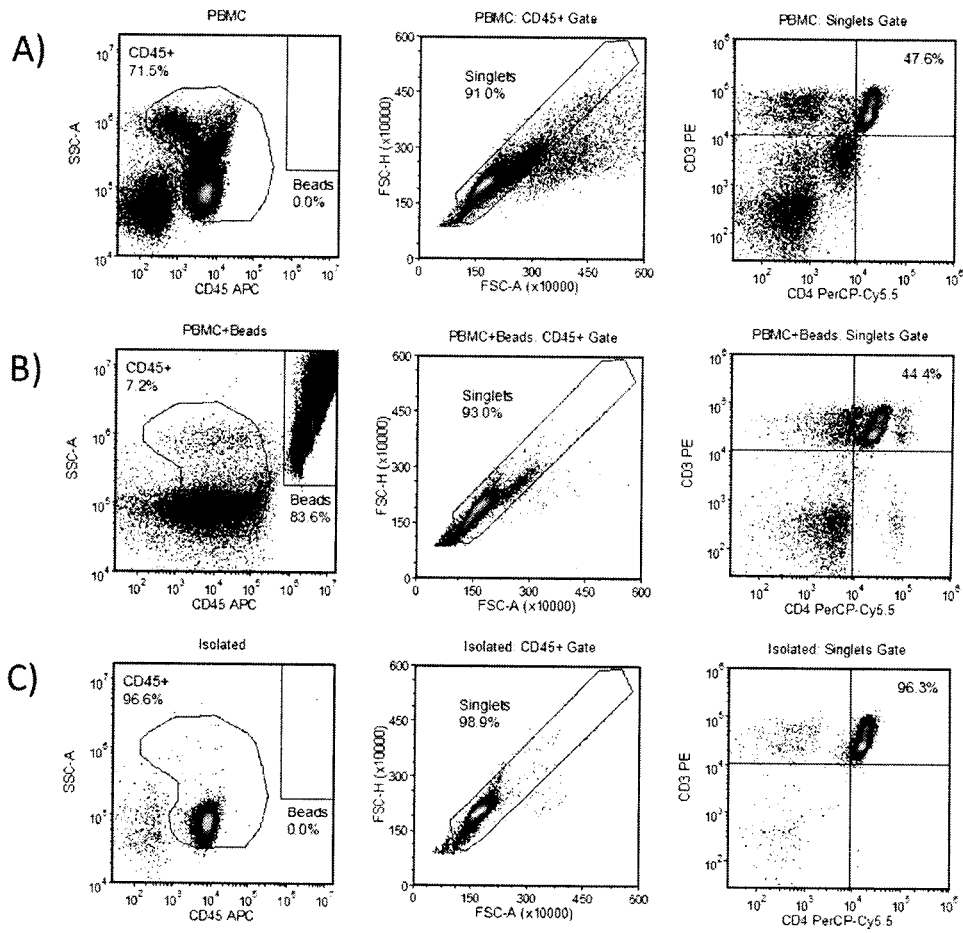


FIGURE 2

Enrichment of CD3+ CD4+ T cells from erythrocyte depleted human PBMCs with pegylated polystyrene beads



**FIGURE 3**

Enrichment of CD3+ CD4+ T cells from human peripheral whole blood using immunomagnetic cell separation of immunorosettes.

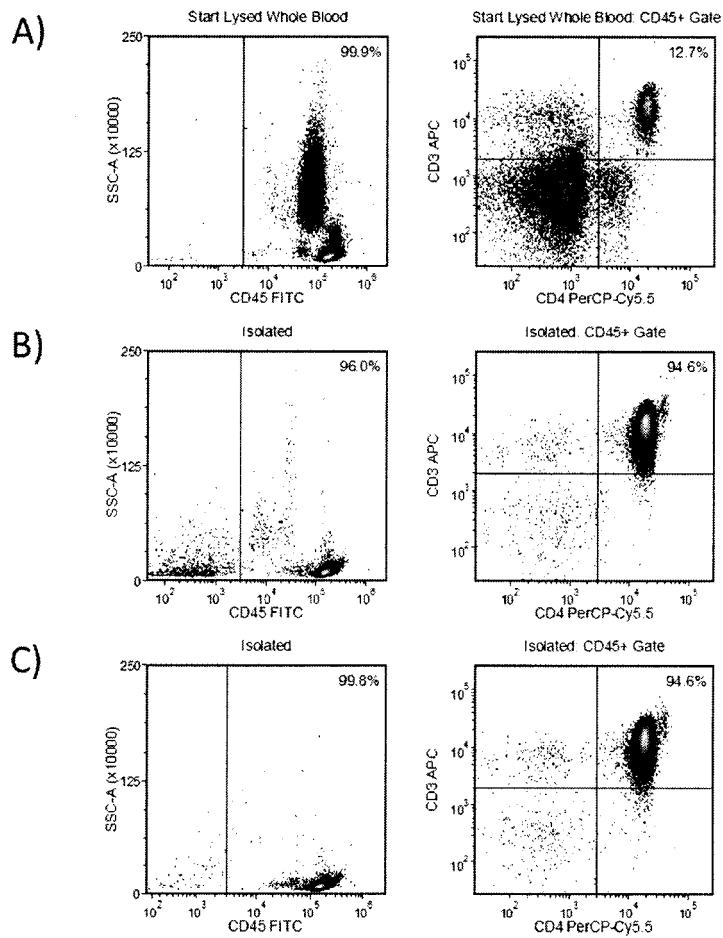


FIGURE 4

Enrichment of lymphocyte populations from human peripheral whole blood using immunomagnetic cell separation of immunorosettes.

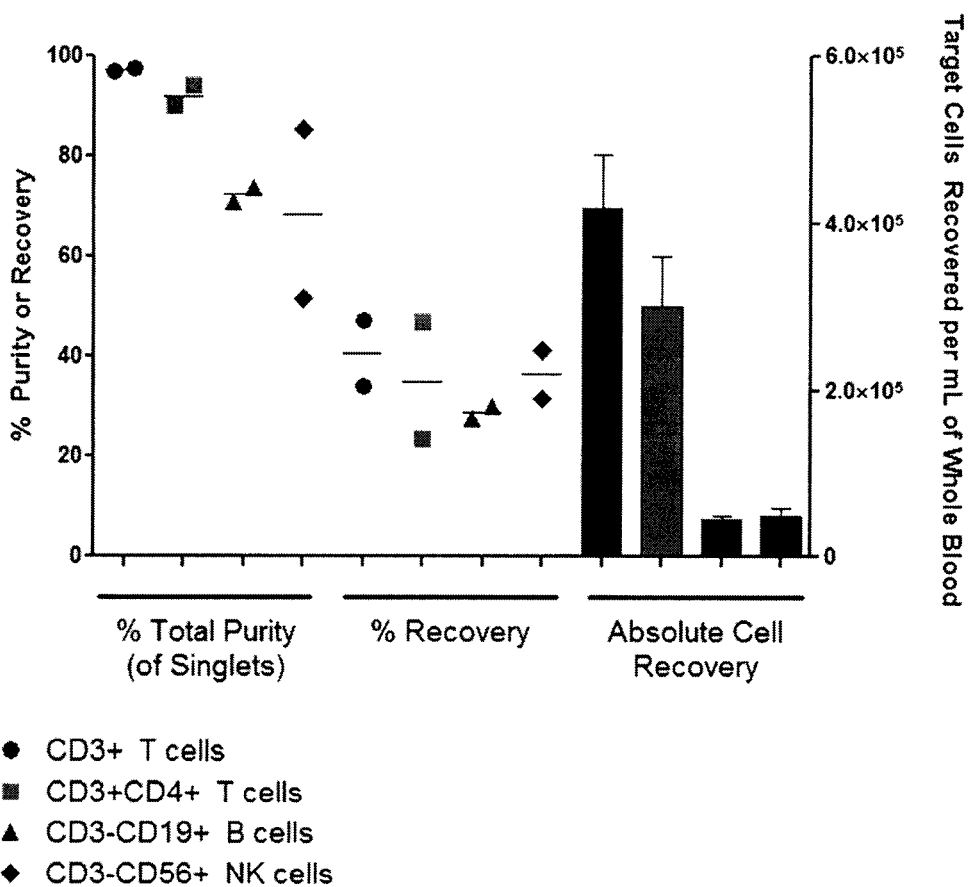
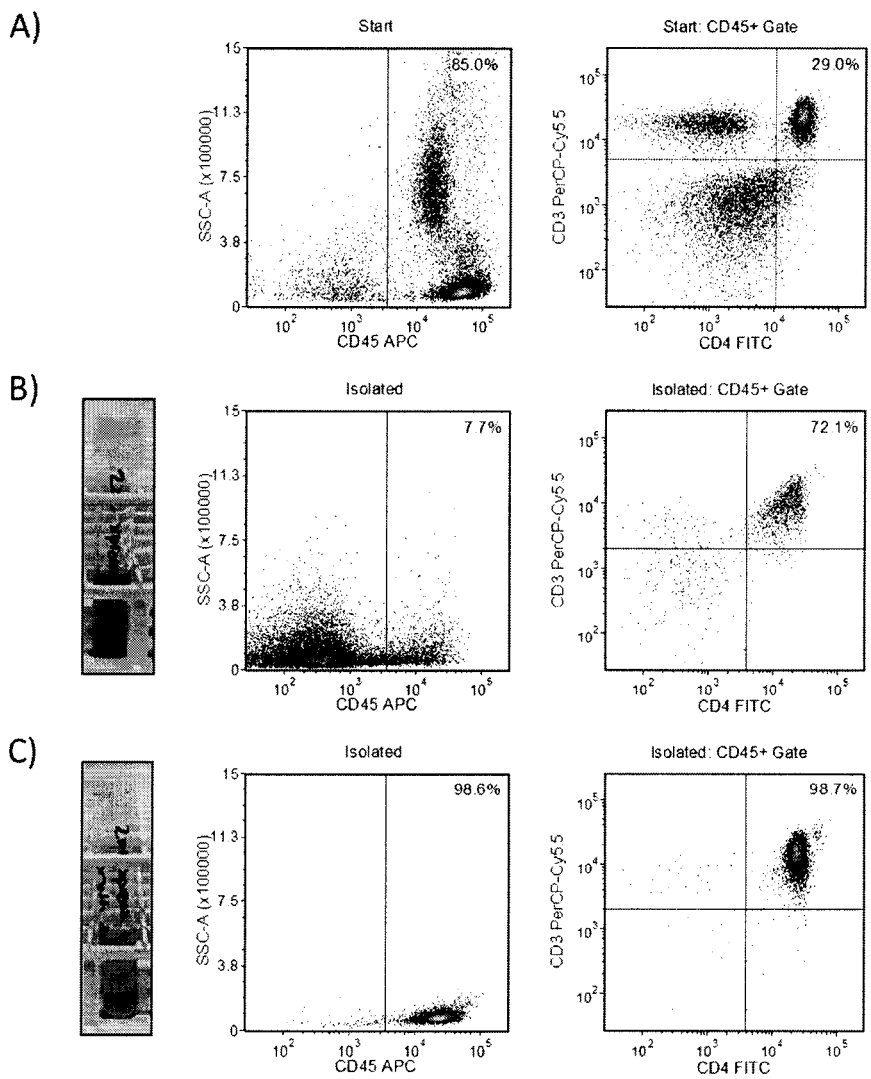


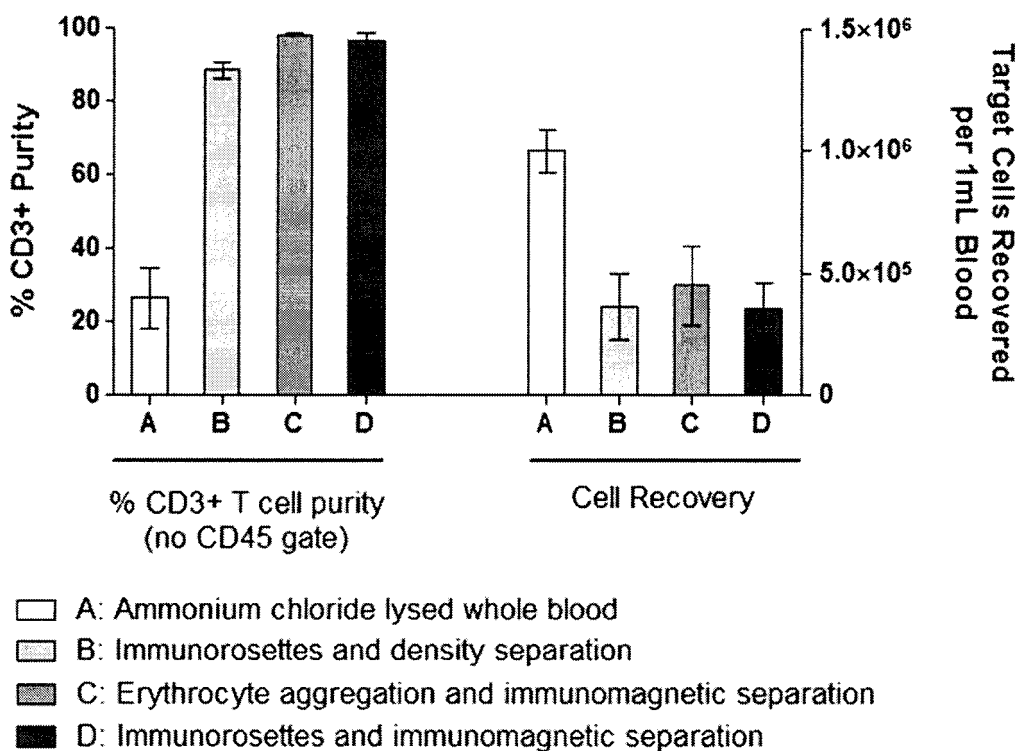
FIGURE 5

Enrichment of human CD3+ CD4+ T cells using MACSxpress with or without additional erythrocyte depletion using immunomagnetic cell separation.



**FIGURE 6**

Enrichment of CD3+ T cells from human peripheral whole blood using immunorosettes and density separation, erythrocyte aggregation and immunomagnetic separation or immunorosettes and immunomagnetic separation.



**REFERENCES CITED IN THE DESCRIPTION**

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专利名称(译)	使用免疫玫瑰花结和磁性颗粒分离细胞的方法		
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[标]申请(专利权)人(译)	干细胞技术公司		
申请(专利权)人(译)	干细胞技术股份有限公司.		
当前申请(专利权)人(译)	干细胞技术股份有限公司.		
[标]发明人	KOKAJI ANDY ISAMU		
发明人	KOKAJI, ANDY ISAMU		
IPC分类号	C12N5/078 C12N5/0783 G01N33/53 C07K16/28		
CPC分类号	A23L2/52 A23L33/10 A23L33/105 A23L33/12 A23L33/15 A23L33/155 A23L33/16 A23L33/175 A61K31/07 A61K31/122 A61K31/197 A61K31/198 A61K31/221 A61K31/355 A61K31/375 A61K31/385 A61K31/4188 A61K31/4415 A61K31/455 A61K31/51 A61K31/519 A61K31/525 A61K31/593 A61K31/714 A61K33/00 A61K33/04 A61K33/06 A61K33/30 C07K16/28 C07K16/2896 C07K16/4283 C07K2317/31 A61K2300/00 C12N5/0634 G01N1/40 G01N33/54326 G01N33/56966 G01N33/56972		
代理机构(译)	HGF LIMITED		
优先权	61/788907 2013-03-15 US 61/929674 2014-01-21 US		
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外部链接	<a href="#">Espacenet</a>		

摘要(译)

本发明涉及使用免疫玫瑰花结和磁性颗粒从非靶细胞分离靶细胞的方法。该方法包括使含有靶细胞和第二靶标(例如红细胞)的样品与抗体组合物接触,所述抗体组合物允许形成靶细胞和第二靶标的免疫玫瑰花结。随后使样品与第二抗体组合物接触,该第二抗体组合物允许磁性颗粒与形成的免疫玫瑰花结和游离的第二靶标结合。使用磁场将用磁性颗粒标记的免疫玫瑰花结和次级靶与非靶细胞分离。抗体组合物任选地含有双功能抗体或四聚体抗体复合物。

FIGURE 1

Cell separation using a combination of non-magnetic and magnetic particles

