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DESCRIPTION

[0001] The invention relates to the field of cancer diagnosis, more specifically to means and method for the monitoring of disease development during and after treatment or for the detection of minimal disseminated disease. Cytostatic or cytotoxic treatment induces remission in the majority of patients with lymphoid malignancies. Nevertheless many of these patients relapse. Apparently the current cytostatic or cytotoxic treatment protocols are not capable of killing all malignant cells in these relapsing patients, although they reached so-called complete remission according to cytomorphological criteria. Since the detection limit of cytomorphological techniques is not lower than 1-5% malignant cells, it is obvious that such techniques can only provide superficial information about the effectiveness of treatment, up to 10^{10} tumor cells still potentially remaining in the body

[0002] Techniques with a higher sensitivity to detect "minimal residual disease" or minimal disease (MRD) are needed to obtain better insight in the reduction of tumor mass during induction treatment and further eradication of the malignant cells during maintenance treatment from one or more than one tissue. The application of flow cytometry for detection of MRD is traditionally based on discrimination between malignant cells and normal leukocytes via malignancy-associated phenotypic characteristics, such as aberrant expression, overexpression, and cross-lineage expression of antigens.

[0003] Current 4-color and 6-color flow cytometry reaches a fair sensitivity of 10^{-3} (to 10^{-4}) in most patients with a hematological malignancy. However, it should be noted that the detection of low frequencies of malignant cells in blood, bone marrow and other body fluids such as cerebrospinal fluid during and after therapy and after hematopoietic stem cell transplantation can be hampered by high frequencies of normal regenerating cells. The extent and the pattern of regeneration differs per treatment protocol, per phase of treatment, per time of sampling, and seems to be dependent on the intensity of the preceding treatment: the more intensive the treatment, the more prominent the regeneration of hematopoietic cells.

[0004] Logically, both the background of regenerating cells and the drug-induced immunophenotypic shifts reduce the sensitivity and specificity of the existing 4-color and 6-color flow cytometric MRD methods. This has mainly lead to approaches in which either multiple combinations of markers are used to evaluate MRD in a patient or, alternatively to use one or a few patient-specific combinations of markers. Recognizing the need for improved diagnostic methods for MRD, the present inventors set out to identify additional markers which could be used to obtain a more sensitive and reliable assay for detecting MRD, particularly based on a fully integrated approach, in which information of multiple markers is combined via multivariate analysis. In addition, this new approach is not anymore limited to individual patients, but is applicable to every patient of a specific disease category, such B-cell precursor acute lymphoblastic leukemia (BCP-ALL), B-cell chronic lymphocytic leukemia (B-CLL) and multiple myeloma (MM).

[0005] After careful selection of the relevant markers, design of appropriate combinations of antibodies in multi-color tubes, and the selection of suited fluorochromes (based on need for brightness, compensation, stability, etc.), a set of antibody reagents was developed. The studies were complemented with extensive multicentric evaluation of the consensus panels in order to reshape and achieve an optimal efficiency. The inventors designed novel ≥ 8-color stainings with carefully selected and thoroughly tested combinations of antibodies, which can reach sensitivities of 10^{-4} to 10^{-5} Based on design-testing-redesign-retesting-redesign (etc.), specific combinations of fluorochrome-conjugated antibodies have been developed per disease category, such as BCP-ALL, B-CLL and MM. One or two ≥ 8-color combinations per patient will allow careful MRD monitoring with sensitivities of at least 10^{-4}. The provided 10-color and 12-color antibody combinations can even better discriminate between normal cells and their malignant counterparts, thereby allowing for MRD detection with sensitivities down to 10^{-5}.

[0006] Here we present novel 8-color 10-color and 12-color antibody combinations for detection of MRD in a sample, e.g. blood or bone marrow, isolated from patients with:

- B-cell precursor acute lymphoblastic leukemia (BCP-ALL)
- B-cell chronic lymphocytic leukemia (B-CLL) or
- Multiple myeloma (MM) and related plasma cell disorders (PCD).

[0007] These multi-color immunostainings can be performed according to the so-called EuroFlow protocols as described by Van Dongen et al. Leukemia 2012; 26: 1908-1075 and by Kalina et al. Leukemia 2012; 26: 1986-2010.

[0008] Accordingly, the invention provides unique reagent compositions for flow cytometric detection of MRD, comprising a

combination of at least eight distinct fluorochrome-conjugated antibodies. In particular, the reagent compositions are of use for detecting MRD in patients with BCP-ALL, B-CLL or MM/PCD. In a preferred embodiment, the composition comprises monoclonal antibodies against a given CD antigen. CD stands for cluster designation and is a nomenclature for the identification of specific cell surface antigens or intracellular antigens defined by monoclonal antibodies. (Monoclonal) antibodies against the indicated markers can be commercially obtained from various companies, including Becton/Dickinson (BD) Biosciences, Dako, Beckman Coulter, CYTOGNOS, Caltag, Pharmingen, Exbio, Sanquin, Invitrogen, and the like.

Flow cytometric MRD detection in BCP-ALL

[0009] In one embodiment, the invention provides a reagent composition for flow cytometric detection of BCP-ALL cells in a human subject, comprising a panel of at least eight distinct fluorochrome-conjugated antibodies. The BCP-ALL panel comprises antibodies against the four "core markers" CD10, CD19, CD20, CD34 and CD45. Preferably, the panel further comprises one or more antibodies selected from the group of antibodies against CD38, CD81, Cylgμ, and deoxynucleotidyl transferase (NuTdT). Very good results are obtained if the panel further comprises one or more sets of antibodies selected from (a) set of antibodies against CD66c and CD123; (b) set of antibodies against CD304 and CD73; and (c) set of antibodies against Smlgκ and Smlgλ, wherein the antibodies within each set are conjugated to the same fluorochrome. In a specific aspect, the BCP-ALL panel comprises antibodies against CD 10, CD 19, CD20, CD34, CD45, one or more antibodies selected from the group of antibodies against CD38, CD81, Cylgμ, NuTdT, and two or more sets of antibodies selected from (a) set of antibodies against CD66c and CD123; (b) set of antibodies against CD304 and CD73; and (c) set of antibodies against Smlgκ and Smlgλ, wherein the antibodies within each set are conjugated to the same fluorochrome.

[0010] For example, a reagent composition comprises distinct fluorochrome-conjugated antibodies directed against one of the following combinations of markers:

1. (i) CD20, CD45, CD81, CD66c, CD123, CD34, CD19, CD10 and CD38, wherein the antibodies against CD66c and CD123 are conjugated to the same fluorochrome;
2. (ii) CD20, CD45, CD81, CD304, CD73, CD34, CD19, CD10 and CD38, wherein the antibodies against CD304 and CD73 are conjugated to the same fluorochrome;
3. (iii) CD20, CD45, NuTdT, Smlgκ, Smlgλ, Cylgμ, CD19, CD34 and CD10,

wherein the antibodies against Smlgκ and Smlgλ are conjugated to the same fluorochrome. See for instance the 8-color BCP-ALL MRD Panel 1A.

[0011] As another example, it comprises distinct fluorochrome-conjugated antibodies directed against the markers CD20, CD45, CD81, NuTdT, CD34, CD19, CD10 and CD38, and one or more sets of antibodies selected from (a) set of antibodies against CD66c and CD123; (b) set of antibodies against CD304 and CD73; and (c) set of antibodies against Smlgκ and Smlgλ, wherein the antibodies within each set are conjugated to the same fluorochrome. See for instance the 10-color tube in Panel 1B comprising antibodies against the markers CD20, CD45, CD81, NuTdT, CD66c, CD123, CD304, CD73, CD34, CD19, CD10 and CD38.

[0012] In a further specific aspect, the composition comprises a combination of fluorochrome-conjugated antibodies directed against the markers CD20, CD45, CD81, NuTdT, CD66c, CD123, CD304, CD73, Smlgκ, Smlgλ, Cylgμ, CD34, CD19, CD10 and CD38, wherein the antibodies against each of the sets CD66c/CD123, CD304/CD73 and Smlgκ/ Smlgλ are conjugated to the same fluorochrome. See for instance the 12-color tube in panel 1C.

[0013] Suitable fluorochromes for conjugating antibodies for use in the present invention against the recited markers are known in the art. As will be understood, the fluorochromes used within a reagent composition should be distinguishable from each other by flow cytometry. The fluorochromes are preferably selected for brightness, limited spectral overlap and limited need for compensation, stability, etc (see: Kalina et al. Leukemia 2012: 26: 1986-2010).

[0014] The following panel of fluorochromes is of particular use in a BCP-ALL reagent composition according to the invention: (1) pacific blue (PacB), brilliant violet 421 (BV421) or Horizon V450, (2) pacific orange (PacO), Horizon V500 (HV500), BV510, Khrome orange (KO) or OC515, (3) fluorescein isothiocyanate (FITC) or Alexa488, (4) phycoerythrin (PE), (5) peridinin chlorophyl protein/cyanine 5.5 (PerCP-Cy5.5), PerCP or PE-TexasRed, (6) phycoerythrin/cyanine7 (PE-Cy7), (7) allophycocyanine (APC) or Alexa647, and (8) allophycocyanine/hilite 7 (APC-H7), APC-Cy7, Alexa680, APC-A750, APC-C750 or Alexa700. After multiple testing rounds, the present inventors observed that very good results can be obtained if the following fluorochromes are chosen: Pacific Blue, brilliant violet 421 or Horizon V450, PacO or Horizon V500, FITC, PE, PerCP-Cy5.5, PE-

Cy7, APC, and APC-H7 or APC-A750 or APC-C750. In a specific aspect, the invention provides for a reagent composition shown in Table 1, panel 1A, panel 1B or panel 1C.

Table 1. Exemplary reagent compositions for MRD detection in BCP-ALL..

Panel 1A. Marker Composition of 8-color BCP-ALL MRD panels of the invention								
tube	**PacB**	**PacO**	**FITC**	**PE**	**PerCPCy5.5**	**PECy7**	**APC**	**APCC750**
1	CD20	CD45	CD81	CD66c and CD123	CD34	CD 19	CD 10	CD38
2	CD20	CD45	CD81	CD304 and CD73	CD34	CD19	CD 10	CD38
2	CD20	CD45	NuTdT	SmIgκ and SmIgλ	CyIgμ	CD 19	CD34	CD10

Panel 1B. Marker Composition of 10-color BCP-ALL MRD panel of the invention									
F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**
CD20	CD45	CD81	NuTdT	CD66c and CD 123	CD304 and CD73	CD34	CD 19	CD10	CD38

Panel 1C. Marker Composition of 12-color BCP-ALL MRD panel of the invention											
F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**	**F11**	**F12**
CD20	CD45	CD81	NuTdT	CD66c and CD 123	CD304 and CD73	SmIgκ and SmIgλ	CyIg μ	CD34	CD 19	CD 10	CD38

Flow cytometric MRD detection in B-CLL

[0015] Also disclosed is a reagent composition for flow cytometric detection of B-cell chronic lymphocytic leukemia (B-CLL) in a human subject, comprising a panel of at least eight distinct fluorochrome-conjugated antibodies, the panel comprising at least antibodies against the seven "core markers" CD5, CD27, CD79b, CD3, CD200, CD81 and CD19. It was found that CD22 and/or Receptor tyrosine kinase-like orphan receptor 1 (ROR1) can be used as valuable additional marker(s). Very good results were obtained in combination with the markers CD43 and CD38.

[0016] Preferred marker combinations for detecting B-CLL are as follows:

1. (a) CD27, CD3, CD79b, CD5, CD22, CD19, CD200 and CD81
2. (b) CD5, CD3, CD79b, ROR1, CD27, CD19, CD200 and CD81
3. (c) CD27, CD3, CD79b, ROR1, CD5, CD22, CD19, CD20, CD200 and CD81
4. (d) CD27, CD3, CD79b, ROR1, CD5, CD22, CD19, CD20, CD200, CD43, CD81 and CD38.

[0017] The following panel of fluorochromes is of particular use in a CLL reagent composition : (1) pacific blue (PacB), brilliant violet 421 (BV421) or Horizon V450, (2) pacific orange (PacO), Horizon V500 (HV500), BV510, Khrome orange (KO) or OC515, (3) fluorescein isothiocyanate (FITC) or Alexa488, (4) phycoerythrin (PE), (5) peridinin chlorophyl protein/cyanine 5.5 (PerCP-Cy5.5), PerCP or PE-TexasRed, (6) phycoerythrin/cyanine7 (PE-Cy7), (7) allophycocyanine (APC) or Alexa647, and (8) allophycocyanine/hilite 7 (APC-H7), APC-Cy7, Alexa680, APC-A750, APC-C750 or Alexa700. After multiple testing rounds, the present inventors observed that very good results can be obtained if the following fluorochromes are chosen: Pacific Blue, brilliant violet 421 or Horizon V450, PacO or Horizon V500, FITC, PE, PerCP-Cy5.5, PE-Cy7, APC, and APC-H7 or APC-A750 or APC-C750.

[0018] In a specific aspect, reagent composition is shown in Table 2.

Table 2. Exemplary reagent compositions for MRD detection in B-CLL

Panel 2A. Composition of 8-color CLL MRD panel							
BV421	**BV510**	**FITC**	**PE**	**PerCPCy5. 5**	**PECy 7**	**APC**	**APCC750**
CD27	CD3	CD79b	CD5	CD22	CD 19	CD200	CD81

Panel 2A. Composition of 8-color CLL MRD panel							
BV421	**BV510**	**FITC**	**PE**	**PerCPCy5. 5**	**PECy 7**	**APC**	**APCC750**
CD5	CD3	CD79b	ROR1	CD27	CD 19	CD200	CD81

Panel 2B. Composition of 10-color CLL MRD tube									
F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**
CD27	CD3	CD79b	ROR1	CD5	CD22	CD 19	CD20	CD200	CD81

Panel 2C. Composition of 12-color CLL MRD tube											
F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**	**F11**	**F12**
CD27	CD3	CD79b	ROR1	CD5	CD22	CD19	CD20	CD200	CD43	CD81	CD38

Flow cytometric MRD detection in multiple myeloma/ptasma cell disease (MM/PCD)

[0019] A still further aspect relates to a reagent composition for detecting MM or PCD cells. The panel comprises antibodies against the four "core markers" CD 138, CD38, CD56 and CD 19, supplemented with at least four additional markers selected from the group consisting of CD27, CD117, CD81, CD229, CD45, Cylgκ and Cylgλ. CD45 is a preferred fifth marker, preferably in combination with CD27, CD 117 and CD81 or CD229, Cylgκ and Cylgλ.

[0020] Provided is a reagent composition with distinct fluorochrome-conjugated antibodies against either one of the following panels of markers:

(n) CD45 CD138CD38 CD56 CD27 CD19 CD117CD81

(o) CD45 CD138CD38 CD56 CD229CD19 Cylgκ Cylgλ

(p) CD138 CD27 CD38 CD56 CD45 CD19 CD117CD81

(q) CD138 CD27 CD38 CD56 CD229CD19 Cylgκ Cylgλ

(r) CD138 CD 27 CD38 CD56 CD45 CD19 Cylgκ Cylgλ

[0021] For example, provided is a reagent composition for flow cytometric detection of MM or PCD in a human subject, comprising a panel of at least eight distinct fluorochrome-conjugated antibodies, the panel comprising at least antibodies against the core markers CD138, CD38, CD56 and CD19, supplemented with at least four additional markers selected from the group consisting of CD27, CD117, CD81, CD229, CD45, Cylgκ and Cylgλ. Preferably, CD45 is the fifth marker, more preferably in combination with the markers CD27, CD117 and CD81, or in combination with the markers CD229, Cylgκ and Cylgλ. Preferred reagent compositions comprise distinct fluorochrome-conjugated antibodies directed against one of the following combinations of markers:

(iv) CD45, CD138, CD38, CD56, CD27, CD19, CD117 and CD81

(v) CD45, CD138, CD38, CD56, CD229, CD19, Cylgκ and Cylgλ

(vi) CD138, CD27, CD38, CD56, CD45, CD19,CD117 and CD81

(vii) CD138, CD27, CD38, CD56, CD229, CD19, Cylgκ and Cylgλ

(viii) CD138, CD27, CD38, CD56, CD45, CD19, Cylgκ and Cylgλ

[0022] See for instance the 8-color PCD MRD Panel 3A.

[0023] Very good results were obtained using fluorochrome-conjugated antibodies directed against the markers CD138, CD27, CD38, CD56, CD45, CD19, CD117, CD81 and one or both set(s) of antibodies selected from (a) set of antibodies against CD229

and CD28; and (b) set of antibodies against Cylgκ and Cylgλ. See for instance the 10-color tube in Panel 3B and the 12-color tube in Panel 3C.

[0024] The following panel of fluorochromes is of particular use in a MM/PCD reagent composition: (1) pacific blue (PacB), brilliant violet 421 (BV421) or Horizon V450, (2) pacific orange (PacO), Horizon V500 (HV500), BV510, Khrome orange (KO) or OC515, (3) fluorescein isothiocyanate (FITC) or Alexa488, (4) phycoerythrin (PE), (5) peridinin chlorophyl protein/cyanine 5.5 (PerCP-Cy5.5), PerCP or PE-TexasRed, (6) phycoerythrin/cyanine7 (PE-Cy7), (7) allophycocyanine (APC) or Alexa647, and (8) allophycocyanine/hilite 7 (APC-H7), APC-Cy7, Alexa680, APC-A750, APC-C750 or Alexa700. After multiple testing rounds, the present inventors observed that very good results can be obtained if the following fluorochromes are chosen: Pacific Blue, brilliant violet 421 or Horizon V450, PacO or Horizon V500, FITC, PE, PerCP-Cy5.5, PE-Cy7, APC, and APC-H7 or APC-A750 or APC-C750.

Table 3. Exemplary reagent compositions for MRD detection in MM/PCD

	Panel 3A. Composition of PCD MRD panel									
Tube	**PacB or BV421 or HV450**		**HV500 or PacO**	**FITC**	**PE**	**PerCPCy5.5**		**PECy7**	**APC**	**APCH7 or APCA750 or APCC750**
1	CD45		CD138	CD38	CD56	CD27		CD19	CD117	CD81
2	CD45		CD138	CD38	CD56	CD229		CD19	Cylgκ	Cylgλ
3	CD138		CD27	CD38	CD56	CD45		CD19	CD117	CD81
4	CD138		CD27	CD38	CD56	CD229		CD19	Cylgκ	Cylgλ
5	CD138		CD27	CD38	CD56	CD45		CD19	Cylgκ	Cylgλ

	Panel 3B. Composition of 10-color PCD MRD panel									
F1	**F2**		**F3**	**F4**	**F5**	**F6**	**F7**		**F8**	**F9**	**F10**
CD138	CD27		CD38	CD56	CD45	CD19	CD117		CD81	CD229	CD28
CD138	CD27		CD38	CD56	CD45	CD19	CD117		CD81	Cylgκ	Cylgλ

10	**Panel 3C. Composition of 12-color PCD MRD tube**										
F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**	**F11**	**F12**
CD138	CD27	CD38	CD56	CD45	CD19	CD117	CD81	CD229	CD28	Cylgκ	Cylgλ

[0025] A further aspect of the invention relates to a diagnostic kit for detecting MRD, in particular BCP-ALL MRD, CLL MRD or MM/PCD MRD comprising one or more of the reagent compositions described herein above, optionally together with instructions for use, buffer, and/or control samples (see: Kalina et al. Leukemia 2012: 26: 1986-2010). In one embodiment, there is provided a BCP-ALL kit comprising one or more reagent compositions of Table 1. Also provided are a CLL kit comprising one or more reagent compositions of Table 2, a PCD kit comprising one or more reagent compositions of Table 3.

[0026] The invention also relates to a method for flow cytometric detection of MRD, comprising the steps of providing a biological sample from a human subject and contacting at least a portion (aliquot) of the sample with a reagent composition provided herein. Any type of sample known or suspected to contain leukocytes may be used directly, or after lysing non-nucleated red cells, or after density centrifugation, or after cell sorting procedures. For example, the sample is peripheral blood, bone marrow, tissue sample such as lymph nodes, adenoid, spleen, or liver, or other type of body fluid such as cerebrospinal fluid, vitreous fluid, synovial fluid, pleural effusions or ascitis. Peripheral blood or bone marrow is preferred.

Provided is a multi-color flow cytometric method for detecting minimal residual disease (MRD) in a biological sample comprising cells, preferably B-lineage cells (B-cell precursors, B-lymphocytes, and plasma cells), comprising the steps of:

1. (i) staining the sample with a reagent composition according to the invention,
2. (ii) subjecting the sample to flow cytometry;
3. (iii) gating on cells for expression of the selected markers detected by the antibodies present in the reagent composition;
4. (iv) distinguishing between normal and malignant cells, based on the expression profile of the multiple markers.

Preferably, the analysis in step (iv) involves multivariate analysis, preferably principal component analysis (PCA), wherein each marker has added value in the distinction process via the principal component analysis. Advantageously, using automated population separation-APS view- is used employing for example Infinicyt software, or multidimensional scaling (MDS) analysis.
PCA is a mathematical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of uncorrelated variables called principal components. The number of principal components is less than or equal to the number of original variables. This transformation is defined in such a way that the first principal component has as high a variance as possible (that is, accounts for as much of the variability in the data as possible), and each succeeding component in turn has the highest variance possible under the constraint that it be orthogonal to (uncorrelated with) the preceding components. Principal components are guaranteed to be independent only if the data set is jointly normally distributed. PCA is sensitive to the relative scaling of the original variables. Depending on the field of application, it is also named the discrete Karhunen-Loève transform (KLT), the Hotelling transform or proper orthogonal decomposition (POD). Alternatively to PCA, MDS or any other type of well-established multivariate analysis, can be used (see: Pedreira et al. Trends Biotechnol 2013).

[0027] In one embodiment, there is provided is a multi-color flow cytometric method for detecting minimal residual disease (MRD) in a biological sample comprising lymphocytes, wherein MRD is BCP-ALL, comprising the steps of:

1. (i) staining the sample with a BCP-ALL reagent composition according to the invention, preferably a composition selected from any one of panels 1A, 1B or 1C,;
2. (ii) subjecting the sample to flow cytometry; (iii) gating on mature B cells and BCP-cells for expression of the markers detected by the antibodies present in the reagent composition; (iv) distinction between normal and malignant BCP cells, based on the application of multiple markers, each having added value in the distinction process via the principal component analysis. See examples in Example 1 and Figures 1 and 2 for an exemplary analysis allowing for detection of MRD in BCP-ALL patients.

[0028] Also provided is a multi-color flow cytometric method for detecting minimal residual disease (MRD) in a biological sample comprising lymphocytes, wherein MRD is CLL, comprising the steps of:

1. (i) staining the sample with a CLL reagent composition according to the invention, preferably a composition selected from any one of panels 2A, 2B or 2C;
2. (ii) subjecting the sample to flow cytometry; (iii) gating B-lymphocytes for expression of the markers detected by the antibodies present in the reagent composition; (iv) distinction between normal and malignant B cells, based on the application of multiple markers, each having added value in the distinction process via the principal component analysis.

See examples in Example 2 and Figures 3 and 4 for an exemplary analysis allowing for detection of MRD in CLL patients.

[0029] Also provided is a multi-color flow cytometric method for detecting minimal residual disease (MRD) in a biological sample comprising lymphocytes, wherein MRD is MM/PCD, comprising the steps of:

1. (i) staining the sample with a MM/PCD reagent composition according to the invention, preferably a composition selected from any one of panels 3A, 3B or 3C;
2. (ii) subjecting the sample to flow cytometry; (iii) gating plasma cells for expression of the markers detected by the antibodies present in the reagent composition; (iv)

distinction between normal and malignant plasma cells, based on the application of multiple markers, each having added value in the distinction process via the principal component analysis.
See examples in Example 3 and Figures 5 and 6 for an exemplary analysis allowing for detection of MRD in MM/PCD patients.

LEGEND TO THE FIGURES

[0030]

Figure 1. Typical example of how to use the CD19 and CD45 identification markers in combination with SSC (Panel A to C) for the distinction between BCP cells and other nucleated cells in a bone marrow sample from a BCP-ALL patient during therapy. In each plot, light grey events correspond to non-B cells in the sample, while dark grey events are mature B-cells and black events BCP cells. In the multivariate analysis representation (APS1) of principal component 1 vs. principal component 2 (Panel D), both mature B cells and BCP cells are clearly separated from all other events based on all informative parameters (e.g. CD19, CD45, SSC).

Figure 2.- Illustrating example of how to use immunophenotypic characterization markers CD10, CD20, CD34, CD66c/CD123, and CD38 in combination with SSC for the distinction between BCP-ALL cells and normal residual B-cells in a bone marrow sample from a BCP-ALL patient during therapy (Panels A to D). Only bone marrow B-cells are displayed, after gated/selected as described in Figure 1. In each plot, black dots correspond to BCP-ALL cells in the sample, while grey dots are normal B-cells. Compared to normal B-cells, BCP-ALL cells show overexpression of CD81 (Panel D), CD 10 (Panels A and D), and CD66c/CD123 (Panel C). In the APS1 (principal component 1 versus principal component 2) representation based on all immunophenotypic markers and scatter characteristics evaluated (Panel E), normal residual B-cells (grey) are clearly discriminated from BCP-ALL cells (black).

Figure 3.- Illustrating example of how to use the CD19 and CD3 identification markers in combination with SSC (Panels A to C) for the distinction between mature B-cells and other nucleated cells in a peripheral blood sample from a CLL patient. In each plot, grey events correspond to non-B-cells in the sample, while black events are total peripheral blood B-cells. In the multivariate analysis representation (APS1) of principal component 1 vs. principal component 2 (Panel D), B-cells are clearly separated from all other events based on all informative parameters (e.g. CD19, CD3, SSC).

Figure 4.- Illustrating example of how to use immunophenotypic characterization markers CD27, CD5, CD22, CD200 and CD79b (panels A to C) for the distinction between CLL cells and normal mature B-cells in a peripheral blood sample from a CLL patient. Only peripheral blood B-cells are displayed, after gated/selected as described in Figure 3. In each plot, black dots correspond to CLL cells in the sample, while grey dots are normal peripheral blood B-cells. Compared to normal B-cells, CLL cells show underexpression of CD22 (Panel B) and CD79b (Panel C) together with overexpression of CD200 (Panel B) and CD5 (Panels A and C). In the APS1 (principal component 1 vs. principal component 2) representation based on all immunophenotypic markers and scatter characteristics evaluated (Panel D), CLL cells are clearly discriminated from normal B-cells, while this degree of discrimination could not be achieved based on individual markers.

Figure 5.- Illustrating example of how to use the CD38 and CD 138 identification markers in combination with SSC (Panel A to C) for the distinction between plasma cells and other nucleated cells in a bone marrow sample of an MM patient after therapy. In each plot, grey dots correspond to non-plasma cells in the sample, while black dots are total bone marrow plasma cells. In the multivariate analysis representation (APS1) of principal component 1 vs. principal component 2 (Panel D), plasma cells (black dots) are clearly separated from all other events (grey dots) based on all informative parameters (e.g. CD138, CD38, SSC).

Figure 6.- Illustrating example of how to use immunophenotypic characterization markers CD81, CD19, CD45, CD56, CD27, CD117 and CD38, in combination with SSC (Panels A to D) for the distinction between myeloma/malignant plasma cells and normal residual plasma cells in a bone marrow sample of an MM patient after therapy. Only bone marrow plasma cells are displayed, after gated/selected as described in Figure 5. In each plot, black dots correspond to myeloma/clonal plasma cells in the sample, while grey dots are normal residual bone marrow plasma cells. Compared to normal plasma cells, myeloma/clonal plasma cells show underexpression of CD81, CD19, CD45, CD27 and CD38 together with higher SSC and overexpression of CD56 and CD117. In the APS1 (principal component 1 vs. principal component 2) representation (Panel E) based on all immunophenotypic markers and scatter characteristics evaluated normal residual plasma cells (grey dots) are clearly discriminated from myeloma/malignant plasma cells (black dots), while this degree of discrimination could not be achieved based on individual markers.

EXPERIMENTAL SECTION

[0031] The power of the EuroFlow approach disclosed herein is based on the combination of sets of markers and the usage of multivariate analyses for both the identification of normal cells (e.g. normal precursor B-cells, normal B-lymphocytes and normal plasma cells) and the distinction between normal/reactive cells vs. clonal/malignant cells. For this purpose, a powerful multivariate analysis of the contribution of individual markers for inclusion and exclusion of each marker in the panel according to its contribution over all other markers in the combination. Such a strategy was used to evaluate the selected combinations of most discriminating markers in multiple sequential rounds of experimental testing. Because of this the final proposed antibody combinations became extremely strong when used in combination with the principal component analysis, specifically with the automated population separation (APS) tool of the Infinicyt software, so that the added (independent) value of each marker is used in a single step of analysis.

[0032] Herewith we provided the summary of the results of the extensive experimental studies for MRD detection in blood and bone marrow of patients with BCP-ALL (Example 1), CLL (Example 2) and Multiple Myeloma (Example 3).

[0033] In the Examples below, lists of markers are provided together with the most frequent phenotypic aberration of these markers in case of BCP-ALL, CLL, and MM/PCD. However, it should be noted that the real discrimination power between normal and malignant cells is based on combinations of markers in the corresponding n-dimensional space, as is clearly visible in the principle component analyses in the figures of Examples 1 to 3. In fact, minor differences of several markers add up to a larger difference in principle component analysis. Therefore the current invention is not about single marker studies for MRD detection, but about carefully selected sets of markers, that allow excellent discrimination between normal cells and their malignant counterparts, such as normal BCP cells vs. BCP-ALL blasts, normal B-lymphocytes vs. B-CLL cells, and normal plasma cells vs. MM/PCD plasma cells.

EXAMPLE 1. Antibody panels and diagnostic method for MRD detection in BCP-ALL patients

Markers for identification of total B cells and B-cell precursors in the bone marrow

List of relevant identification markers: CD 19, CD45

[0034] How to use them: Pre-gating using the CD19 marker is essential for identifying a pure B-cell population. To focus on normal B-cell precursors (BCP), CD45-negativity or weak positivity can be used to discriminate BCP from CD45-positive mature B-cells. In case of CD19-directed therapies, CD19 might be replaced by CD22. These markers may be used in combination also with sideward light scatter (SSC) or forward light scatter (FSC) or both FSC and SSC to identify B-cells in peripheral blood or bone marrow or other types of samples (e.g. bone marrow, tissue biopsy, spinal fluid). Of note, other markers, like CD10, CD20, CD38 and CD34, which are used for discriminating BCP-ALL cells from normal BCP cells (see below), may also contribute to the gating of the total BCP cell population (e.g. CD34+, CD10+, CD20-to dim, CD38+).

Markers for distinguishing normal vs. malignant B-cell precursor cells

List of markers and most frequent phenotypic aberration:

[0035]

CD38:
underexpressed in BCP-ALL/malignant vs. normal B-cell precursor cells

CD10:
over- or underexpressed in BCP-ALL/malignant B-cell precursor cells

CD45:
underexpressed (usually negative) in BCP-ALL/malignant vs. normal B-cell precursor cells

CD20:
under- or overexpressed in BCP-ALL/ malignant vs. normal B-cell precursor cells

CD81:
over- or underexpressed in BCP-ALL/ malignant vs. normal B-cell precursor cells

CD66c:
overexpressed in BCP-ALL/ malignant vs. normal B-cell precursor cells (particularly BCR-ABL positive ALL; generally negative in TEL-AML1-posiitve or MLL-AF4-positive ALL)

CD123:
overexpressed in BCP-ALL/ malignant vs. normal B-cell precursor cells (particularly in hyperdiploid ALL)

CD304:
overexpressed in BCP-ALL/ malignant vs. normal B-cell precursor cells

CD73:
overexpressed in BCP-ALL/ malignant vs. normal B-cell precursor cells

CD34:
under- or overexpressed in BCP-ALL/ malignant vs. normal B-cell precursor cells

SSC:
increased or decreased intensity in BCP-ALL/ malignant vs. normal B-cell precursor cells.
FSC:
increased or decreased intensity in BCP-ALL/ malignant vs. normal B-cell precursor cells.

EXAMPLE 2. Antibody nanels and diagnostic method for MRD detection in CLL patients.

Markers for identification of total B-cells in peripheral blood and bone marrow:

List of identification markers: CD19, CD3 (exclusion marker)

[0036] How to use them: Pre-gating using this marker combination is essential for identifying a pure B-cell population, and removing T-cell/B-cell doublets. These markers may be used in combination also with sideward light scatter (SSC) or forward light scatter (FSC) or both FSC and SSC to identify B-cells in peripheral blood or bone marrow or other types of samples (e.g. tissue biopsy, spinal fluid). For a more refined gating with better enrichment of CLL cells, both CD5 and CD27 may be used.

Markers for distinguishing normal B-cells from CLL cells:

List of markers and most frequent phenotypic aberration:

[0037]

CD27: positive on CLL cells and a small fraction of normal B-cells

CD5: positive on CLL cells and a small fraction of normal B-cells

CD79b: underexpressed on CLL cells as compared to normal transitional and mature B-lymphocytes

CD22: underexpressed on CLL cells as compared to normal transitional and mature B-lymphocytes

CD20: underexpressed on CLL cells as compared to normal transitional and mature B-lymphocytes

CD200: overexpressed on CLL cells as compared to normal transitional and mature B-lymphocytes

ROR1: overexpressed on CLL cells as compared to normal transitional and mature B-lymphocytes

CD43: overexpressed on CLL cells as compared to normal transitional and mature B-lymphocytes

CD81: underexpressed on CLL cells as compared to B-cell precursors and both transitional and mature B-lymphocytes

CD38: underexpressed on CLL cells as compared to B-cell precursors

EXAMPLE 3. Antibody panels and diagnostic method for MRD detection in MM/PCD patients

Markers for identification of total plasma cells in the bone marrow:

[0038] List of identification markers: CD38, CD 138 and CD229
How to use them: Any combination of the three markers in any fluorochrome position works; also it is possible to use any

combinations of two of the three markers or in a subset of cases (not all) even one of the three markers alone. Preferable combinations are order as follows: 1) CD138/CD38/CD229; 2) CD138/CD38, 3) CD138/CD229; 4) CD38/CD229; 5) CD138; 6) CD38); 7) CD229. Note that any of these markers individually and in combination may be used in combination also with sideward light scatter (SSC) or forward light scatter (FSC) or both FSC and SSC to identify plasma cells in the bone marrow or other types of samples (e.g. peripheral blood, tissue biopsy, spinal fluid).

Markers for distinguishing normal vs clonal/malignant plasma cells:

List of markers and most frequent phenotypic aberration:

[0039]

CD38: underexpressed in malignant plasma cells compared to normal plasma cells

CD27: underexpressed in malignant plasma cells compared to normal plasma cells

CD45: underexpressed in malignant plasma cells compared to normal plasma cells

CD 19: underexpressed (usually negative) in malignant plasma cells compared to normal plasma cells

CD81: underexpressed in malignant plasma cells compared to normal plasma cells

CD56: overexpressed in malignant plasma cells compared to normal plasma cells

CD28: overexpressed in malignant plasma cells compared to normal plasma cells

CD 117:overexpressed in malignant plasma cells compared to normal plasma cells

Cylgk and Cylglambda: expression restricted to either one or the other Ig light chains in malignant plasma cells while showing a balanced distribution (Cylgk/Cylglambda ratio in normal plasma cells usually ranging between ratios 3 and 0.5).

SSC: increased or decreased intensity in malignant plasma cells compared to normal plasma cells.

FSC: increased or decreased intensity in malignant plasma cells compared to normal plasma cells.

REFERENCES CITED IN THE DESCRIPTION

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Non-patent literature cited in the description

- **VAN DONGEN et al.**Leukemia, 2012, vol. 26, 1908-1075 [0007]
- **KALINA et al.**Leukemia, 2012, vol. 26, 1986-2010 [0007] [0013] [0025]
- **PEDREIRA et al.**Trends Biotechnol, 2013, [0026]

Patentkrav

1. Reagenssammensætning til flow-cytometrisk detektering af B-celle precursor ALL (BCP-ALL) hos et menneskeligt individ, hvilken sammensætning omfatter et panel på mindst otte forskellige fluorchrom-konjugerede antistoffer, hvilket panel omfatter mindst antistoffer mod kernemarkørerne CD10, CD19, CD20, CD34 og CD45, og hvor panelet endvidere omfatter et eller flere antistoffer, der er udvalgt fra gruppen af antistoffer mod CD38, CD81, CyIgμ og deoxynucleotidyl-transferase (NuTdT), og hvor panelet endvidere omfatter et eller flere sæt antistoffer, der er udvalgt fra
   (a) sæt antistoffer mod CD66c og CD123;
   (b) sæt antistoffer mod CD304 og CD73 og
   (c) sæt antistoffer mod SmIgκ og SmIgλ,
   hvor antistofferne i hvert sæt er konjugeret til det samme fluorchrom, og hvor fluorchromerne kan skelnes mellem de forskellige sæt.

2. Reagenssammensætning ifølge krav 1, hvor panelet omfatter to eller flere sæt antistoffer udvalgt fra
   (a) sæt antistoffer mod CD66c og CD123;
   (b) sæt antistoffer mod CD304 og CD73 og
   (c) sæt antistoffer mod SmIgκ og SmIgλ.

3. Reagenssammensætning ifølge krav 1 eller 2, der omfatter forskellige fluorchrom- konjugerede antistoffer, der er rettet mod én af følgende kombinationer af markører:
   (i) CD20, CD45, CD81, CD66c, CD123, CD34, CD19, CD10 og CD38, hvor antistofferne mod CD66c og CD123 er konjugeret til det samme fluorchrom;
   (ii) CD20, CD45, CD81, CD304, CD73, CD34, CD19, CD10 og CD38, hvor antistofferne mod CD304 og CD73 er konjugeret til det samme fluorchrom;
   (iii) CD20, CD45, NuTdT, SmIgκ, SmIgλ, CyIgμ, CD19, CD34 og CD10, hvor antistofferne mod SmIgκ og SmIgλ er konjugeret til det samme fluorchrom.

4. Reagenssammensætning ifølge krav 3, der omfatter forskellige fluorchrom-konjugerede antistoffer, der er rettet mod markørerne CD20, CD45, CD81, NuTdT, CD66c, CD123, CD304, CD73, CD34, CD19, CD10 og CD38, og et eller flere sæt antistoffer udvalgt fra
   (a) sæt antistoffer mod CD66c og CD123;
   (b) sæt antistoffer mod CD304 og CD73 og
   (c) sæt antistoffer mod SmIgκ og SmIgλ,
   hvor antistofferne i hvert sæt er konjugeret til det samme fluorchrom.

5. Reagenssammensætning ifølge krav 4, der omfatter forskellige fluorchrom-konjugerede antistoffer dirigeret mod markørerne CD20, CD45, CD81, NuTdT, CD66c, CD123, CD304, CD73, SmIgκ, SmIgλ, CyIgμ, CD34, CD19, CD10 og CD38, hvor antistofferne mod hvert sæt CD66c/CD123, CD304/CD73 og SmIgκ/SmIgλ er konjugeret til det samme fluorchrom.

6. Diagnostisk kit til flow-cytometrisk detektering af minimal restsygdom (MRD), der omfatter mindst én reagenssammensætning ifølge kravene 1-5, eventuelt sammen med anvendelsesinstruktionerne, buffer og/eller kontrolprøver.

7. Multifarvet flow-cytometrisk fremgangsmåde til detektering af minimal restsygdom (MRD) i en biologisk prøve, der omfatter celler, fortrinsvis lymfocytter, der omfatter trinnene med:

(i) farvning af prøven med en reagenssammensætning ifølge et hvilket som helst af kravene 1-5,

(ii) udsættelse af prøven for flow-cytometri;

(iii) sortering og gruppering af celler til ekspression af de udvalgte markører, der er detekteret af antistofferne til stede i reagenssammensætningen; og

(iv) skelnen mellem normale og maligne celler baseret på de multiple markørers ekspressionsprofil.

8. Fremgangsmåde ifølge krav 7, hvor trin (iv) involverer multivariat analyse, fortrinsvis principal komponentanalyse (PCA).

DRAWINGS

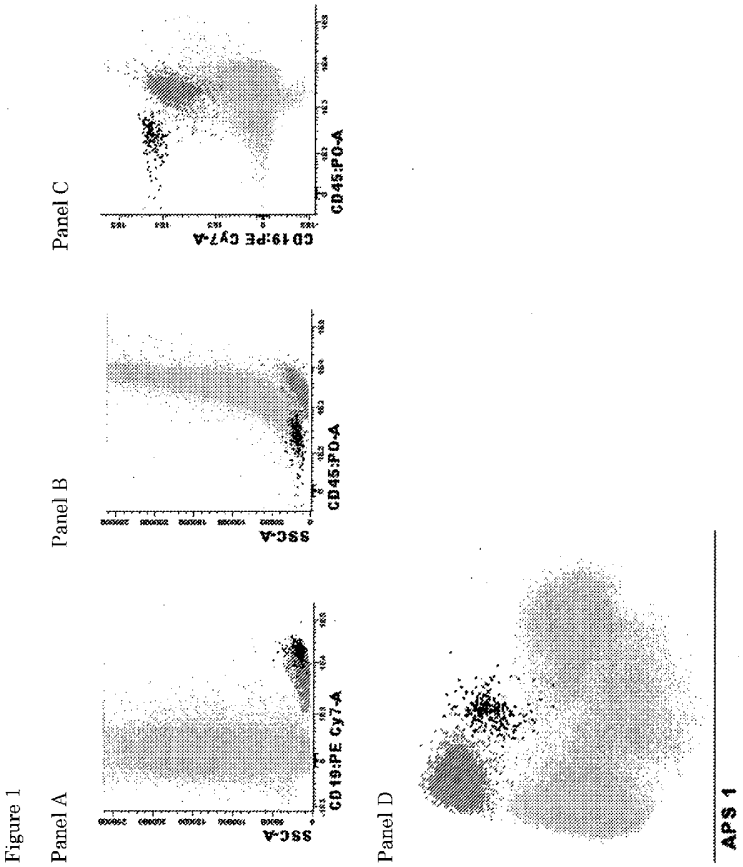
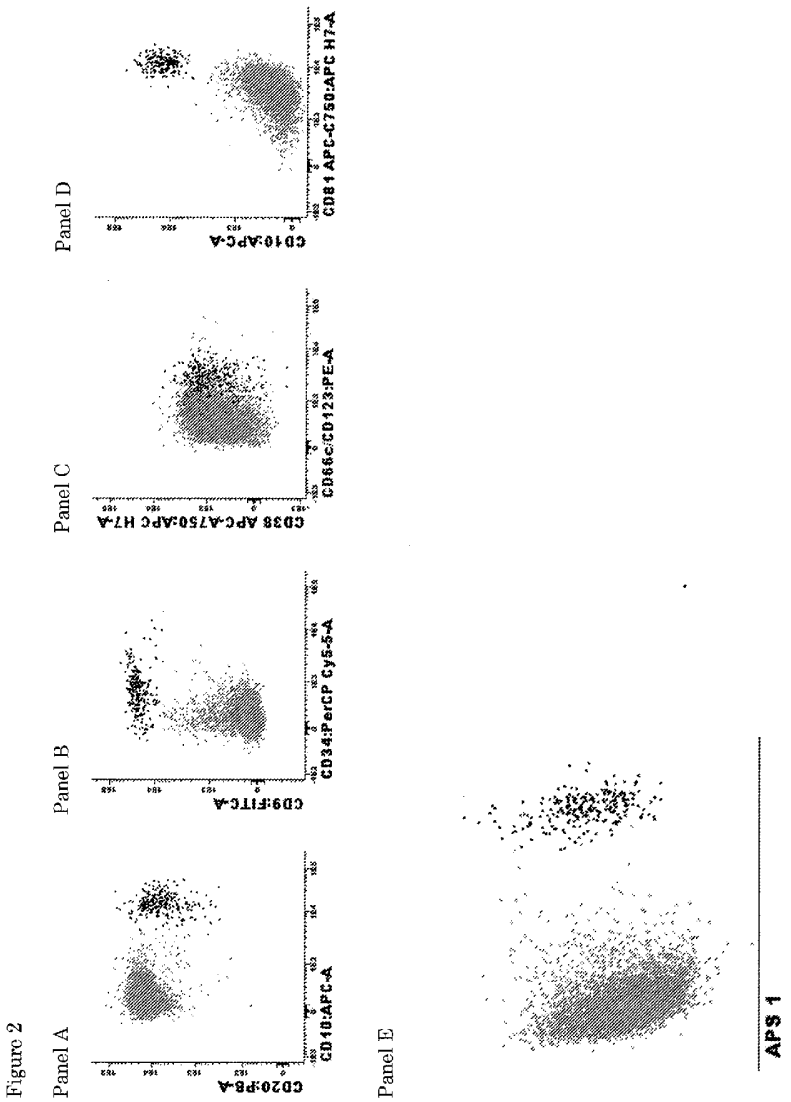


Figure 1

Figure 2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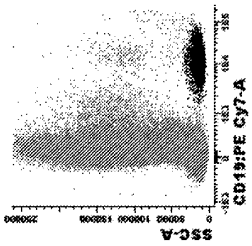
CD10-APC-A
Panel B
CD9-FITC-A
CD34-PerCP Cy5-5-A
Panel C
CD38 APC-A750-APC H7-A
CD66c/CD123-PE-A
Panel D
CD10-APC-A
CD81 APC-C750-APC H7-A
Panel E
APS 1

Figure 3

Panel A



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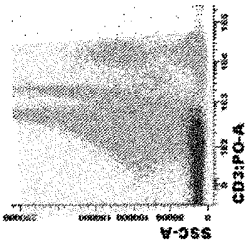

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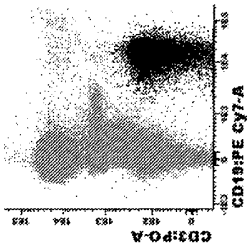


Panel D

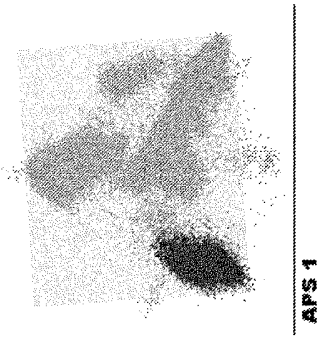
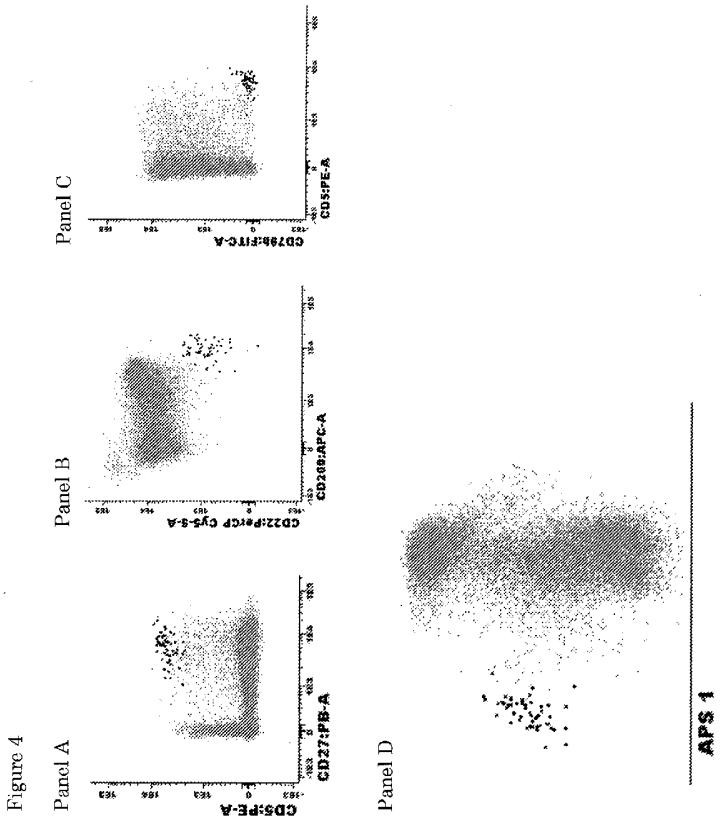


Figure 4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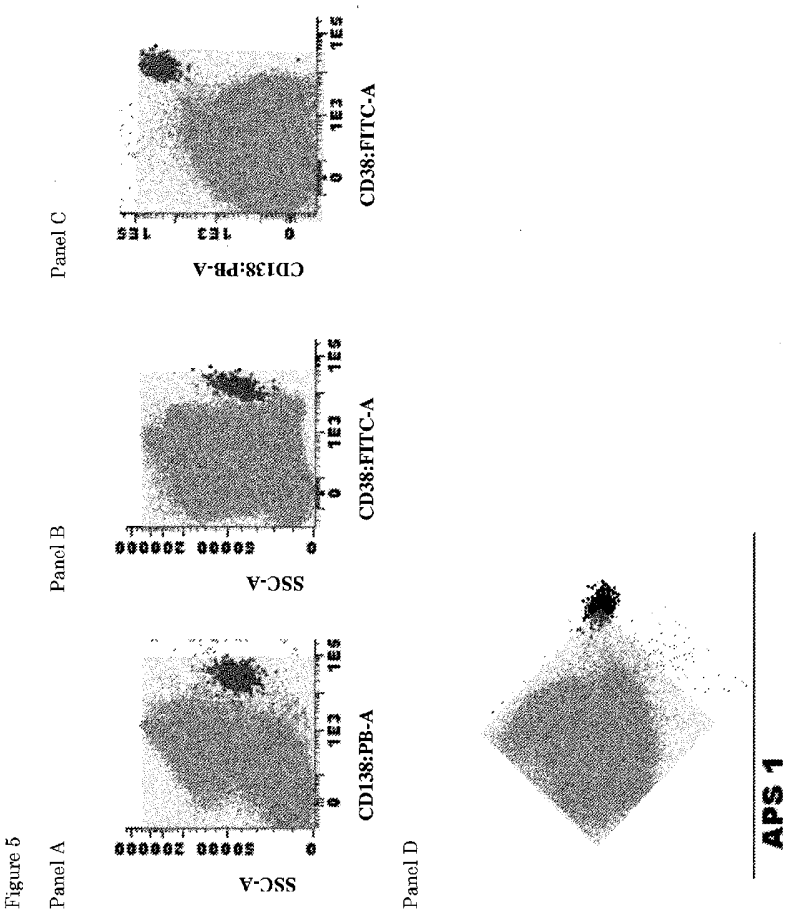
CD27:PB-A
Panel B
CD22:PerCP Cy5-5-A
CD268:APC-A
Panel C
CD79b:FITC-A
CD5:PE-A
Panel D
APS 1

Figure 5


Panel A
SSC-A
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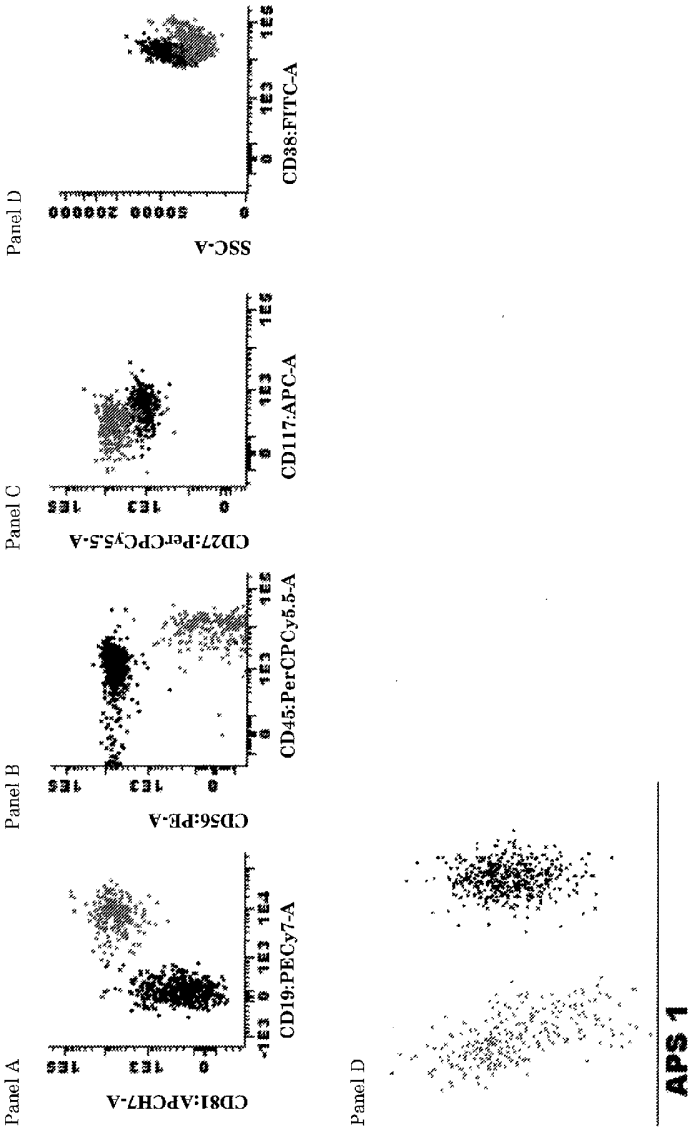
Panel B
SSC-A
CD38:FITC-A
Panel C
CD138:PB-A
CD38:FITC-A
Panel D
APS 1

Figure 6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Panel B
CD56:PE-A
CD45:PerCPCy5.5-A
Panel C
CD27:PerCPCy5.5-A
CD117:APC-A
Panel D
SSC-A
CD38:FITC-A
Panel D
APS 1