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(54) **CELLULES SOUCHES HEMATOPOIETIQUES**

(54) **HEMATOPOIETIC STEM CELLS**

(57) La présente invention concerne des cellules souches hématopoïétiques humaines appelées CD34-Lin- et CD34-CD38-Lin- et des procédés d'isolement et d'utilisation de ces cellules dans des compositions et dans des méthodes de reconstitution d'une population cellulaire déficiente ou absente. La présente invention concerne également une population de cellules souches hématopoïétiques humaines pouvant être isolées et modifiées génétiquement et destinées à être introduites chez un patient humain, afin de corriger divers dysfonctionnements génétiques, mises en culture et encore différenciées in vitro pour obtenir une nouvelle population de cellules.

(57) The present invention relates to human hemotopoietic stem cells characterized as CD34-Lin- and CD34-CD38-Lin- and to methods of isolating and using such cells in compositions and in methods for the reconstitution of a deficient or missing cell population. The present invention also provides a population of human hematopoietic stem cells that can be isolated and genetically altered for introduction in a human patient, to correct various genetic disorders and cultured and further differentiated in vitro to provide a new population of cells.

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(21) International Application Number: PCT/CA98/01012 (22) International Filing Date: 30 October 1998 (30.10.98) (30) Priority Data: 2,219,869 31 October 1997 (31.10.97) CA (71) Applicant (for all designated States except US): HSC RESEARCH AND DEVELOPMENT LIMITED PART- NERSHIP [CA/CA]; Suite 5270, 555 University Avenue, Toronto, Ontario M5G 1X8 (CA). (72) Inventors; and (75) Inventors/Applicants (for US only): DICK, John, E. [CA/CA]; Suite 5270, 555 University Avenue, Toronto, Ontario M5G 1X8 (CA). BONNET, Dominique [CA/CA]; Suite 5270, 555 University Avenue, Toronto, Ontario M5G 1X8 (CA). BHATIA, Mickie [CA/CA]; Suite 5270, 555 University Avenue, Toronto, Ontario M5G 1X8 (CA). (74) Agent: RAE, Patricia, A.; Sim & McBurney, 6th floor, 330 University Avenue, Toronto, Ontario M5G 1R7 (CA).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: HEMATOPOIETIC STEM CELLS (57) Abstract The present invention relates to human hemotopoietic stem cells characterized as CD34-Lin- and CD34-CD38-Lin- and to methods of isolating and using such cells in compositions and in methods for the reconstitution of a deficient or missing cell population. The present invention also provides a population of human hematopoietic stem cells that can be isolated and genetically altered for introduction in a human patient, to correct various genetic disorders and cultured and further differentiated <i>in vitro</i> to provide a new population of cells.		

Hematopoietic Stem Cells

Field of the Invention

The present invention relates to human hematopoietic stem cells and to
5 methods of isolating and using such cells in compositions and in methods for
the reconstitution of a deficient or missing cell population. The present
invention also provides a population of human hematopoietic stem cells that
can be isolated and genetically altered for introduction in a human patient to
correct various genetic disorders.

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Background of the Invention

The mammalian hematopoietic system consists of a heterogeneous array
of cells ranging from large numbers of differentiated cells with defined function
to rare pluripotent stem cells with extensive developmental and proliferative
15 potential (1, 2, 3). The defining feature of a stem cell is its ability to repopulate
the hematopoietic system of a recipient after transplantation. Stem cells are
playing an increasingly important role in clinical and commercial applications,
as the role of stem cells in transplantation widens. Identification and
purification of stem cells is essential both to determine the cellular and
20 molecular factors that govern stem cell development and for the application of
clinical procedures including stem cell transplantation and gene therapy.

Cell surface expression of the CD34 antigen was thought to be the
distinguishing feature of stem cells because CD34 is downregulated as stem
cells differentiate into more abundant mature cells (4), and CD34 has been used
25 as a basis for isolation of stem cells. CD34, however, does not mark stem cells
exclusively, since 1% of bone marrow cells are CD34+ and include clonogenic
progenitors that are not able to repopulate the hematopoietic system after
transplantation. Other markers such as Thy-1 can be combined with CD34 to
positively select for a cell fraction more enriched in stem cells (5, 6, 7).

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Conversely, the CD34+ cell fraction can be enriched by eliminating cells that express markers that are expressed on non-repopulating cells (e.g. lineage antigens). Nevertheless, all current clinical and experimental protocols utilizing human stem cells, including *ex vivo* culture, gene therapy and bone marrow transplantation, focus on CD34+ cells.

There have been reports (8, 9, 10) of murine CD34- hematopoietic stem cells which are capable of long term repopulation. For human hematopoietic stem cells, however, the CD34+ antigen has been regarded as a stem cell marker without exception.

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Summary of the Invention

The present inventors have identified and isolated a population of human hematopoietic stem cells which do not express CD34 (CD34-), CD38 (CD38-) or lineage specific markers (Lin-) and which are able to generate, by proliferation and differentiation, multiple lineages of the human hematopoietic system, as evidenced by their ability to produce multilineage human hematopoietic engraftment of immune-deficient NOD/SCID mice after transplantation. Moreover, the repopulative capacity and the differentiative capacity of the CD34-CD38-Lin- cells can be stimulated by *in vitro* culture of these cells.

20

According to an object of the present invention there is provided a substantially homogeneous population of human hematopoietic stem cells which are CD34-Lin-.

According to another object of the present invention there is provided a substantially homogeneous population of human hematopoietic stem cells which are CD34-CD38-Lin-.

25

According to another object of the present invention there is provided a therapeutic composition comprising an effective amount of CD34- Lin- human hematopoietic stem cells and a pharmaceutically acceptable carrier.

According to another object of the present invention there is provided a therapeutic composition comprising an effective amount of CD34-CD38-Lin-human hematopoietic stem cells and a pharmaceutically acceptable carrier.

According to yet a further object of the present invention there is
5 provided a method for obtaining a substantially enriched population of CD34-Lin- human hematopoietic cells comprising the steps of:

- removing mononuclear cells expressing some lineage-specific antigens from a sample of hematopoietic cells;
- combining the resultant hematopoietic cells with labeled antibodies to
10 which bind specifically to CD34+; and
- isolating the unbound CD34-Lin- cells.

According to still another object of the present invention there is provided a method for reconstituting hematopoiesis in an immunocompromised subject, the method comprising administering to a subject a composition
15 comprising an enriched population of CD34- Lin- stem cells.

According to a further object of the present invention there is provided a method for introducing CD34-Lin- stem cells in a mammal, said method comprising the steps of:

- providing an enriched population of CD34-Lin- stem cells; and
20 - introducing said stem cells into said mammal.

According to another object of the present invention there is provided a method of treating a hematopoietic disorder in a subject, comprising:

- providing an enriched population of human CD34-CD38-Lin- stem cells;
- 25 administering said stem cells to the subject in need of treatment.

According to another object of the present invention there is provided a method for the production of CD34+ stem cells, said method comprising:

- providing an enriched population of human CD34-CD38-Lin- stem cells;

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- culturing said stem cells *in vitro* under suitable conditions for a time sufficient to allow said cells to differentiate into CD34+ cells; and
- isolating CD34+ stem cells.

According to still a further object of the present invention there is
5 provided a method for expanding a population of CD34-Lin- stem cells, said method comprising the steps of:

- isolating CD34-Lin- stem cells from suitable hematopoietic source;
- culturing said isolated cells *in vitro* for a time sufficient and under culture conditions to result in the expansion of said cells.

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Brief Description of the Drawings

Certain embodiments of the invention are described, reference being made to the accompanying drawings, wherein:

15 Figure 1A shows cell surface expression of CD34 on cord blood cells depleted for lineage markers (Lin-). (Panel I) Lin- cells were stained with a class III monoclonal antibody for CD34 (581) conjugated to FITC (Becton Dickinson, BD). Cells residing in R1 were considered CD34 negative (CD34-). (Panel II) CD34- cells were purified using standard cell sorting techniques and
20 re-analyzed using the same CD34-581 antibody. (Panel III and Panel IV) Purified R1 cells were stained and re-analyzed using a class I monoclonal antibody for CD34 (Immun-133) (Coulter) and a class II monoclonal antibody for CD34 (Q-Bend-10) (Becton Dickinson, BD).

Figure 1B shows cell populations immunostained for CD34 expression.
25 Representative cells are shown from a total of 25-75 cells examined for each treatment (n=2).

Figure 1C shows a comparison of cell surface markers between CD34-Lin- versus CD34+Lin- cord blood cells.

Figure 2 shows level of human cell engraftment in NOD/SCID mice transplanted with highly purified CD34-Lin- cells at various indicated doses.

Figure 3 shows the multilineage differentiation of human CD34-Lin- cells in NOD/SCID mice. Bone marrow from a highly engrafted mouse transplanted with 120,000 CD34-Lin- cord blood cells was stained with various human-specific monoclonal antibodies and analyzed by flow cytometry. Approximately 10^6 mononuclear cells collected from mouse bone marrow were prepared for multilineage analysis (12). (A) Cells with medium to high forward scatter (region R1) were gated and further analyzed. (B) Histogram of CD45 (pan-leukocyte marker) expression indicating that 2.5% of the cells present in the murine bone marrow are human, gated R2. All further lineage markers were examined on cells within gate R2 (CD45+). (C) Isotype control for non-specific IgG staining of PE and FITC fluorescence. (D) Expression of myeloid marker CD33 and granulocyte marker CD15; (E) pan-B cell markers CD19 and CD20; (F) CD38 and the immature hematopoietic marker CD34; (G and H) T-cell markers CD2, CD3, CD4 and CD8.

Figures 4A and 4B show frequency analyses of CD34-Lin- cells and CD34-CD38-Lin- cells found in human hematopoietic tissue. Figure 4A: Column I: Fetal liver collected from 8 week old human fetus, n=3; Column II: Fetal blood aspirated from 19 week old fetus, n=1; Column III: Cord blood collected from placenta at time of birth, n=3. Figure 4B: Column I: Normal Adult Bone marrow, n=2; Column II: Bone marrow from a normal adult donor after 5 days of G-CSF administration, n=2; Column III: Peripheral blood collected from a normal adult donor after 5 days of G-CSF administration, n=2.

Figure 5 shows the percentage of input cells after *in vitro* culture of CD34-Lin- cells. Purified cells were counted and seeded (250-2000) in wells containing serum free media (SF) (solid bars) or SF supplemented with 25% HUVEC- conditioned media (shaded bars). Cells were counted each day and the percentage of input cells was calculated (n=3).

Figure 6 shows an analysis of CD34 and CD38 expression of highly purified cell populations after *in vitro* culture. A representative experiment (n=4) of CD34 and CD38 cell surface expression performed on initially purified CD34-CD38-Lin- cells, and purified cells after 2 and 4 days of culture in SF, SF supplemented with 5% FCS or 25% HUVEC-CM. The entire contents of individual wells was collected at 2 and 4 days (5000-10,000 cells), stained with monoclonal antibodies CD34 and CD38 directly conjugated to FITC and PE respectively (Becton Dickinson, BD). Stained populations were then washed and analyzed using standard flow cytometric techniques, followed by the display of histograms using the Cell Quest software program (BD).

Figure 7A shows levels of engraftment of non expanded CD34-CD38-Lin- or CD34-CD38+Lin- cell fractions in NOD/SCID mice.

Figure 7B shows the capacity of CD34-Lin-, CD34-CD38+Lin- and CD34-CD38-Lin- cell fractions to engraft NOD/SCID after *ex vivo* culture. (A) The level of human cell engraftment is the BM of 136 mice transplanted with either CD34-Lin-, CD34-CD38-Lin- or CD34-CD38+Lin- cell fractions seeded at the indicated cell doses after day 2 or 4 of *ex vivo* culture from 43 CB and 3BM samples.

Figures 8A to 8H show the multilineage differentiation of human CD34-Lin- cells in NOD/SCID mice after *ex vivo* culture represented in histograms. Figure 8A: Histogram of CD45 (human-specific pan-leukocyte marker) expression indicating that 7% of the cells present in the murine bone marrow are human. Figure 8B: Forward and Side scatter of the CD45 human cells. Subsequent analysis of lineage markers was done on CD45+ cells within gate R1 (lymphoid and blast cells) or R2 (myeloid cells) gates. Figure 8C: Analysis for the presence of immature cells using the CD34 and CD38 markers. Figure 8D: Analysis for the presence of human B cell lineage cells using CD19 and CD20 markers. Figures 8E to 8F: Analysis for the presence of human T lymphocytes using the panel of T cell markers: CD2, CD3, CD4 and CD8.

Figures 8G to 8H: Analysis for the presence of myeloid cells using CD33, CD14, CD15 and CD13 markers.

Figure 9 shows DNA analysis of NOD/SCID mice transplanted with CD34-Lin- or CD34+CD38-Lin- cells purified from the same cord blood sample and cultured in the presence of 5% FCS. The southern blot was hybridized with a human chromosome 17-specific α -satellite probe.

Detailed Description of the Invention

The identification of the CD34-Lin- and CD34-CD38-Lin- human hematopoietic stem cell has important implications for understanding the origin of hematopoietic diseases such as leukemia and for clinical procedures such as stem cell transplantation and gene therapy for the treatment of various diseases. The stem cell of the present invention is also important for the treatment or prophylaxis against disease or infection, for the reconstitution of deficient or missing cell populations, as for example in cancer patients after myeloablative therapy, and for the treatment of congenital or acquired genetic abnormalities and defects by the introduction of desired genetic information into the patient.

The CD34-Lin- and CD34-CD38-Lin- human hematopoietic stem cells of the present invention can be isolated using standard techniques of cell sorting in order to rapidly identify and isolate the cells so that they may be isolated *ex vivo* and cultured *in vitro* to provide an expanded population of the cells for use as a therapeutic composition for humans requiring such. Such *ex vivo* cultured cells may be genetically manipulated as required prior to being reintroduced back into a patient.

In accordance with one embodiment, the invention provides a substantially homogeneous population of human hematopoietic stem cells which are characterized as CD34-Lin- and CD34-CD38-Lin-.

In accordance with a further embodiment, the invention provides a method for preparing a substantially homogeneous population of human hematopoietic stem cells which are CD34-Lin- and CD34-CD38-Lin-.

In accordance with a further embodiment, the invention provides a
5 therapeutic composition comprising CD34-CD38-Lin- human hematopoietic stem cells. The invention also provides a therapeutic composition comprising CD34-CD38-Lin- human hematopoietic stem cells. Such compositions can be used to treat hematopoietic disorders such as malignancies (i.e. leukemia), immune disorders and diseases resulting from a failure or a dysfunction of
10 normal blood cell production or maturation. Diseases may include but are not restricted to congenital disorders, severe combined immunodeficiency, Wiskott-Aldrich syndrome, Fanconi's anemia, congenital red cell aplasia, lysosomal storage disease, thalassemia major, sickle cell anemia, aplastic anemia, acute lymphoblastic leukemia, acute myelogenous leukemia,
15 megakaryoblastic leukemia, hematologic melanomas, lymphoma, multiple myeloma, myelodysplastic syndromes, carcinomas, neuroblastomas, arthritis and neurological genetic diseases (e.g. Gaucher Disease).

The novel stem cells of the present invention may be used in various clinical procedures such as stem cell transplantation for the reconstitution of a
20 deficient or a missing cell population, therapy, gene therapy and for combating infection.

In accordance with another aspect of the invention there is provided a method for the treatment of hematopoietic disorders such as leukemia comprising the use of human hematopoietic cells characterized as CD34-Lin-
25 and CD34-CD38-Lin-.

In accordance with another aspect of the present invention is a method for the *ex vivo* generation of human hematopoietic cells using CD34-Lin- cells and/or CD34-CD38-Lin- cells.

In accordance with another aspect of the present invention is a gene therapy method for providing genetically altered human hematopoietic cells characterized as CD34-Lin- or CD34-CD38-Lin- to a patient.

5 In accordance with another aspect of the present invention is a method for reconstituting deficient or missing human hematopoietic cell populations comprising the use of CD34-Lin- cells or CD34-CD38-Lin- cells.

In accordance with another aspect of the present invention is a method for transplanting human hematopoietic cell populations to a patient comprising the use of CD34-Lin- and CD34-CD38-Lin- cells.

10 In accordance with another aspect of the present invention is a method for combatting infection in a patient comprising administering an effective amount of human hematopoietic cells characterized as CD34-Lin- or CD34-CD38-Lin-.

15 In accordance with yet a further aspect of the present invention there is provided a method utilizing human hematopoietic cells characterized as CD34-Lin- or CD34-CD38-Lin- for the production of CD34+ human hematopoietic cells.

20 In accordance with a further aspect of the present invention is a method for increasing the repopulating capacity of human hematopoietic cells characterized as CD34-Lin- or CD34-CD38-Lin- by culturing such cells *in vitro* for several days.

In accordance with a further aspect of the present invention is a method for screening candidate compounds affecting proliferation or differentiation of stem cells characterized as CD34-Lin- and CD34-CD38-Lin-.

25

Characterization of Hematopoietic Stem Cells

It was determined, using CD34 class III fluorescent monoclonal antibodies, that CD34- cells that do not express lineage markers exist in human hematopoietic tissues (Fig. 1A). The possibility that CD34 protein was

produced in the CD34-Lin- cells but not transported to the cell surface was excluded using permeabilized, stained cytopins of purified CD34-Lin- that were further conjugated with fluorescent monoclonal antibodies. This confirmed that no CD34-FITC signal was detected in the CD34-Lin- cells (Fig. 1B).

Heterogeneity within human CD34+Lin- cells is well documented and further subdivision for the most primitive cells is typically based on the cell surface markers CD38, c-kit, Thy-1 and HLA-DR. The expression of these markers on both the CD34+Lin- and CD34-Lin- cells was compared (Fig. 1C). CD34-Lin- cells displayed a bi-modal distribution of CD38, clearly dividing the population into two fractions, in contrast to the high proportion of CD34+Lin- cells that express CD38 (Fig. 1C). Cell surface expression of c-kit was similar between the two populations, while CD34-Lin- cells were almost exclusively Thy-1- and HLA-DR- (Fig. 1C). Both the absence of HLA-DR expression and the presence of Thy-1 have been proposed as defining more primitive subfractions within the CD34+Lin- population. Therefore, the CD34-Lin- population derived from cord blood is a distinct population which differs from primitive CD34+Lin- cells not only in CD34 expression but also in phenotypic heterogeneity based on additional stem-cell associated markers.

Using clonogenic methylcellulose assays, the hematopoietic progenitor activity of CD34-Lin-, CD34-CD38-Lin- and CD34-CD38+Lin- cells was determined by comparing their CFC and LTC-IC content. The clonogenic capacity of CD34-Lin- cells was extremely low in comparison to that of CD34+CD38-Lin- cells (Table I). As many as 10,000 cells needed to be seeded on MS-5 stroma to detect a single LTC-IC within the CD34-Lin- cell fraction, while further purification demonstrated that detection of LTC-IC in the CD34-CD38-Lin- fraction required seeding of at least 2000 cells. By contrast, as few as 10 CD34+CD38-Lin- cells contain an LTC-IC. The CD34-CD38+Lin- cells were devoid of LTC-IC activity (limit of detection at 10,000 cells) but

contained a much higher capacity to form CFC (colony-forming cells) specifically committed to the erythroid lineage (Table I). The low efficiency of production of myeloid and erythroid committed progenitors, as well as the more primitive LTC-IC, were similar to observations made with murine CD34-Lin-
5 cells in the same assay systems and suggested that functional similarities may exist between the CD34-Lin- cells from these two species.

The only conclusive method for detecting stem cells is to determine their ability to repopulate recipient hosts. The repopulation capacity of primitive human cells can be assayed by their ability to initiate multilineage human
10 engraftment in immune-deficient NOD/SCID mice. Based on cell purification and gene marking, cells capable of repopulating NOD/SCID mice (termed the SCID-Repopulating Cell, SRC) were established as distinct from, and more primitive than, the majority of progenitors detected in *in vitro* assays. CD34+CD38-Lin- cells, and not CD34+CD38+Lin- cells, gave rise to
15 multilineage engraftment. Transplantation of as many as 10^6 CD34-Lin+ cells did not give engraftment. To determine whether highly purified CD34-Lin- cells had SRC activity, and to determine the frequency of any repopulating cells, CD34-Lin- cells were transplanted at varying cell doses into NOD/SCID mice using standard protocols, and bone marrow was analyzed for the presence
20 of human cells after 8-12 weeks. The level of human cell engraftment in 23 NOD/SCID mice was quantitated by FACS and DNA analysis for the presence of human cells and results are summarized in Fig. 2. A large proportion of transplanted mice were engrafted with human cells, indicating that CD34-Lin- cells were able to repopulate NOD/SCID mice. This cell was designated the
25 CD34NEG-SCID Repopulating Cell (CD34NEG-SRC). The frequency was 1 CD34NEG-SRC in 125,000 CD34-Lin- cells. The differentiative and proliferative capacity of the CD34NEG-SRC cell was assessed by flow cytometric analysis. A representative engrafted NOD/SCID mouse 10 weeks after the transplant of 120,000 CD34-Lin- cells is shown in Fig. 3. Cells with

medium to high forward scatter (region R1, Fig. 3A) were gated and further analyzed, based on CD45 expression, a human specific pan-leukocyte marker (Fig. 3B). The isotype control is shown in Fig. 3C. The bone marrow of this mouse contained 2.5% CD45+ human cells (Fig. 3B), or at least 106 total human cells indicating that the CD34-Lin- cells have extensive proliferative capacity. Human granulocytes (CD15+) were present among myeloid cells (CD33+) (Fig. 3D). Human B-lymphoid cells were also present in the murine bone marrow as shown by staining for CD19 and CD20 (Fig. 3E). Interestingly, human T-cells expressing both CD2 and CD3 (Fig. 3G), along with CD4 and CD8 positive cells (Fig. 3H), were also identified. NOD/SCID mice transplanted with highly purified primitive CD34+CD38-Lin- cells never gave rise to engraftment containing T-cells demonstrating the unique *in vivo* repopulation behavior of the CD34-Lin- cells. In addition to multilineage engraftment, immature CD34+ and CD34+CD38- cells were detected (Fig. 3F). It was concluded that human CD34-Lin- cells have the ability to repopulate NOD/SCID mice and differentiate *in vivo* into multiple lineages of myeloid and lymphoid cells. The production of CD34+CD38- and CD34+CD38+ cells *in vivo* suggests that CD34-Lin- cells are developmentally earlier than CD34+ cells in the hierarchy of human hematopoiesis.

There is evidence that the frequency of primitive cells changes during ontogeny with the highest proportions seen in the fetus. A variety of fetal, neonatal, and adult sources of human hematopoietic tissue were analyzed in an attempt to identify and quantify the CD34-Lin- population. The results indicate (Fig. 4A,4B) that CD34-Lin- cells are produced early in human ontogeny and can persist throughout adult life and that the mechanisms that operate during the mobilization of CD34+ cells by G-CSF also affect CD34-Lin- cells.

This data provides the first identification of a novel human hematopoietic stem cell that does not express CD34 or lineage-specific markers. As determined by all available monoclonal antibodies, this population

is not only distinct by the absence of CD34, but also by the lack of HLA-DR and Thy-1 markers. In addition to these phenotypic differences, several lines of evidence functionally distinguish these two stem cell populations. While the CD34-Lin- cells have limited hematopoietic activity *in vitro*, CD34+CD38-Lin- cells are highly clonogenic based on their ability to produce CFC and LTC-IC. Although both stem cell fractions are capable of repopulation, the presence of T-cells within the multilineage engraftment is a unique characteristic of CD34-Lin- transplantation; human T cells have not been detected in mice transplanted with CD34+CD38-Lin- cells (n=25). It is unlikely, therefore, that NOD/SCID repopulation was derived from contamination of CD34-Lin- cells by CD34+CD38-Lin- cells. Furthermore, based on LTC-IC frequency, a minimum of 1 LTC-IC resides within 10 highly purified CD34+CD38-Lin- cells. In contrast to flow cytometry, by which it has been determined that the CD34-Lin- population is 99% (or in some cases 100%) pure, the LTC-IC assay allows detection of a smaller number of contaminating CD34+CD38-Lin- cells. Using this assay, only a single LTC-IC could be detected in as many as 10 000 CD34-Lin- cells. If this LTC-IC activity came from a CD34+CD38-Lin- cell, a maximum of 10 CD34+CD38-Lin- cells could be contained in the CD34-Lin- purified fraction (0.1% contamination). In addition, repopulated mice have not been observed when only 10 CD34+CD38-Lin- cells were transplanted (12). Since the frequency of SRC derived from CD34-Lin- cells is 1 in 125 000, a maximum of 125 CD34+CD38-Lin- cells could have been transplanted, again less than the number needed to repopulate CD34+CD38- cells.

The *in vivo* differentiation of human CD34-Lin- cells into CD34+ and lineage positive cells after murine engraftment suggests that CD34-Lin- cells precede CD34+ cells in the hierarchy of human hematopoiesis.

It is also possible that the CD34-Lin- cells upon further phenotypic evaluation may contain certain subpopulations of cells with have further different phenotypic antigenic expression. It is also possible that CD34-Lin-

cells have further phenotypic antigenic expression themselves that will further help to isolate these new class of stem cells in a more efficient and rapid manner.

The identification of these novel repopulating cells, termed CD34^{neg}-SCID repopulating cells (CD34^{neg}-SRC), provides an opportunity to examine the differentiation and proliferation potential of these cells and to establish their relationship to other cells within the human stem cell hierarchy. These CD34^{neg}-SRC are found within both the CD34-Lin⁻ cell fraction and the CD34-CD38-Lin⁻ fraction. CD38⁺ cells identified within the CD34-Lin⁻ fraction do not have repopulation potential.

Many studies based on *ex vivo* culture have demonstrated that there is heterogeneity within the CD34⁺ cell fraction. Subfractionation of the CD34⁺ cells on the basis of Thy1, CD38, and HLA-DR expression together with *in vitro* clonogenic and LTC-IC assays have demonstrated the progenitor-progeny relationship of the various cell types that make up the stem cell hierarchy (4, 14). The availability of the SRC assay to detect even earlier cell types has added more information about the organization of cells within this hierarchy (14). Moreover, it was demonstrated that the SRC (derived from CD34⁺CD38⁻ cells) can be expanded for 4 days in serum-free cultures without inducing their differentiation. However, all SRC are lost within an additional 4 days of culture concomitant with the appearance of more differentiated CD38⁺ cells (11). At the same time, both colony-forming cells (CFC) and long-term culture initiating cells (LTC-IC) could be greatly expanded during 8 days of culture, demonstrating that the majority of the SRC are a distinct population (11), but may be closely related to ELTC-IC (18). Thus, *in vitro* culture systems can be used to identify very fine transitions in the developmental program by combining both flow cytometry and functional CFC, LTC-IC and SRC assays. It is now demonstrated that *ex vivo* culture of CD34-Lin⁻ cells can induce the appearance of CD34⁺ cells and can increase the proportion of CD34-Lin⁻ cells

that have SRC activity. These studies provide new insight into the developmental program of human hematopoietic stem cells.

Earlier studies demonstrated that the CD34-Lin⁻ cell fraction expressed a bimodal distribution of CD38, allowing for further purification into CD38⁻ and CD38⁺ subpopulations (12). To determine whether the CD34-Lin⁻, CD34-CD38-Lin⁻, and CD34-CD38⁺Lin⁻ cells could be induced to proliferate and/or differentiate, these cells were cultured in defined serum-free (SF) conditions that have been previously shown to support expansion of Lin³⁴⁺³⁸⁻ cells and maintenance and modest increase of POS CD34 POS-SRC (11, 17). Cells were plated in methlycellulose assays at day 0, and after day 4 of liquid culture, (Table II) to determine the effect of cytokine stimulation on the clonogenic progenitors present in CD34-Lin⁻, CD34-CD38-Lin⁻ and CD34-CD38⁺Lin⁻ cell fractions. Both the CD34-Lin⁻ and more purified CD34-CD38-Lin⁻ cells have a low plating efficiency (PE), 1 in 89 and 1 in 297 CFC respectively, whereas a higher PE of CD34-CD38⁺Lin⁻ cells (1 in 10.4 cells) was seen. Interestingly, the clonogenic capacity of the CD34-CD38⁺Lin⁻ cells was restricted to the erythroid lineage. After 4 days of culture in SF media, or SF media supplemented with the addition of 25% conditioned medium obtained from primary human umbilical vein endothelial cells (HUVEC-CM), the PE of all the sub-populations examined had decreased, whereas the addition of 5% of FCS increased the clonogenicity of CD34-Lin⁻ cells and, to a greater extent, CD34-CD38⁺Lin⁻ cells (Table II). This difference in clonogenicity may reflect heterogeneity within CD34-Lin⁻ cells and demonstrates that the CD38⁺ subfraction is already committed to the erythroid lineage, suggesting that the CD34^{neg}-SRC resides in the CD34-CD38-Lin⁻ subfraction.

To determine whether the CD34-CD38-Lin⁻ cells could be stimulated to proliferate, changes in cell number were recorded between day 0 and 4 of culture with SF or 25% HUVEC-CM (Fig. 5). The total number of cells decreased by 2 fold at day 4 in SF media. Supplementation of 5% fetal calf

serum (FCS) showed no increase in the viability of these cells (data not shown). However, the addition of HUVEC-CM to SF media maintained or slightly increased the total cellularity (Fig.5). These results demonstrate that culture conditions that are optimal for CD34+CD38-Lin- cells (11) are unable to
5 support CD34-CD38-Lin- cells, while soluble components present in primary HUVEC-CM seem to permit their survival.

The effect of culture on the differentiation program of CD34-CD38-Lin- cells, from individual wells was analyzed by flow cytometry after 2 and 4 days of culture in various conditions (Fig. 6). Surprisingly, CD34-CD38-Lin-
10 cells seeded in SF media began expression of CD34, which could be enhanced with the addition of 5% serum (Fig. 6). In contrast, the majority of cells obtained after 2 or 4 days of culture in the presence of 25% HUVEC conditioned medium still maintained the original CD34-CD38-Lin- phenotype. The stimulation of CD34-CD38-Lin- cells to differentiate and produce
15 CD34+CD38- cells suggests that CD34-CD38- cells precede the CD34+CD38- population in the hierarchy of human hematopoiesis. Moreover, these results indicate that the CD34-CD38-Lin- cells respond to signals present in SF or 5% serum conditions and that HUVEC-CM can inhibit this stimulation.

To confirm which fraction contained CD34neg-SRC cells, both CD34-
20 CD38-Lin- and CD34-CD38+Lin- cells were transplanted into NOD/SCID mice. Transplantation with as few as 10,000 or 4,000 CD34-CD38-Lin- cells, derived from cord blood or bone marrow respectively, resulted in engraftment (Fig. 7A), whereas as many as 180,000 CD34-CD38+Lin- cells were incapable of repopulation (Fig 7A). These data indicate that the CD34neg-SRC cells
25 present in the CD34-Lin- fraction are restricted to the CD38- subfraction. However, since an entire cord blood sample contains only 1 or 2 CD34neg-SRC (e.g. frequency is 1 CD34neg-SRC in 125,000 CD34-Lin- cells and one cord blood sample contains up to 250,000 CD34-Lin- cells), the losses

associated with subselecting based on CD38 expression resulted in only 9% of samples which repopulated NOD/SCID mice.

To evaluate the repopulating activity of CD34-Lin- and more highly purified CD34-CD38-Lin- cell fractions after *ex vivo* culture, cultured cells were injected into NOD/SCID mice and the level of human engraftment evaluated after 8 to 10 weeks. Purified cell fractions from 44 CB and 3 BM samples cultured for 2 and 4 days in SF, SF supplemented with 5% FCS or 25% HUVEC-CM were transplanted at various doses into 144 recipient NOD/SCID mice and the level of human cell engraftment was determined (Figure 7B). A total of 15 of 35 mice were engrafted following transplantation with CD34-Lin- cells that had been cultured for 4 days in SF or 5% FCS at cell doses below the calculated frequency of CD34-SRC. For example, 13 of 29 mice were engrafted following transplantation of 50,000 to as few as 4,000 cultured CD34-Lin- cells. By contrast, only 1 of 7 mice were engrafted when 100,000 uncultured CD34-Lin- cells were transplanted (Figure 2). Similarly, a higher proportion of mice (33%) transplanted with cultured CD34-CD38-Lin- cells were engrafted (Figure 7B) compared to mice transplanted with similar doses of uncultured CD34-CD38-Lin- (Figure 7B). Quantitative analysis using Poisson statistics indicated a frequency of 1 CD-SRC in 38,000 cultured CD34-CD38-Lin- cells (range 1/22,000 to 1/71,000). It was not possible to calculate the frequency of CD34-SRC in uncultured CD34-CD38-Lin- cells (Figure 7A) because there was infrequent engraftment despite injection of as many as 50,000 cells. This result indicates that the actual stem cell frequency of freshly isolated cells must be much lower compared to cultured cells. The human lineage distribution in mice transplanted with expanded CD34-Lin- and CD34-CD38-Lin- cells is similar to that with unstimulated purified CD34-Lin- cells (Figure 8). In addition, the inability of cultured CD34-CD38+Lin- cells to engraft mice confirms the absence of repopulating cells within this fraction. It

was evident that culture conditions, especially HUVEC-CM, induce an increase in the number of CD34-SRC in the absence of cell proliferation.

The bone marrow of engrafted mice was analyzed by multiparameter flow cytometry to determine whether cultured CD34-Lin- repopulating cells possessed the same *in vivo* proliferative and differentiative capacity as uncultured cells. A representative analysis of the bone marrow of a NOD/SCID mouse transplanted with an initial population of 40,000 CD34-Lin- cells after 4 days of culture is shown in Figure 8. The bone marrow of this mouse contained 7% human cells as detected by expression of CD45, a human specific pan-leukocyte marker (Fig. 8). Both B and T-lymphoid cells were present in the murine bone marrow as shown by staining for CD19, CD20 and CD4, CD3 antigens (Fig. 8D-F). The presence of CD33+, CD14+, CD15+ and CD13+ cells indicated the differentiation potential of CD38-CD38-Lin- cells to the myeloid lineages (Fig. 8G-H). The engraftment pattern of mice transplanted with expanded CD34-CD38-Lin- cells is similar to that observed with unstimulated purified CD34-Lin- cells. The presence of human T-cells is a unique feature of CD34-Lin- engraftment, since T-cells have not been detected in mice transplanted with purified CD34+CD38-Lin- cells either before or after *ex vivo* culture.

We had previously found that the CD34+SRC are lost if CD34+CD38-Lin- or CD34+Lin- cells are cultured in the presence of serum suggesting that the CD34-SRC are biologically distinct from CD34+SRC. To test this directly, CD34+CD38-Lin- and CD34-Lin- cells from the same human CB sample were cultured for 4 days under the same serum containing conditions. In a representative experiment, 3 out of 6 mice were engrafted following transplantation with cultured CD34-Lin- cells (Figure 9). In contrast, 5,000 and 10,000 CD34+CD38-Lin- cells, containing 10-20 CD34+SRC, cultured under the same conditions were unable to engraft NOD/SCID mice. These results provide independent confirmation that the repopulating cells derived from the

CD34-Lin- subfraction are biologically distinct from those derived from CD34+CD38-Lin- cells.

It is believed that the repopulating cells of the human hematopoietic system are the CD34-CD38-Lin- sub-population and short term *ex vivo* culture of this fraction has been observed to increase the proportion of repopulating cells. The ability of CD34-CD38-Lin- cells to produce CD34+CD38- cells *in vitro* and *in vivo* demonstrates the developmental capacity of these cells and further suggests that this population of cells is more primitive than the CD34 positive fraction. In addition, conditions evaluated here provide the foundation for future gene transfer and *ex-vivo* expansion of this novel population and for the identification of factors that stimulate their proliferation and differentiation.

Furthermore, the knowledge that CD34-Lin- cells exist and can repopulate the human hematopoietic system provides a novel therapeutic composition and a method for the treatment of hematopoietic disorders. In particular, the composition and method can be used to treat hematopoietic disorders such as leukemia and for several clinical procedures such as stem cell transplantation, therapy, for combatting infection and for cell reconstitution. These cells can also be used to generate CD34+ cells and possibly other cell types.

The new class of stem cells of the present invention provide a new method of treatment for various disorders that is very advantageous. From a small sample of bone marrow, peripheral blood or cord blood, the CD34-Lin- cells can be isolated, maintained in culture, enriched and expanded and stored for later use or further stimulated in culture to differentiate and provide other sources of cell types.

It is understood by those skilled in the art that the stem cells of the present invention can be identified and isolated from bone marrow, peripheral blood and cord blood. The most clinically advantageous source is peripheral blood due to the fact that the procedure for obtaining such is easy and non-

invasive. Collection of peripheral blood also has no health effect on the donor. While peripheral blood is the most convenient and least invasive source for use in isolating the stem cells of the present invention, it is understood by those skilled in the art that the bone marrow and cord blood are more ideal as a
5 starting point due to the larger percentage of stem cells present in such.

The stem cells of the present invention can be isolated using standard techniques known in the art to identify classes of cells and to select subsets of cells of the hematopoietic systems. One such technique is flow cytometry. A flow cytometer can identify different cells by measuring the light they scatter or
10 the fluorescence they emit as they flow through a laser beam. This it can sort out cells of a particular type from a mixture. A fluorescence activated cell sorter or FACS, can select one cells from thousands of other cells. FACS utilizes a multicolour flow cytometer to detect and separate cells bound with fluorescent conjugated antibodies to the specific antigens that identify the
15 development stage or lineage stage of the cells of the hematopoietic system. Detectable fluorescent signals are generating by hitting the cells with a laser beam as they pass through a flow sheath. A nonfluorescent forward scatter signal is used to represent volume and a side scatter signal detects cellular texture and granularity. The colour signals of the fluorochromes used to
20 conjugate with the antibodies detect the cell specific antigens. FACS analysis can analyze two or more colours simultaneously and generate data which is inputted into a computer program to generate colour plots, histograms and perform statistical analysis.

The stem cells of the present invention can also be isolated using a
25 method of negative selection by depletion of lineage positive cells. In this technique, the undesired cells are selected for and depleted leaving behind the desired cells.

Other techniques which may be used to isolate the stem cells of the present invention include immunoseparation where antibodies against specific

receptor molecules are used together with immunoaffinity columns to bind cells having the specific target receptor. The targeted cells are then removed from the antibody complex by the use of shear fluid force.

The cells of the present invention can be used to screen compounds
5 which may affect their proliferation and/or differentiation into other cell types. For example, an isolated population of CD34-Lin- cells may be suitably cultured *in vitro* to which selected growth factors, cytokines, chemicals, peptides and other agents are added individually or in specific combination. One skilled in the art would readily comprehend the conditions and procedures
10 for preparing such a cell culture and the amounts of agent to add for such testing. After a period of time the CD34-Lin- cells may be phenotypically characterized and counted in order to determine the effect of the added agent(s). In this manner, one may establish a simple method for producing a specific cell type for a clinical application.

15 The cells of the present invention can be used for understanding the origin of hematopoietic diseases such as leukemia and for clinical procedures such as stem cell transplantation and gene therapy for the treatment of various diseases. The stem cell of the present invention is also important for the treatment or prophylaxis against disease or infection, for the reconstitution of
20 deficient or missing cell populations, as for example in cancer patients after myeloablative therapy, and for the treatment of congenital or acquired genetic abnormalities and defects by the introduction of desired genetic information into the patient.

In an embodiment of the present invention, the CD34-Lin- or CD34-
25 CD38-Lin- stem cells can be utilized for stem cell transplantation in order to reconstitute missing or deficient cell populations. Bone marrow transplants are typically done in order to restore hematopoiesis in cancer patients receiving high doses of chemotherapy and/or radiation therapy as well as in leukemia patients and aplastic anemia patients. Cord blood has recently been used in

order to reconstitute hematopoiesis as an alternative to bone marrow transplants. However, there are several disadvantages with bone marrow transplants as they are highly invasive and require major surgery. One also must find a suitable phenotypically matched donor. The patient's body must be
5 rid of all tumor cells which involves the use of cytotoxic chemicals leaving the patient hematopoietically deficient. A source of bone marrow sample is then engrafted after which it takes 3 to 4 weeks for engraftment to occur.

In contrast to bone marrow transplants, the present invention allows one to identify and purify CD34-Lin- or CD34-CD38-Lin- cells from a suitable
10 source such as bone marrow, cord blood or peripheral blood. These cells can then be enriched *ex vivo* and transplanted or infused back into a patient missing or deficient in a cell population. The cells may also be treated genetically or chemically prior to transplantation back into the patient. The procedure for reinfusion of the stem cells is less harmful to a patient than the reintroduction of
15 bone marrow and requires a substantially smaller volume of cells than bone marrow transplants. It is understood by those skilled in the art that the cells of the present invention can be differentiated into various types of mature cells which may be used for transplantation.

Peripheral blood transplantation can also be used to isolate and provide
20 back an enriched culture of CD34-Lin- or CD34-CD38-Lin- cells.

The invention provides a therapeutic composition comprising CD34-Lin- human hematopoietic stem cells. The invention also provides a therapeutic composition comprising CD34-CD38-Lin- human hematopoietic stem cells. Such compositions can be used to treat hematopoietic disorders
25 such as malignancies (i.e. leukemia), immune disorders and diseases resulting from a failure or a dysfunction of normal blood cell production or maturation. Diseases may include but are not restricted to congenital disorders, severe combined immunodeficiency, Wiskott-Aldrich syndrome, Fanconi's anemia, congenital red cell aplasia, lysosomal storage disease, thalassemia major, sickle

cell anemia, aplastic anemia, acute lymphoblastic leukemia, acute myelogenous leukemia, megakaryoblastic leukemia, hematologic melanomas, lymphoma, multiple myeloma, myelodysplastic syndromes, carcinomas, neuroblastomas, arthritis and neurological genetic diseases (e.g. Gaucher Disease).

5 It is understood by those skilled in the art that the compositions of the present invention may comprises substantially pure populations of CD34- Lin- human hematopoietic stem cells or CD34-CD38-Lin- human hematopoietic stem cells. The compositions may also comprise enriched cultures of CD34- Lin- or CD34-CD38-Lin- cells. Whether substantially pure or enriched, the
10 compositions of the present invention may additionally comprise cells selected from the group consisting of CD34+ cells, Thy-1 cells, CD4+ cells, CD56+ cells, CD33+ cells, CD9+ cells, CD11+ cells, CD41+ cells, CD45 cells and mixtures thereof. The type of composition made depends on the end use. For example, for the treatment of leukemia it is desired to provide a composition
15 comprising substantially homogenous populations of CD34-Lin- or CD34-CD38-Lin- cells whereas for a standard stem cell transplant for other cancers or autoimmune diseases, the composition may comprise a mixture of CD34-Lin- or CD34-CD38-Lin- cells together with CD34+ cells.

 It is also possible to utilize as therapeutic compositions substantially
20 enriched populations of cells characterized phenotypically as CD34-Lin- or CD34-CD38-Lin- but which also may be further phenotypically characterized by the presence or absence of other antigenic markers.

 It is also within the scope of the present invention to produce a composition comprising CD34-Lin- or CD34-CD38-Lin- cells to which
25 infection fighting cells (B lymphocytes, T lymphocytes and their precursors) blood clotting cells (platelets), or any of the myeloid lineage cells (erythrocytes, granulocytes, macrophages, monocytes, basophils, eosinophils and their precursors) may be added to provide a "specialized" composition specific for a particular disease. It is also understood by those skilled in the art that various

pharmaceutical agents may be added to the compositions of the invention in order to treat specific disease states. For example, such pharmaceutical agents may include antibiotics, chemotherapeutic agents, cytokines, etc.

The stem cells of the present invention can be used to fight against both
5 disease and infection. Cell populations to be transplanted can be screened and treated for infections such as viral (e.g. AIDS, herpes, hepatitis, etc.), bacterial (e.g. staphylococcus, streptococcus, etc.) and fungal *in vitro*. A sample of a patient's blood or bone marrow containing the infected cells can be purged of infection and the stem cells isolated and enriched *ex vivo*. In order to destroy
10 the bacteria or virus which may affect such cells, the cultured cells may be treated with a suitable antiviral agent or antibacterial agent. Alternatively, the isolated cells can be individually screened to select for uninfected cells and the resulting population enriched. Prior to the implantation of the treated or untreated selected uninfected cells, the patient to receive such transplant is
15 treated with a suitable chemical agent, drug or radiation treatment to eliminate all infected cells. The enriched sample of stem cells can then be transplanted into the patient. If desired, prior to transplantation into the patient, other cell types may be added to the culture of stem cells depending on the disease condition to be treated. For example, in the case of AIDS, a fresh supply of
20 uninfected T-cells may also be added to the enriched culture of CD34-Lin- or CD34-CD38-Lin- cells.

In another embodiment, the CD34-Lin- or CD34-CD38-Lin- cells may be used as a method of gene therapy. The CD34-Lin- or CD34-CD38-Lin- cells may be isolated and enriched in *in vitro* culture where a desired genetic
25 sequence can be inserted into the cells prior to their reintroduction into a patient. The genetic element introduced can simply be one to correct a defect in the cells themselves or to target a specific recombinant gene sequence to a specific area of the patient. Examples of diseases which may be treated with genetically altered stem cells of the present invention include but are not

restricted to hemophilia A, thalassemia, sickle-cell anemia, SCID and Gaucher's disease.

Methods of gene therapy are well known by those skilled in the art. Briefly, a sample of CD34-Lin- or CD34-CD38-Lin- cells are isolated from a source and cultured according to the method of the present invention. The cell culture is maintained under suitable conditions and the cells are subjected to techniques for the introduction and stable incorporation of a desired genetic sequence into the cells. Such introduction techniques may include transfection (calcium-mediated or microsome-mediated transfection), cell fusion, electroporation, microinjection or infection using recombinant vaccinia or retrovirus vectors. The cells which acquire the selected genetic sequence are then screened for, and reintroduced into a patient. Alternatively, the identified cells may be further cultured to allow the cells to enrich and/or further differentiate to another cell type prior to being reintroduced into a patient.

The cells of the present invention may be transfected with a selected DNA sequence encoding for a therapeutic agent such as an antibiotic, anticancer agents, peptides, cytotoxic compounds and antisense RNA. Alternatively, the cells may be transfected with an antigenic or immunogenic product which creates an immune response in a patient and reintroduction. In this manner, such cells would produce a vaccine like effect.

In a further embodiment of the invention, the cells of the present invention can be used for fetal genetic testing. The stem cells may be isolated from samples of peripheral blood taken from a pregnant woman which contain some fetal cells. Isolated fetal cells may be cultured and the stem cells isolated and tested for genetic abnormalities.

In still a further embodiment of the present invention, the stem cells of the present invention may be isolated and cultured *in vitro* and treated with specific factors and/or cytokines in order to produce a specific cell lineage such as immune cells, granulocytic, megakaryocytic, etc. which carry out a

specific function. Such specialized cells may be generated in large numbers and transplanted back into patients in order to treat them of a disease such as for example an autoimmune disease or one of the diseases listed *supra*.

5

Examples

The examples are described for the purposes of illustration and are not intended to limit the scope of the invention.

Methods of synthetic chemistry, protein and peptide biochemistry and immunology referred to but not explicitly described in this disclosure and
10 examples are reported in the scientific literature and are well known to those skilled in the art.

Example 1 - Analysis of CD34-Lin- cells found in human hematopoietic tissue

Mononuclear cells were isolated from various human hematopoietic cell
15 sources and stained with monoclonal antibodies for CD2, CD3, CD4, CD7, CD13, CD14, CD15, CD16, CD19, CD20, and glycophorin conjugated to FITC, CD38 conjugated to PE and CD34 conjugated to Cy-5 . Cells gated R1 did not express lineage associated markers (Lin-) and were further analyzed for the expression of CD34 and CD38.

20

Identification of CD34- cells with no lineage markers in human hematopoietic tissues

To determine whether CD34- cells that do not express lineage markers exist in human hematopoietic tissues, human cord blood cells were first
25 depleted of mononuclear cells that express 15 different lineage-specific antigens from human cord blood. This Lin- population was 99% pure (data not shown). The Lin- cells were then stained with the most widely used CD34 class III monoclonal antibody conjugated to FITC. Flow cytometric analysis showed two distinct populations of CD34+ and CD34- cells (Fig 1A panel I). The

CD34- cells (gated R1, Fig. 1A, I) were collected by flow sorting and reanalysis demonstrated their high purity (99%; Fig. 1A, II). To confirm that these cells did not express any surface CD34 antigen, the sorted cells were re-stained with two other CD34 Class I and II antibodies that recognize different epitopes of the CD34 molecule (Fig. 1A, III and IV). No CD34+ cells were detected, attesting to the high purity and lack of CD34 cell surface expression of this CD34-Lin- population.

To exclude the possibility that CD34 protein was produced in the CD34-Lin- cells but not transported to the cell surface, cytopins of purified CD34-Lin- cells were permeabilized, stained with CD34 monoclonal antibodies conjugated to FITC and counter stained with DAPI (Fig. 1B). No CD34-FITC signal was detected in the CD34-Lin- cells. The specificity of the procedure was shown by the detection of cell surface and intracellular expression of CD34 on a population of purified CD34+Lin- cells under similar conditions. Background fluorescence was indicated by staining cells with IgG conjugated to FITC as isotype control (Fig. 1B). These results indicate that a population of Lin- cells exist in human cord blood that does not produce intracellular or cell surface CD34.

Heterogeneity within human CD34+Lin- cells is well documented and further subdivision for the most primitive cells is typically based on the cell surface markers CD38, c-kit, Thy-1 and HLA-DR. The expression of these markers on both the CD34+Lin- and CD34-Lin- cells was compared (Fig. 1C). CD34-Lin- cells displayed a bi-modal distribution of CD38, clearly dividing the population into two fractions in contrast to the high proportion of CD34+Lin- cells that express CD38 (Fig. 1C). Cell surface expression of c-kit was similar between that two populations, while the CD34-Lin- cells are almost exclusively Thy-1- and HLA-DR- (Fig. 1C). Both the absence of HLA-DR expression and the presence of Thy-1 have been proposed to define more primitive subfractions within the CD34+Lin- population. Therefore, the CD34-Lin-

population derived from cord blood is a distinct population which differs not only in CD34 expression from primitive CD34+Lin- cells but also in phenotypic heterogeneity based on additional markers associated with stem cells.

5

Example 2- Cell Immunostaining

Cord blood cells purified by flow cytometry as done previously (11, 12) were cytopun onto slides, permeabilized, and incubated in a BSA solution. The results are shown in Figure 1B. (Column I) CD34+CD38-Lin- cells were
10 stained with isotype control antibody conjugated to FITC (Becton Dickinson) and counterstained with DNA binding DAPI as a control for non-specific background fluorescence. (Column II) CD34+CD38-Lin- cells and (Column III) CD34-Lin- cells were stained with CD34 monoclonal antibodies followed by DAPI counter stain. All slides were examined using a fluorescent
15 microscope utilizing the appropriate filters for DAPI to detect the nucleus of cells and FITC for the presence of CD34 protein.

Both CD34-Lin- and CD34+Lin- purified cells were stained with monoclonal antibodies conjugated to fluorochromes for CD38 (Becton Dickinson, BD), c-kit (BD), Thy-1 (Coulter) and HLA-DR (BD). Stained
20 populations were then washed and analyzed using standard flow cytometric techniques (J. Exp Med, PNAS) followed by the display of histograms using the Cell Quest software program (BD) (n=3).

Example 3 - Cell Engraftment

25 Purified cell populations at the indicated dose were transplanted by tail vein injection into sublethally irradiated mice (375 cGy using a ¹³⁷Cs g-irradiator) according to a standard protocol as previously described (16, 17). Mice were sacrificed 8 to 12 weeks post transplant and the bone marrow from the femurs, tibiae and iliac crests of each mouse were flushed into IMDM

containing 10% FCS. Mouse bone marrow was analyzed using FACS analysis and by southern analysis using genomic DNA extracted by standard protocols in which the level of human cell engraftment was determined by comparing the characteristic 2.7 kb band with those of human:mouse DNA mixtures as
5 controls (limit of detection 0.05% human DNA) (16, 17). The results are shown in Figures 2 and 7.

Example 4 - Determination of Hematopoietic Progenitor Activity of CD34-Lin-,
CD34+CD38-Lin- and CD34-CD38+Lin-

10 Highly purified cells were plated in clonogenic methlycellulose assays and seeded on MS-5 stroma in order to quantitate the CFC and LTC-IC content, respectively. Clonogenic capacity of CD34-Lin- cells was extremely low in comparison to CD34+CD38-Lin- cells (250 CFC vs. 8.9 CFC per 800 cells) (Table I). As many as 10,000 cells needed to be seeded on MS-5 stroma to
15 detect a single LTC-IC within the CD34-Lin- cell fraction, while further purification demonstrated that detection of LTC-IC in the CD34-CD38-Lin- fraction required seeding of at least 2000 cells. By contrast, as few as 10 CD34+CD38-Lin- cells contain an LTC-IC. The CD34-CD38+Lin- cells were devoid of LTC-IC activity (limit of detection at 10,000 cells) but contained a
20 much higher capacity to form CFC specifically committed to the erythroid lineage (Table I). The low efficiency of production of myeloid and erythroid committed progenitors, as well as the more primitive LTC-IC, is similar to observations made with murine CD34-Lin- cells in the same assay systems and suggest that functional similarities may exist between the CD34-Lin- cells from
25 these two species (8, 10).

Example 5 - Multilineage Differentiation of Human CD34-Lin- cells in
NOD/SCID mice after *ex vivo* Culture

A representative mouse was transplanted with 50,000 expanded CD34-Lin- cord blood cells after 2 days of *ex-vivo* culture in the presence of SF medium supplemented with 5% FCS. Mouse bone marrow was extracted 10 weeks after transplant and analyzed by multiparameter flow cytometry (11, 12).
5 The results are shown in Figure 8.

CD34- Cell Culture with Growth Factors

CD34-Lin- cells were incubated in 50 ml of SF condition consisting of IMDM supplemented with 1% BSA (Stem Cell Technologies), 5 mg/ml of
10 human insulin (Humulin R from Eli Lilly and Co.), 100 mg/ml of human transferrin (Gibco, BRL), 10 mg/ml of low density lipoproteins (Sigma Chemical Co.), 10⁻⁴ M Beta-mercaptoethanol and growth factors (GF). GF cocktail was used at final concentrations of 300 ng/ml of SCF (Amgen) and Flt-3 (Immunex), 50 ng/ml of G-CSF (Amgen), 10 ng/ml of IL-3 (Amgen) and IL-
15 6 (Amgen). 25% of condition media obtained from a fresh umbilical vein endothelial cell culture in a low percentage of serum (10%) and passaged four times, was added in some wells. Cells were cultured in flat bottomed suspension wells of 96-well plates (Nunc), incubated for 2 and 4 days at 37°C and 5% CO₂ and 50 ml of fresh GF cocktail was added to each well every other
20 day.

Example 6 - Effect of *ex vivo* culture on the number of clonogenic progenitors present in the CD34-Lin-, CD34-CD38-Lin- and CD34-CD38+Lin- cell fractions

25 An aliquot of 800 to 2, 500 CD34-Lin-, CD34-CD38-Lin- or CD34-CD38+Lin- cells were plated in clonogenic progenitor assays under standard conditions at the initiation of *ex vivo* cultures (day 0). Cells present after 4 days of culture in the presence of SF or SF supplemented with 5% FCS or 25% of HUVEC-CM were plated in the same conditions. The number of CFC/800

input cells were estimated (mean $\pm$ SEM ; n=number of experiment). The results are seen in Table II.

Example 7 - The effect of liquid culture on the development and potential
 5 differentiation of CD34-CD38-Lin- cells

Individual wells were analyzed by flow cytometry after 2 and 4 days of culture in various conditions (Fig. 6). CD34-CD38-Lin- cells seeded in SF media began to express CD34 which could be enhanced with the addition of serum (Fig. 6). In contrast, the majority of cells obtained after 2 or 4 days of
 10 culture in the presence of 25% HUVEC (human umbilical vein endothelial cell) conditioned medium still maintained the same phenotype. The acquisition of CD34 demonstrates the differentiation capacity of CD34-CD38-Lin- cells *in-vitro*. The production of CD34+CD38- cells suggests that CD34-CD38-Lin- cells precede CD34+CD38- population in the hierarchy of human
 15 hematopoiesis.

Example 8- Cytokine stimulation of CD34-CD38-Lin- Repopulating Activity

To evaluate the repopulating activity of CD34-Lin- and more highly purified CD34-CD38-Lin- cell fractions after *ex vivo* culture, cultured cells
 20 were injected into NOD/SCID mice and the level of human engraftment evaluated after 8 to 10 weeks. Purified cell fractions from 44 CB and 3 BM samples cultured for 2 and 4 days in SF, SF supplemented with 5% FCS or 25% HUVEC-CM were transplanted at various doses into 144 recipient NOD/SCID mice and the level of human cell engraftment was determined (Figure 7B). A
 25 total of 15 of 35 mice were engrafted following transplantation with CD34-Lin- cells that had been cultured for 4 days in SF or 5% FCS at cell doses below the calculated frequency of CD34-SRC. For example, 13 of 29 mice were engrafted following transplantation of 50,000 to as few as 4,000 cultured CD34-Lin- cells. By contrast, only 1 of 7 mice were engrafted when 100,000

uncultured CD34-Lin⁻ cells were transplanted (Figure 2). Similarly, a higher proportion of mice (33%) transplanted with cultured CD34-CD38-Lin⁻ cells were engrafted (Figure 7B) compared to mice transplanted with similar doses of uncultured CD34-CD38-Lin⁻ (Figure 7B). Quantitative analysis using

5 Poisson statistics indicated a frequency of 1 CD-SRC in 38,000 cultured CD34-CD38-Lin⁻ cells (range 1/22,000 to 1/71,000). It was not possible to calculate the frequency of CD34-SRC in uncultured CD34-CD38-Lin⁻ cells (Figure 7A) because there was infrequent engraftment despite injection of as many as

10 50,000 cells. This result indicates that the actual stem cell frequency of freshly isolated cells must be much lower compared to cultured cells. The human lineage distribution in mice transplanted with expanded CD34-Lin⁻ and CD34-CD38-Lin⁻ cells is similar to that with unstimulated purified CD34-Lin⁻ cells (Figure 8). In addition, the inability of cultured CD34-CD38⁺Lin⁻ cells to engraft mice confirms the absence of repopulating cells within this fraction. It

15 was evident that culture conditions, especially HUVEC-CM, induce an increase in the number of CD34-SRC in the absence of cell proliferation.

The bone marrow of engrafted mice was analyzed by multiparameter flow cytometry to determine whether cultured CD34-Lin⁻ repopulating cells possessed the same *in vivo* proliferative and differentiative capacity as

20 uncultured cells. A representative analysis of the bone marrow of a NOD/SCID mouse transplanted with an initial population of 40,000 CD34-Lin⁻ cells after 4 days of culture is shown in Figure 8. The bone marrow of this mouse contained 7% human cells as detected by expression of CD45, a human specific pan-leukocyte marker (Fig. 8). Both B and T-lymphoid cells were present in the

25 murine bone marrow as shown by staining for CD19, CD20 and CD4, CD3 antigens (Fig. 8D-F). The presence of CD33⁺, CD14⁺, CD15⁺ and CD13⁺ cells indicated the differentiation potential of CD38-CD38-Lin⁻ cells to the myeloid lineages (Fig. 8G-H). The engraftment pattern of mice transplanted with expanded CD34-CD38-Lin⁻ cells is similar to that observed with

unstimulated purified CD34-Lin- cells. The presence of human T-cells is a unique feature of CD34-Lin- engraftment, since T-cells have not been detected in mice transplanted with purified CD34+CD38-Lin- cells either before or after *ex vivo* culture.

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Example 9 - Testing of Whether CD34-SRC are Biologically Distinct from CD34+SRC.

To test whether the CD34+SRC are lost if CD34+CD38-Lin- or CD34+Lin- cells are cultured in the presence of serum, CD34+CD38-Lin- and
10 CD34-Lin- cells from the same human CB sample were cultured for 4 days under the same serum containing conditions. In a representative experiment, 3 out of 6 mice were engrafted following transplantation with cultured CD34-Lin- cells (Figure 9). In contrast, 5,000 and 10,000 CD34+CD38-Lin- cells, containing 10-20 CD34+SRC, cultured under the same conditions were unable
15 to engraft NOD/SCID mice. These results provide independent confirmation that the repopulating cells derived from the CD34-Lin- subfraction are biologically distinct from those derived from CD34+CD38-Lin- cells.

For the DNA analysis shown in Figure 9, NOD/SCID mice were transplanted with CD34-Lin- or CD34+CD38-Lin- cells purified from the same
20 cord blood sample and cultured in the presence of 5% FCS. A southern blot was performed using standard techniques and was hybridized with a human chromosome 17-specific α -satellite probe.

The present invention is not limited to the features of the embodiments described herein, but includes all variations and modifications within the scope
25 of the claims.

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Table 1

Hematopoietic Progenitor Activity of CD34-Lin-, CD34-CD38-Lin-
and CD34-CD38+Lin-

	<u>Phenotype of purified population</u>	<u>CFC/800 cells</u>	<u>Frequency of LTC-IC</u>
5	CD34-Lin-	8.9±3 (n=4)	<1/10,000 (n=2) exp #1 5,501±1640(n=2) exp#2
10	CD34-Lin-CD38-	2.7±2 (n=36)	<1/2,000 (n=4)
15	CD34-Lin-CD38+	77±31 (n=4)	<1/10,000 (n=10)
20	CD34+CD38-	250±46 (n=4)	>1/10 (n=4)

Table 2

Effect of *ex vivo* culture on the number of clonogenic progenitors
 5 present in the CD34-Lin-, CD34-CD38-Lin- and CD34-CD38+Lin- cell
 fractions.

	<u>Phenotype of purified population</u>	<u>Day</u>	<u>Medium</u>	<u>Number</u> [?]
10	CD34-Lin-	8.9 ±3 (n=4)	Serum Free 5% Serum	10 ±0.9 (n=4) 76 ±21 (n=2)
15	CD34-Lin-CD38-	2.7± 2 (n=36)	5% Serum Serum Free 25% HUVEC0.5% ±0.5 (n=7)	6.4 ±4.5 (n=7) 1.2 ±1 (n=9)
20	CD34-Lin-CD38+	77 ±31 (n=10)	Serum Free 5% Serum	20 ±10 (n=4) 134± 41 (n=4)
	*CFC/800 Input Cells			

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We claim:

1. A substantially homogeneous population of human hematopoietic stem cells which are CD34-Lin-.
- 5 2. A substantially homogeneous population of human hematopoietic stem cells which are CD34-CD38-Lin-.
3. The population of claim 1 or 2, wherein said stem cells are further characterized by the presence or absence of other antigenic phenotypic markers.
- 10 4. A therapeutic composition comprising an effective amount of CD34-Lin- human hematopoietic stem cells and a pharmaceutically acceptable carrier.
5. A therapeutic composition comprising an effective amount of CD34-
15 CD38-Lin- human hematopoietic stem cells and a pharmaceutically acceptable carrier.
6. The composition of claims 4 or 5, wherein said composition additionally comprises cells selected from the group consisting of CD34+ cells, Thy-1 cells,
20 CD4+cells, CD56+ cells, CD33+ cells, CD9+ cells, CD11+ cells, CD41+ cells, CD45+ cells and mixtures thereof.
7. The composition of claim 6, wherein said composition additionally comprises cells selected from the group consisting of B lymphocytes, T
25 lymphocytes, platelets and myeloid lineage cells.
8. The composition of claim 6, wherein said composition additionally comprises at least one pharmaceutical agent.

9. A method for obtaining a substantially enriched population of CD34-Lin- human hematopoietic cells comprising the steps of:
- removing mononuclear cells expressing some lineage-specific antigens from a sample of hematopoietic cells;
 - 5 - combining the resultant hematopoietic cells with labeled antibodies to which bind specifically to CD34+; and
 - isolating the unbound CD34-Lin- cells.
10. The method of claim 9, wherein said sample of hematopoietic cells is
10 selected from the group consisting of fetal cells, bone marrow, cord blood and peripheral blood.
11. A method for reconstituting hematopoiesis in an immunocompromised subject, the method comprising administering to a subject a composition
15 comprising an enriched population of CD34- Lin- stem cells.
12. A method for introducing CD34-Lin- stem cells in a mammal, said method comprising the steps of:
- providing an enriched population of CD34-Lin- stem cells; and
 - 20 - introducing said stem cells into said mammal.
13. The method of claim 12, wherein said enriched population of CD34-Lin- stem cells are characterized as CD34-CD38-Lin-.
- 25 14. The method of claim 12, wherein said enriched population of CD34-Lin- stem cells are isolated from bone marrow, cord blood, fetal blood, peripheral blood or bone marrow.

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15. The method of claim 12, wherein said enriched population of CD34-Lin-stem cells are genetically altered *in vitro* prior to introducing said stem cells into said mammal.
- 5 16. The method of claim 12, wherein said enriched population of CD34-Lin-stem cells are pharmaceutically or chemically treated *in vitro* prior to introducing said stem cells into said mammal.
- 10 17. The method of claim 12, wherein said enriched population of CD34-Lin-stem cells are differentiated into CD34+ cells prior to introducing said stem cells into said mammal.
- 15 18. The method of claim 12, wherein said enriched population of CD34-Lin-stem cells is introduced into a mammal having a disorder selected from the group consisting of leukemia, infection, immune disorders, Wiskott-Aldrich syndrome, Fanconi's anemia, severe combined immunodeficiency, congenital disorders, congenital red cell aplasia, lysosomal storage disease, thalassemia major, sickle cell anemia, aplastic anemia, acute lymphoblastic leukemia, acute myelogenous leukemia, megakaryoblastic leukemia, hematologic melanomas, lymphoma, multiple myeloma, myelodysplastic syndromes, carcinomas, neuroblastomas, arthritis and neurological genetic disease.
- 20 19. The method of claim 12, wherein said enriched population of CD34-Lin-stem cells is introduced into a mammal in order to replace a deficient cell type.
- 25 20. A method of treating a hematopoietic disorder in a subject, comprising:
- providing an enriched population of human CD34-CD38-Lin- stem cells;
administering said stem cells to the subject in need of treatment.

21. The method of claim 20, wherein the population of stem cells is cultured *in vitro* before administering the cells to the subject.
- 5 22. A method for the production of CD34+ stem cells, said method comprising:
- providing an enriched population of human CD34-CD38-Lin- stem cells;
 - culturing said stem cells *in vitro* under suitable conditions for a time
 - 10 sufficient to allow said cells to differentiate into CD34+ cells; and
 - isolating CD34+ stem cells.
23. A method for expanding a population of CD34-Lin- stem cells, said method comprising the steps of:
- 15 - isolating CD34-Lin- stem cells from suitable hematopoietic source;
 - culturing said isolated cells *in vitro* for a time sufficient and under culture conditions to result in the expansion of said cells.
24. The method of claim 23, wherein certain agents are added to said cell
- 20 culture to allow said cells to differentiate into a mature cell type.

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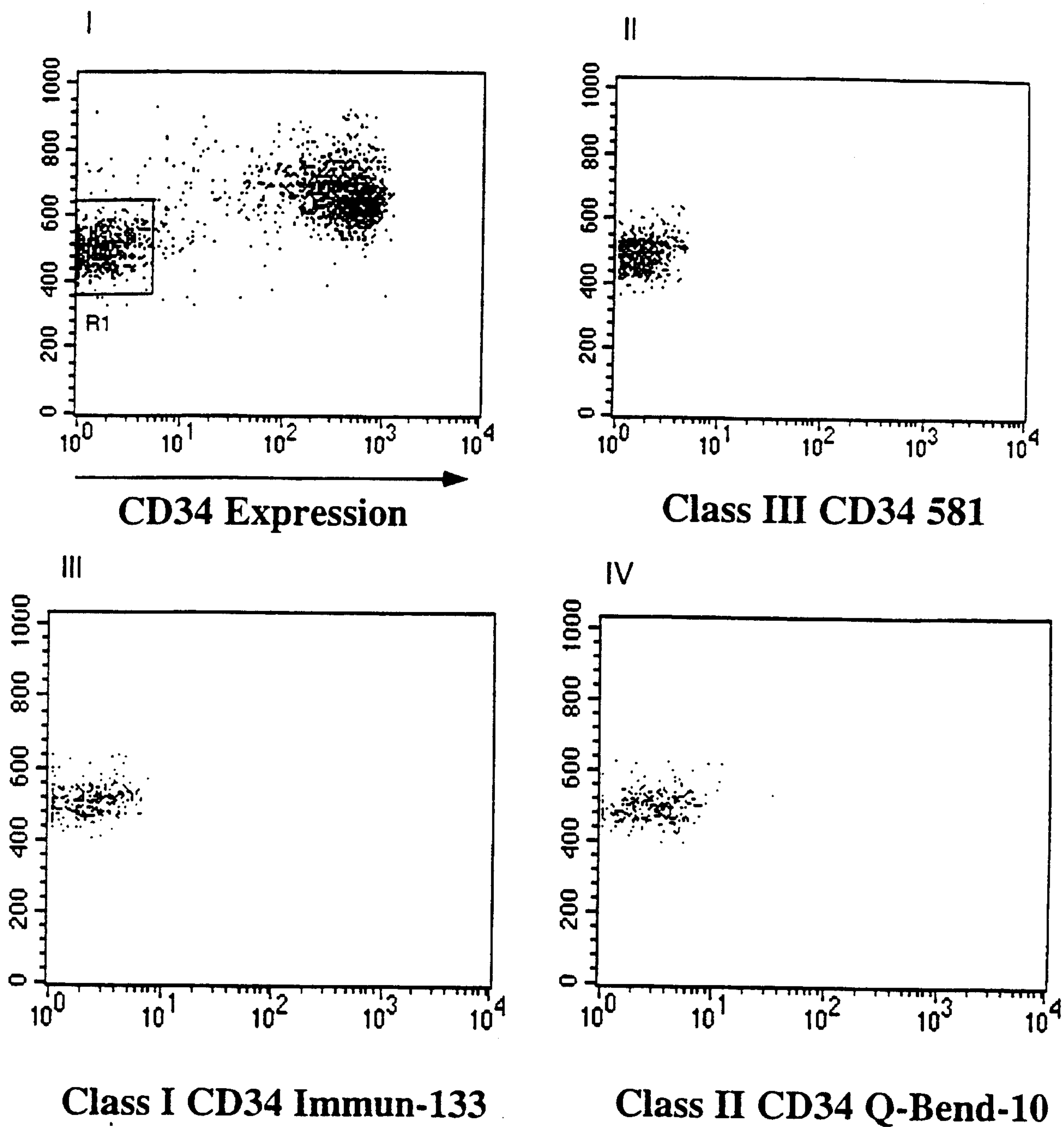


FIGURE 1A

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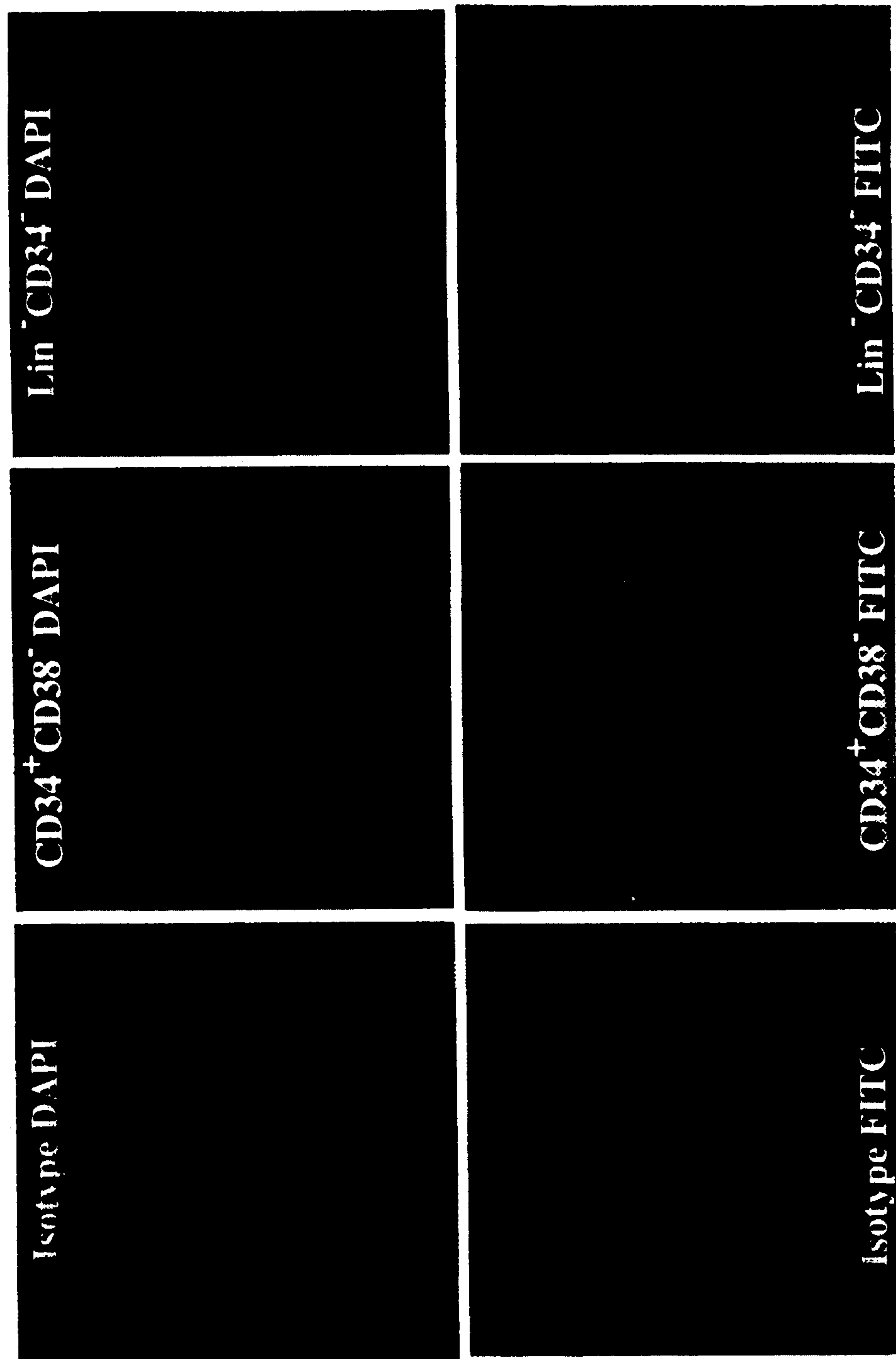


FIGURE 1B

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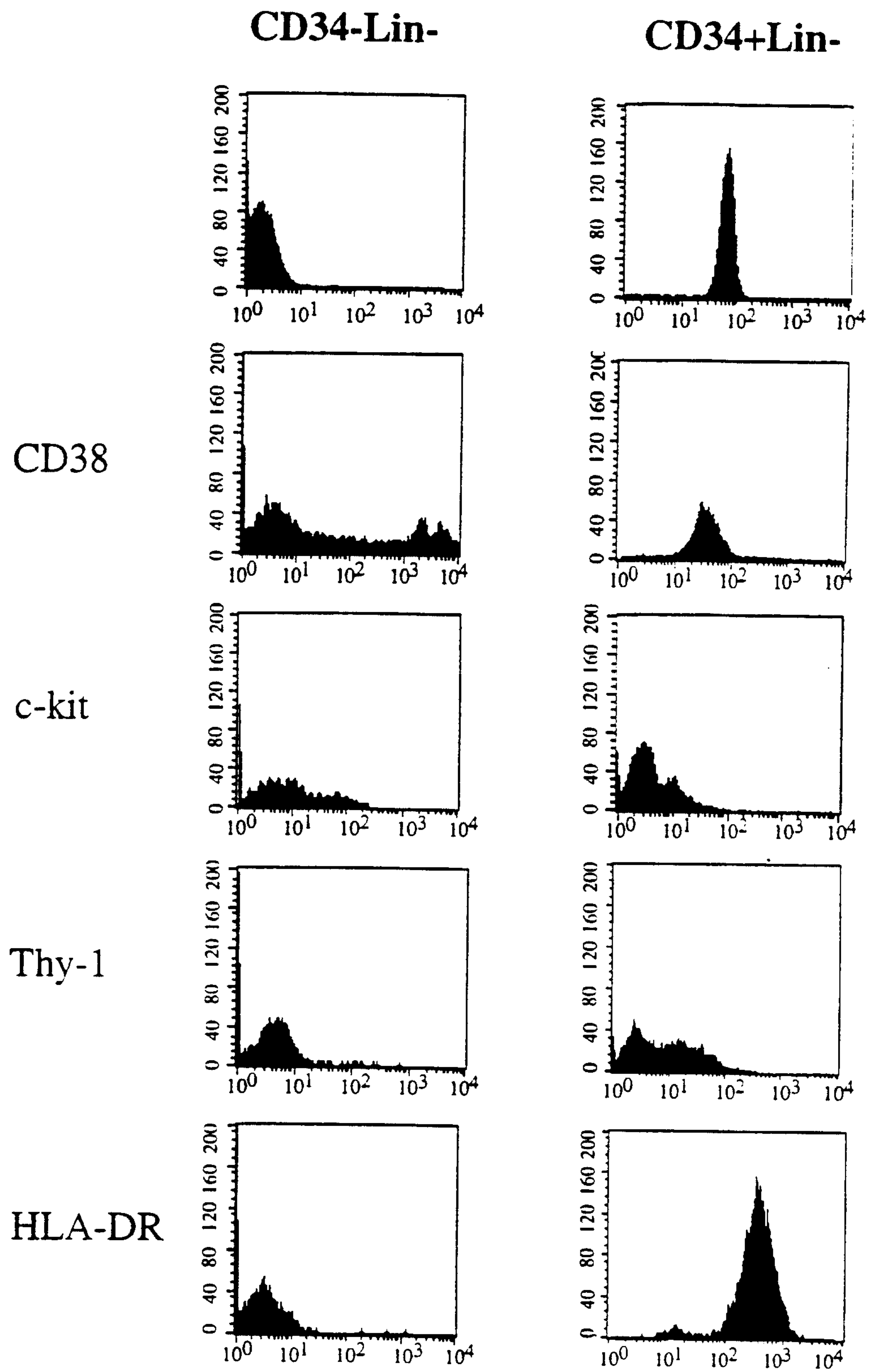


FIGURE 1C

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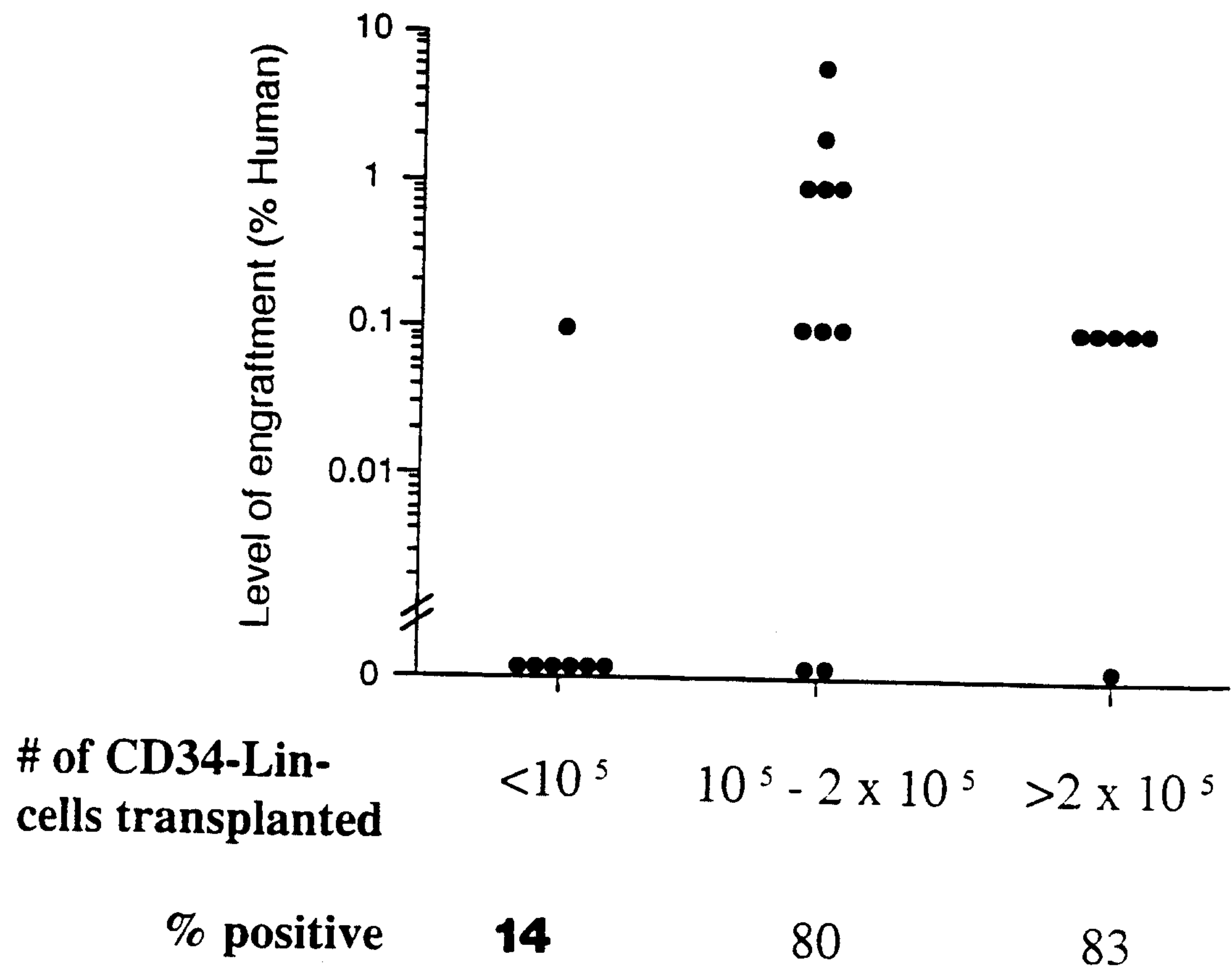


FIGURE 2

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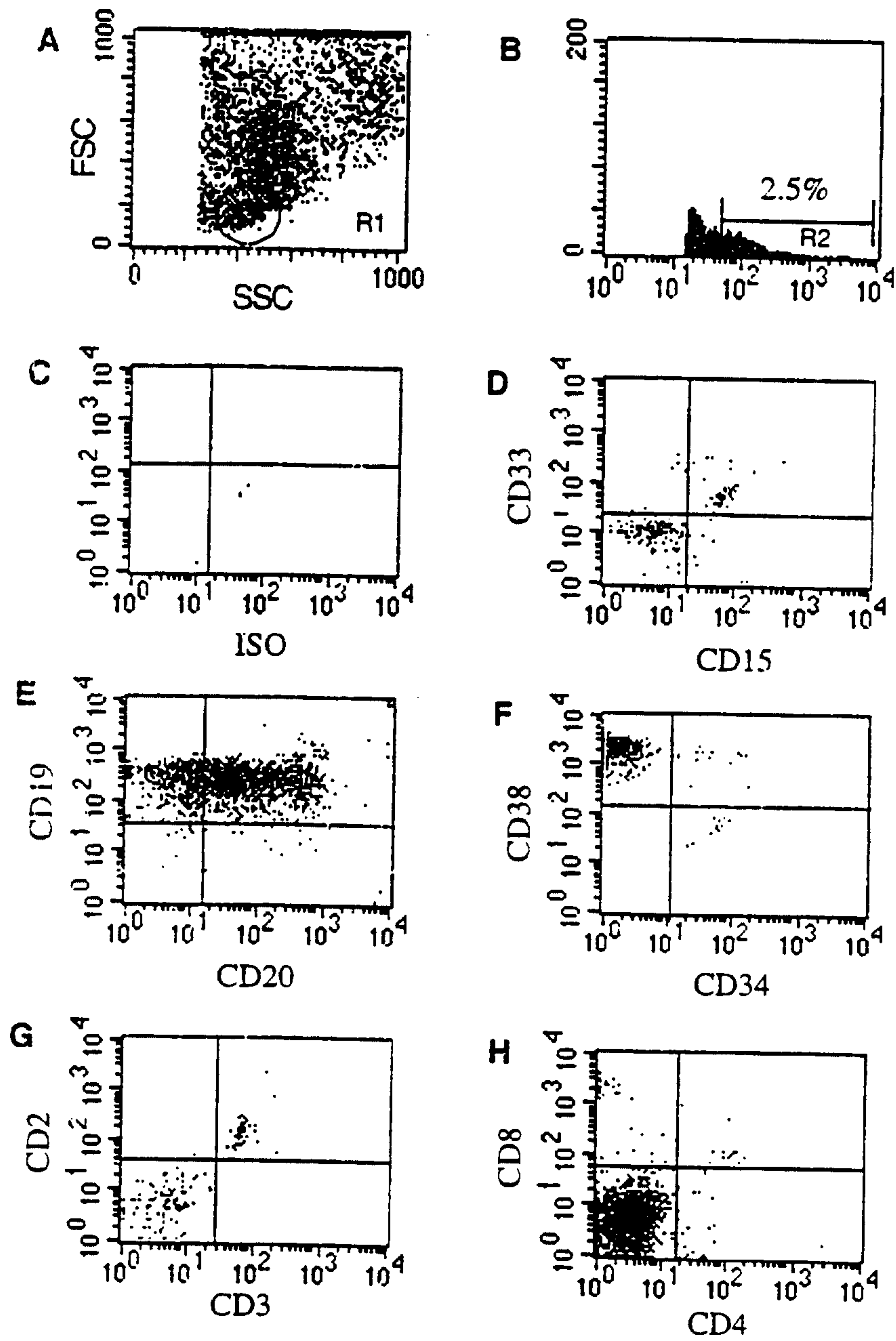


FIGURE 3

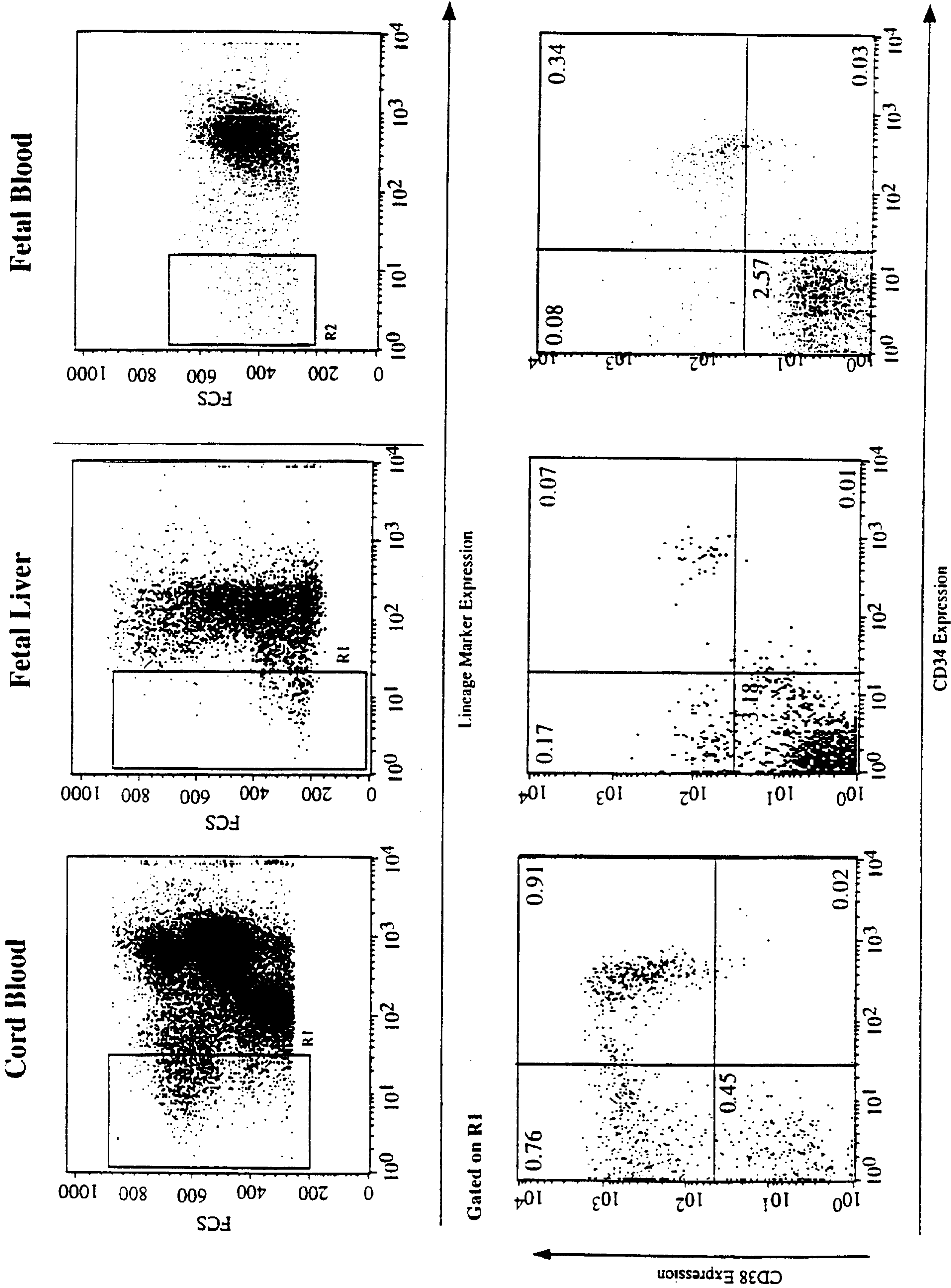


Figure 4A

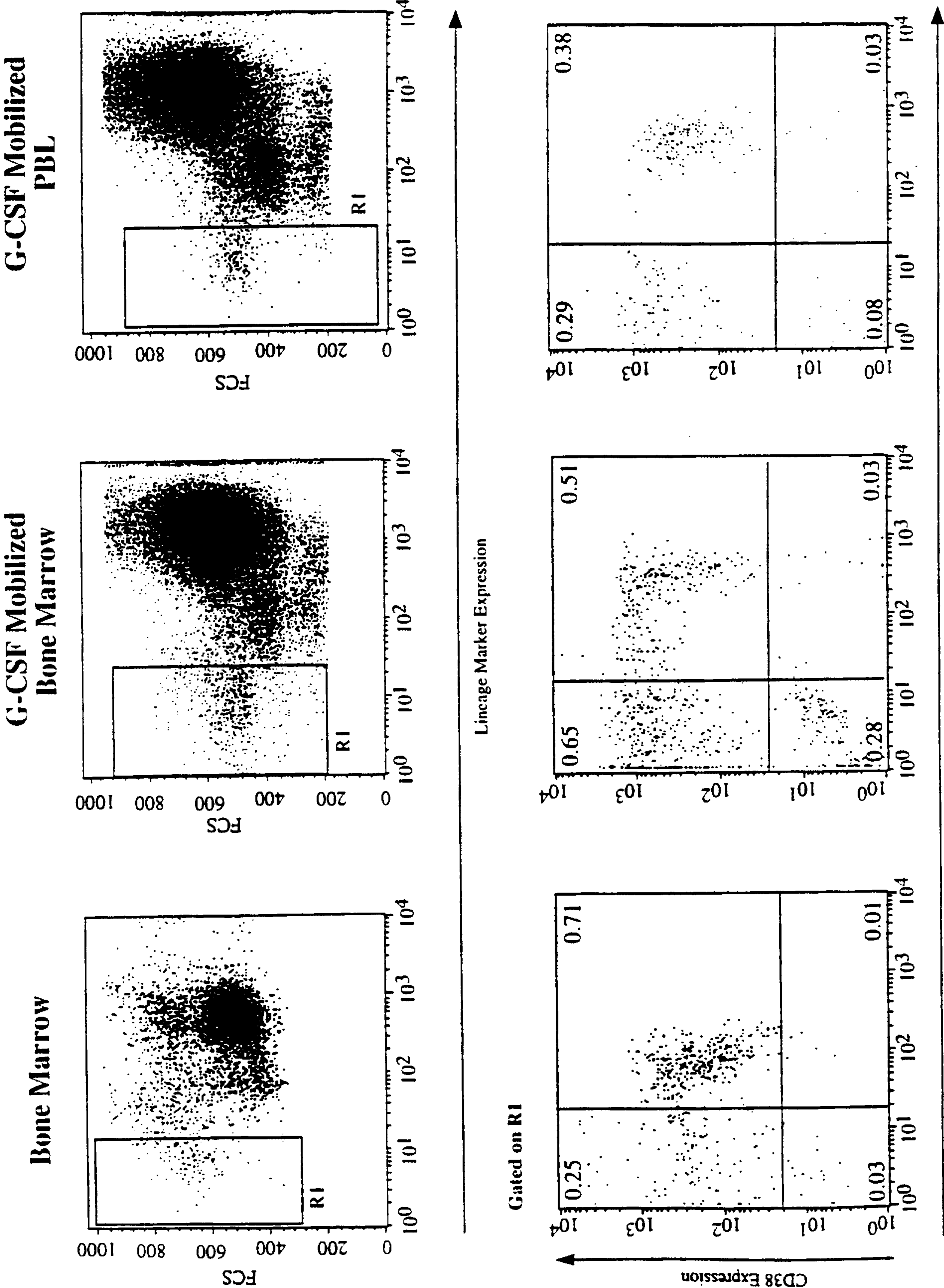


Figure 4B

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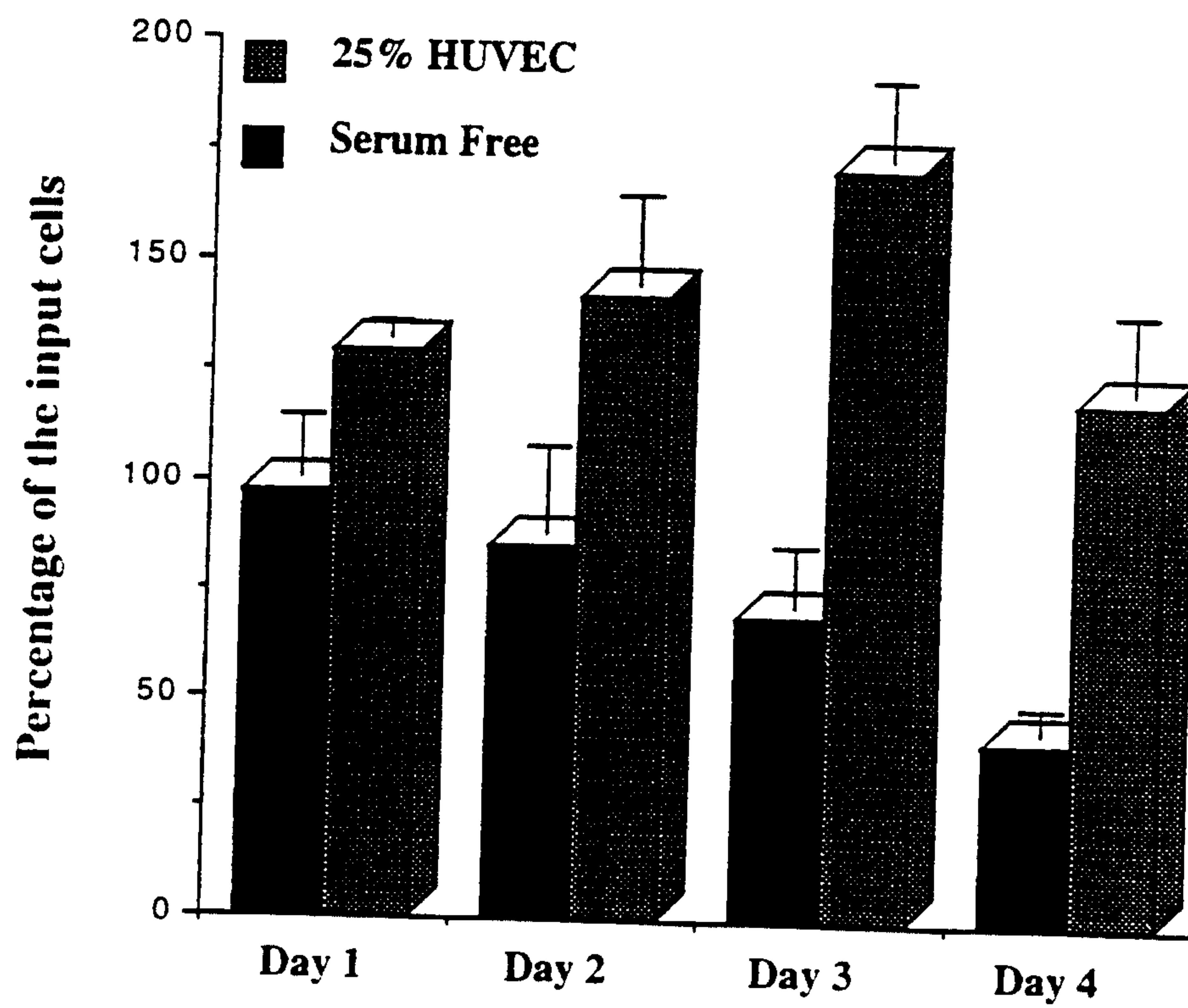


FIGURE 5

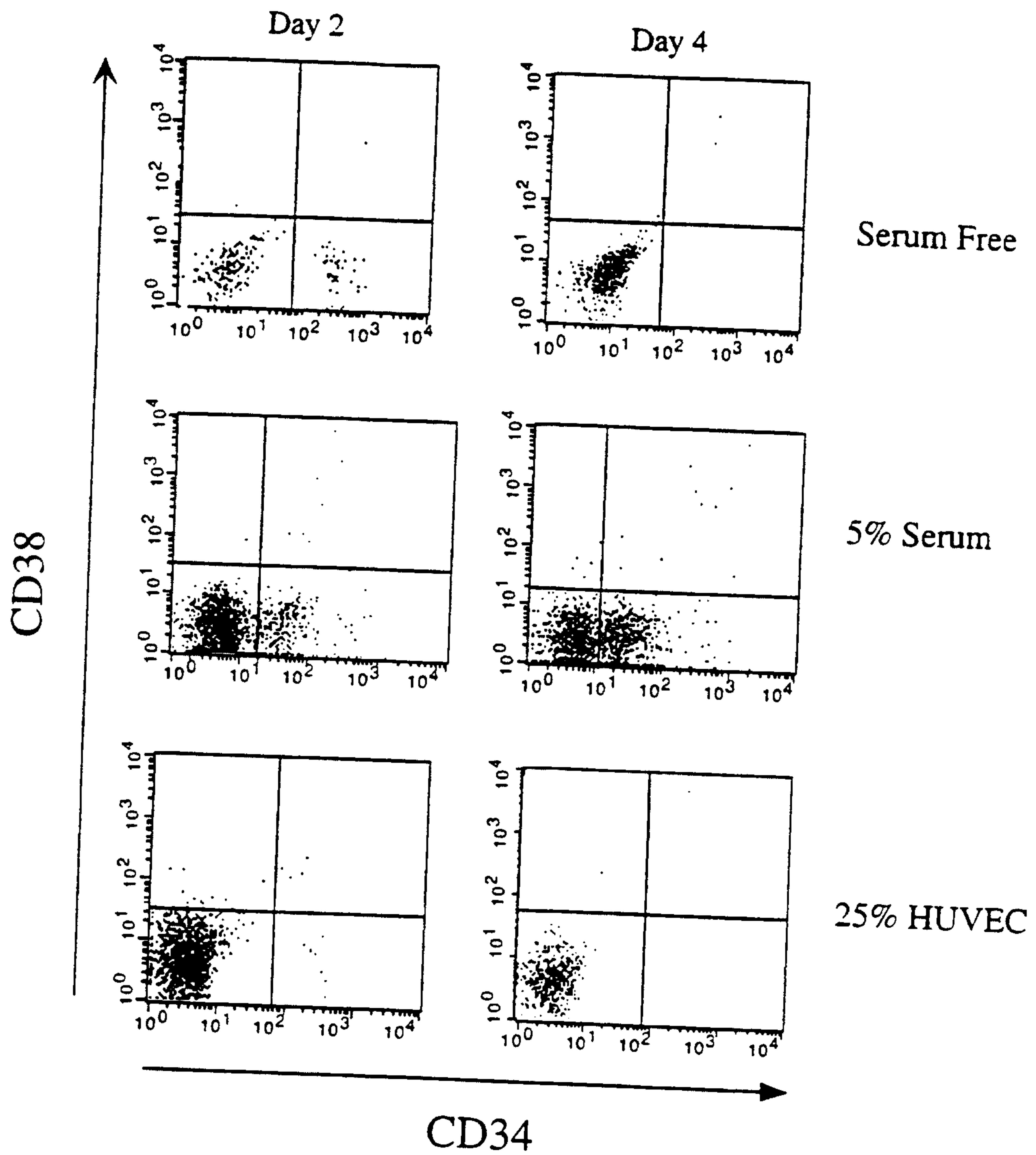


FIGURE 6

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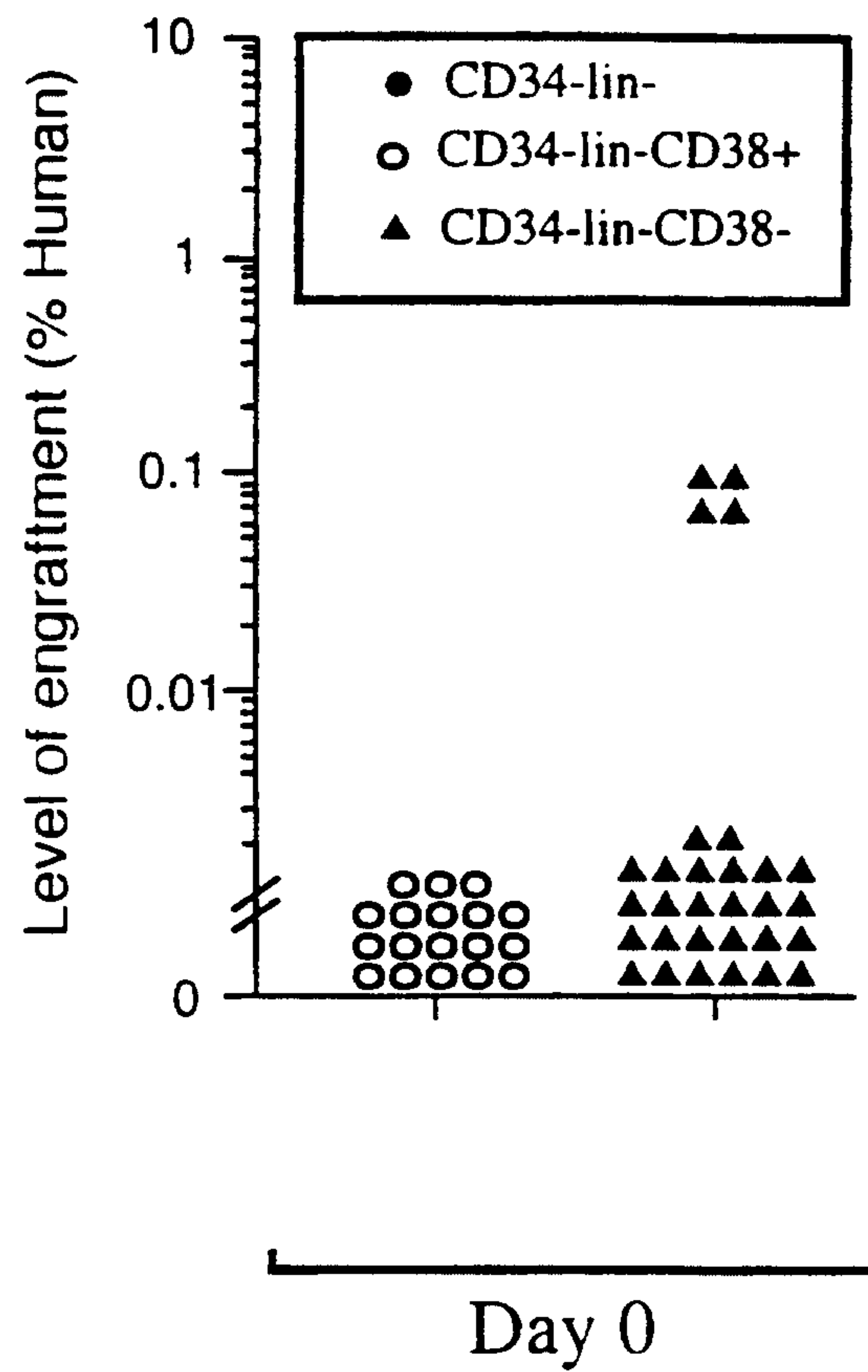


Figure 7A

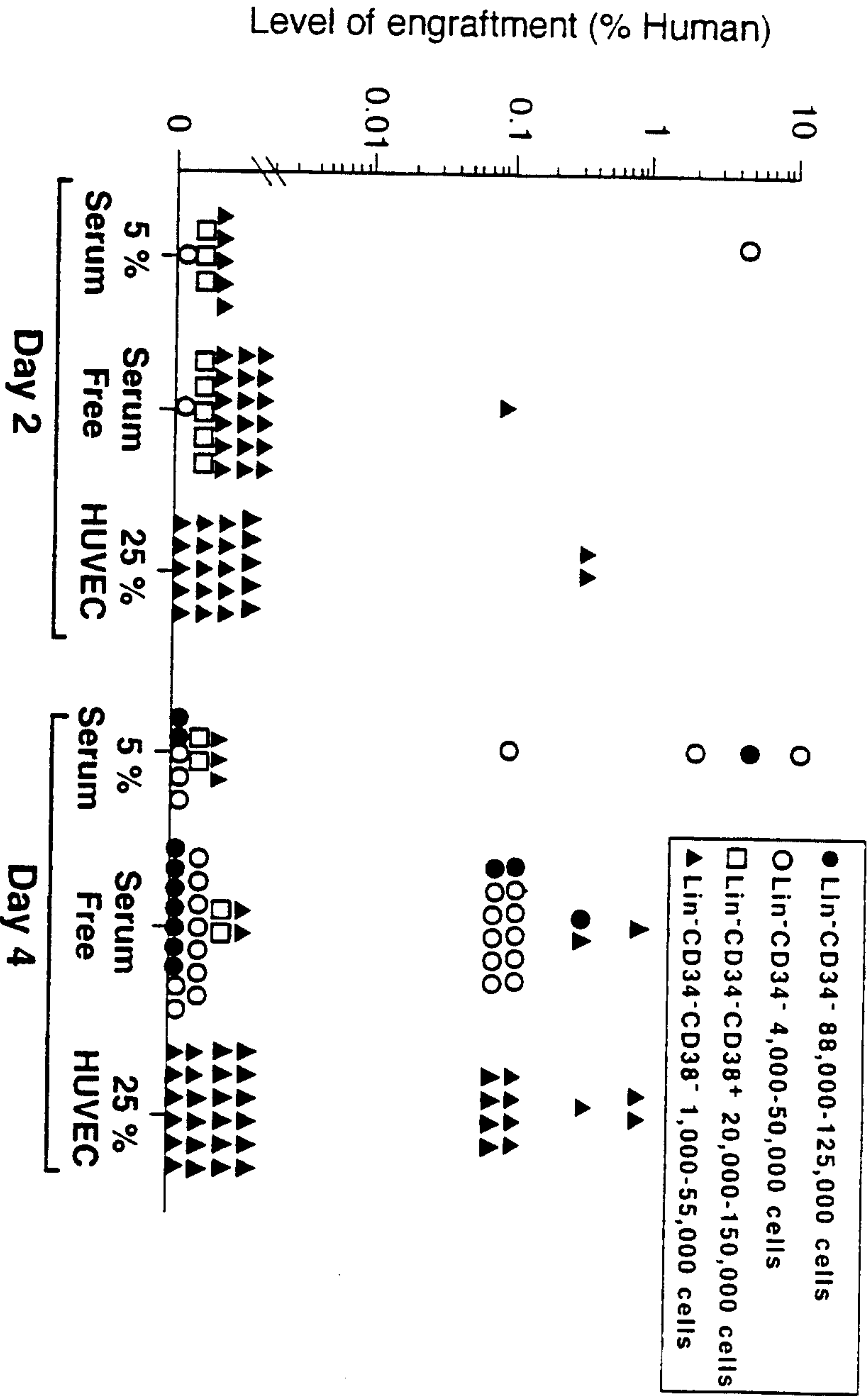


FIGURE 7B

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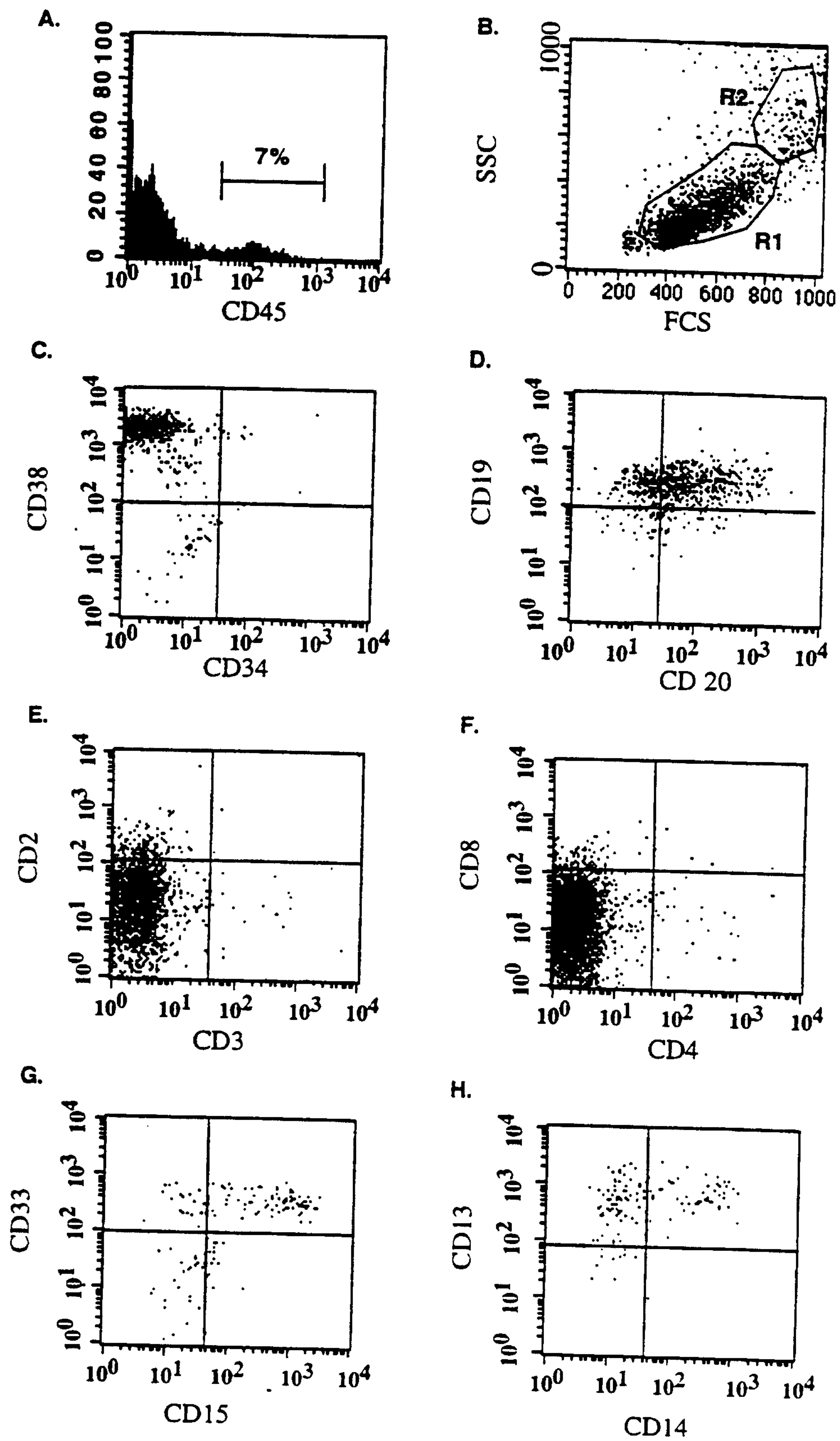


FIGURE 8

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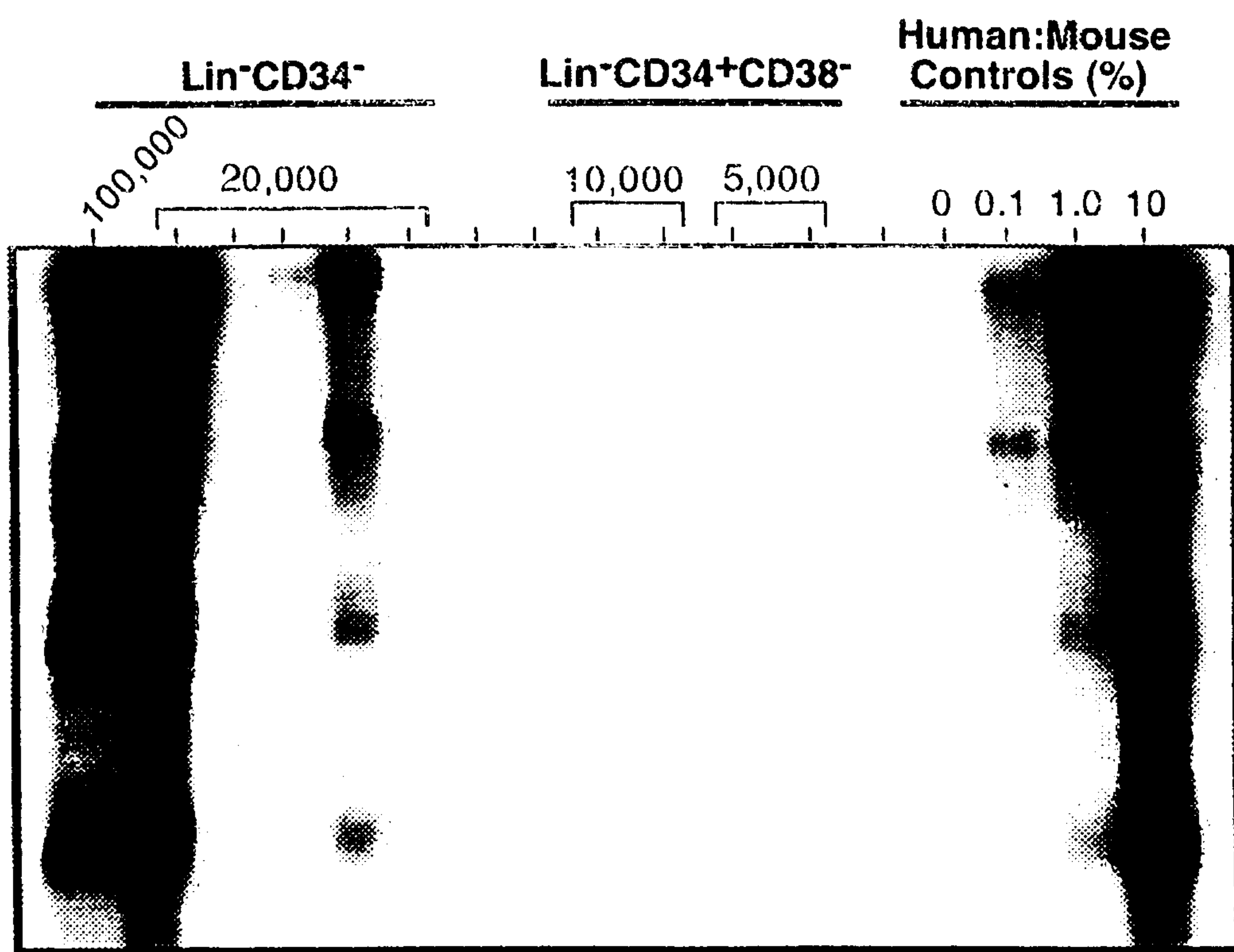


FIGURE 9