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(54) Title: METHODS OF INDUCING CELL DEATH OF PRIMITIVE HEMATOPOIETIC CELLS AND COMPOSITIONS FOR INDUCTION THEREOF

(57) Abstract

The present invention encompasses methods for inducing cell death in hematopoietic cells expressing CD43. The methods can be used in conjunction with therapeutic treatments such as chemotherapy and hematopoietic stem cell transplantation. The method is also useful in obtaining a highly enriched population of hematopoietic stem cells.

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METHODS OF INDUCING CELL DEATH OF PRIMITIVE HEMATOPOIETIC CELLS AND COMPOSITIONS FOR INDUCTION THEREOF

Technical Field

This invention is related to hematopoietic cell malignancies, isolation and purification techniques for sorting various hematopoietic cell populations, and assays for the identification and characterization of CD43 ligands and potential novel factors which stimulate proliferation of primitive hematopoietic cell populations.

Background Art

Mammalian hematopoietic cells provide a diverse range of physiological activities. These cells are divided into lymphoid, myeloid and erythroid lineages. The lymphoid lineage, comprising B cells and T cells, provides for the production of antibodies, regulation of the cellular immune system, detection of foreign agents in the blood, detection of cells foreign to the host, and the like. The myeloid lineage, which includes monocytes, granulocytes, megakaryocytes as well as other cells, monitors for the presence of foreign bodies, provides protection against neoplastic cells, scavenges foreign materials and produces platelets. The erythroid lineage provides the red blood cells, which act as oxygen carriers.

Despite the diversity of the nature, morphology, characteristics and function of the blood cells, it is

presently believed that these cells are derived from a single HPC population, termed "hematopoietic stem cells" (HSCs). Unlike more "mature" blood cells, HSCs are capable of self-regeneration but may also develop into hematopoietic progenitor cells (HPCs) that are no longer pluripotent and capable of self-regeneration. These HPCs divide repeatedly to form more mature cells which eventually become terminally differentiated to form the various mature hematopoietic cells. Thus the large number of mature hematopoietic cells is derived from a small reservoir of HSCs by a process of proliferation and differentiation. As used herein, "HSCs" refers to hematopoietic stem cells and not stem cells of other cell types.

A highly purified population of HSCs can be used for a variety of *in vitro* experiments and *in vivo* indications. For instance, a purified HSC population will allow for identification of growth factors associated with their self-regeneration. In addition, there may be as yet undiscovered growth factors associated (1) with the early steps of dedication of HSC to a particular lineage; (2) the prevention of such dedication; and (3) the negative control of HSC proliferation.

HSCs find use: (1) in regenerating the hematopoietic system of a host deficient in HSCs; (2) in a host that is diseased and can be treated by removal of bone marrow, isolation of HSCs and treatment of individuals with drugs or irradiation prior to re-engraftment of HSCs; (3) producing various hematopoietic cells; (4) detecting and evaluating growth factors relevant to HSC self-regeneration; (5) in the development of hematopoietic cell lineages and assaying for factors associated with hematopoietic development; and, (6) as targets for gene therapy to endow blood cells with useful properties.

Highly purified HSCs are essential for hematopoietic engraftment, including but not limited to, that in cancer

patients and transplantation of other organs in association with hematopoietic engraftment. HSCs are important targets for gene therapy, where expression of the recombinant genes promotes the health of the individual into whom the HSCs are transplanted. In addition, the ability to isolate HSCs may allow one to separate the HSCs from tumor cells in the bone marrow or peripheral blood of patients with cancer. These HSCs will then be reinfused into a patient after myelosuppressive or myeloablative chemotherapy. Thus, there have been world-wide efforts toward isolating the human HSC in substantially pure or pure form.

HSCs constitute only a small percentage of the total number of hematopoietic cells. Hematopoietic cells are identifiable by the presence of a variety of cell surface protein "markers." Such markers may be either specific to a particular lineage or HPC or be present on more than one cell type. The markers also change with stages of differentiation. Currently, it is not known how many of the markers associated with differentiated cells are also present on stem and HPCs. U.S. Pat. No. 4,714,680 describes a composition comprising human HSCs. Characterizations and isolation of human HSCs are reported in a variety of references. See e.g., Baum et al. (1991) *Proc. Natl. Acad. Sci. USA;* and Tsukamoto et al. U.S. Patent No. 5,061,620.

One marker, CD34, was previously indicated as present solely on HSCs. The original antibodies to CD34 were generated using the leukemic cell line KG1a. Since the original claim that CD34 was a HSC marker, numerous experiments have shown that it is also found on a significant number of lineage committed HPCs. In fact, 80-90% of the $CD34^+$ population is marked by other lineage specific and non-specific markers. Therefore, in view of the small proportion of the total number of cells in the bone marrow or peripheral blood which are HSCs, the uncertainty of the markers associated with the HSC as distinct from more differentiated

cells, and the general difficulty in assaying for human HSCs biologically, the identification and purification of HSCs has been elusive.

Mouse HSCs have been obtained in at least highly concentrated, if not a purified form, where fewer than about 30 cells obtained from bone marrow were able to reconstitute all of the lineages of the hematopoietic system of a lethally irradiated mouse. Each assayed cell is multipotent for all blood lineages, while self-renewal is variable amongst these cells. Spangrude et al. (1988) *Science 241*:58-62; Smith et al. (1991) *Proc. Natl. Acad. Sci. USA 88*:2788-2792; Uchida (1992) *Ph.D. Thesis Stanford U.*; and see also, EPA 89.304651.6 and the references cited therein which describe the isolation of mouse HSCs.

CD43 is a highly sialylated glycoprotein with a unique primary structure and is expressed in a high density on almost all mature leukocytes. Shelley et al. (1989) *Proc. Natl. Acad. Sci. USA 86*:2819; and Pallant, et al. (1989) *Proc. Natl. Acad. Sci. USA 86*:1328. Expression of this molecule has been reported to be defective in lymphocytes of patients with the X chromosome-linked immunodeficiency disorder, Wiskott-Aldrich syndrome. Although CD43 has been implicated in delivering a co-stimulatory signal to T-cells which promotes their proliferation, its function still remains elusive. Park et al. (1991) *Nature 350*:706.

Human CD43, designated sialophorin, has been cloned and sequenced. Shelley et al. (1989); and Pallant et al. (1989). The DNA sequence predicts an integral membrane polypeptide with an N-terminal hydrophobic signal peptide followed by a mucin-like 235-residue extracellular region with a uniform distribution of 46 serine, 47 threonine, and 24 proline residues. This is followed by a 23-residue transmembrane region and a 123-residue C-terminal intracellular region. These latter regions have been highly conserved during evolution; the intracellular region contains a number of

potential phosphorylation sites that might mediate transduction of activation signals.

CD43 has also been called gpL115, LSGP and leukosialin. CD43 is a heavily glycosylated mucin-type (acidic) glycoprotein (>50% carbohydrate) with one N-linked and multiple O-linked carbohydrate units. Cell-type specific glycosylation patterns give rise to two forms of CD43 with apparent molecular weights of 115,000 and 135,000. CD43 is expressed early in thymus ontogeny and, therefore, may play a role in the regulation of T-cell maturation and the development of the thymic repertoire. It has been suggested that the maintenance of lymphocytes in circulation, as well as their surface morphology, is dependent on the presence on the cell surface of intact native CD43 molecules bearing a high density of negative charge. In addition, monoclonal antibodies to CD43 activate human T lymphocytes independently of the T-cell receptor-CD3 complex, suggesting that CD43 is a critical component of an independent T-lymphocyte activation pathway. Studies with phorbol 12-myristate 13-acetate indicate that CD43 is subject to phosphorylation by protein kinase C.

Anti-CD43 monoclonal antibodies have a co-stimulatory effect on T cells, natural killer cells, B cells and monocytes, and one such antibody has been shown to activate T cells directly. To investigate a possible physiological role for CD43, a complementary DNA encoding the human protein was introduced into an antigen-responsive murine T-cell hybridoma. It was observed that CD43 enhances the antigen-specific activation of T cells and that the intracellular domain of CD43, which is hyperphosphorylated during T-cell activation, is required for this function. It was also found that antigen-presenting cells can bind specifically to immobilized purified CD43 and that the binding can be inhibited by liposomes containing CD43 as well as by anti-CD43 monoclonal antibodies.

CD43 has been reported to function as an alternative receptor for ICAM-1. Rosenstein et al. (1991) *Nature 354*:233. However, its ICAM-1-binding capacity could not be confirmed by others. de Fougerolles et al. (1993) *J. Exp. Med. 179*:619.

Hematopoiesis, maintained by HSCs and occurring in a bone marrow stroma microenvironment, is a tightly regulated process. Although adhesion molecules are generally thought to be involved in the regulation of HSC growth, their specific roles in this process are poorly understood. The intimate contact of primitive HSC with this microenvironment is mediated by adhesion molecules which are proposed not only to support attachment of these cells to stroma, but also to transduce signals involved in the regulation of their growth. Nonetheless, specific roles of these individual adhesion molecules remain virtually unknown. The only exceptions are murine $\alpha_4\beta_1$ integrin (Miyake et al. (1991) *J. Exp. Med. 173*:599) and CD44 (Miyake et al. (1990) *J. Exp. Med. 171*:477) which have been shown to participate in the regulation of HSC proliferation, since monoclonal antibodies recognizing these molecules abrogate hematopoiesis during *in vitro* stromal cell-dependent long-term cultures. Due to the complexity of the experimental system used in these studies, however, it is not clear whether the observed effects result from inhibition of 1) "passive" cellular contacts responsible for keeping HSCs in a proper environment supplied with necessary growth factors; 2) adhesive interactions coupled with HSC membrane signaling, directly involved in the regulation of their growth; or 3) indirect regulatory functions of accessory cells.

Cell surface molecules are known to play crucial roles in the growth control of lymphocytes. Such molecules may function as receptors for growth-stimulating cytokines or be associated with receptors and transmit signals essential for the regulation of growth. Receptor blockade or removal of

the stimulating cytokines can lead to decreased lymphocyte growth. For example, withdrawal of interleukins slows human lymphocyte growth and ultimately leads to a characteristic form of cell death called "programmed cell death" or apoptosis. Duvall et al. (1986) *Immun. Today* 7:115.

Apoptosis is the most common form of eukaryotic cell death and occurs in embryogenesis, metamorphosis, tissue atrophy and tumor regression. Wyllie et al. (1980) *Int. Rev. Cytol. 68*:251. It is also induced by cytotoxic T lymphocytes and natural killer and killer cells; by cytokines such as tumor necrosis factor (TNF) and lymphotoxin (LT); and by glucocorticoids. The characteristic signs of apoptosis are segmentation of the nucleus, condensation of the cytoplasm, membrane blebbing, and DNA fragmentation into multimers of about 180 base pairs in length. It has been demonstrated that anti-CD3 antibodies induce apoptosis in immature thymocytes *in vitro*. Smith et al. (1989) *Nature 337*:181. In addition, it appears that CD3-triggered apoptosis might be responsible for negative selection of T cells in the thymus.

The selective induction of apoptosis in cells, such as diseased cells, could prove a useful therapeutic tool. It has now been shown that a molecule, CD43, expressed on primitive HPCs may be directly involved in the regulation of their growth, delivering, under certain conditions, a signal for apoptosis.

All publications cited herein are hereby incorporated herein by reference in their entirety.

Summary of the Invention

The present invention encompasses methods of inducing apoptosis in hematopoietic cells including tumor cells. The method comprises administering an anti-CD43 antibody or other CD43 cross-linking agent under conditions whereby apoptosis is induced in the cells.

In another aspect, the present invention provides methods of depleting tumor cells from an *in vitro* population of hematopoietic cells, which methods comprise treating the *in vitro* population of cells with a CD43 antibody of sufficient amount and under conditions whereby apoptosis is induced in a substantial amount of the tumor cells and other more mature cells, while allowing HSCs to survive.

Thus, in one aspect, the present invention provides methods of enriching an *in vitro* population of hematopoietic cells for HSCs, comprising adding to the *in vitro* population of hematopoietic cells an amount of a CD43 antibody (αCD43) sufficient to induce cell death, while allowing HSCs to survive.

In addition, the present invention encompasses methods of treating a patient with a hematopoietic cell malignancy, comprising the steps of: (a) removing a population of hematopoietic cells from a patient with a malignancy to obtain an *in vitro* population of hematopoietic cells; (b) enriching the *in vitro* population of hematopoietic cells for HSCs according to a method of the present invention, to obtain a population of HSCs and HPCs containing a smaller tumor burden; (c) administering to the patient with a malignancy a sufficient dose of chemotherapeutic agents or radiation treatment to significantly reduce the level of malignancy; and (d) re-engrafting the population of HSCs into the patient following chemotherapeutic or radiation treatment.

The present invention further encompasses compositions comprising an enriched population of HSCs obtained by the methods of the invention. The population is termed ΔR-CD43 as it is obtained by depletion of hematopoietic cells expressing CD43 susceptible to CD43 mediated cell death. The remaining cells, while still expressing CD43, are resistant to αCD43-mediated apoptosis, thus, the designation ΔR-CD43. Note that after inducing cell death with αCD43, the

remaining, ΔR-CD43, hematopoietic cell population will usually still express CD43 and is not $CD43^-$. This population, enriched for HSCs, is resistant to the apoptotic effects of anti-CD43 antibodies.

In a further embodiment, the present invention encompasses compositions comprising a CD43 apoptosis-stimulating antibody, useful in the enrichment of an *in vitro* population of hematopoietic cells for HSCs.

In a further embodiment, the present invention encompasses methods of screening compounds to identify a potential apoptosis-stimulating CD43 ligand, which method comprises: (a) obtaining a composition comprising CD43, or a functional portion thereof; (b) contacting the CD43 to a test compound under conditions sufficient to allow binding of the test compound to CD43; (c) measuring binding of the test compound to CD43; (d) administering a test compound which specifically binds to CD43 to a population of HPCs; and (e) measuring the rate of apoptosis in the HPCs.

In a further embodiment, the ΔR-CD43 cells are used to screen for novel, stem cell specific, cytokines. The ΔR-CD43 cells are grown under conditions suitable to maintain viability and the effects of known or putative cytokines monitored for their effects on growth, differentiation and proliferation of the cells.

Brief Description of the Drawings

Figure 1 (A and B) is a series of FACS analyses depicting expression of CD43 by primitive HPCs.

Figure 2 is a graph depicting the effect of immobilized MEM-59 on the growth of $CD34^{hi}LIN^-$ cells.

Figure 3 is a series of FACS analyses depicting induction of cell death by MEM-59.

Figure 4 (A and B) shows both photomicrographs and FACS analyses of the detection of apoptotic cells in the MEM-59-treated $CD34^{hi}LIN^-$ cell population.

Figure 5 (A through D) is a series of FACS analyses depicting the effect of MEM-59 and cytokines on HPCs.

Figure 6 (A through D) is a series of FACS analyses depicting the effect of MEM-59 on the viability of peripheral lymphocytes after 48 hours (A and B) and 5 days (C and D).

Detailed Description of the Invention

It has now been found that CD43 is another cell surface molecule, in addition to the T-cell receptor FAS/APO-1, receptors for tumor necrosis factor, CD4, CD30 etc., which mediates apoptosis in certain types of cells whereas, in other cell types, proliferation is initiated or enhanced. The type of modulation depends upon the stage of cell maturation and activating condition. Previous studies have indicated only that an anti-CD43 monoclonal antibody (αCD43) stimulates proliferation of T-cells. Park et al. (1991).

The physiologic circumstances under which CD43 ligand-initiated apoptosis occurs remain to be elucidated. While not being bound by any one theory, it appears that induction of apoptosis requires the formation of multimers of cell surface CD43, and that actively growing cells and cells undergoing DNA synthesis are more susceptible to the induction of apoptosis than quiescent cells. The exact number of molecules in the multimer has not been elucidated but it appears that more than two molecules are involved.

This process may function *in vivo* as a negative regulatory mechanism eliminating highly proliferative cells which are either displaced from the appropriate environment or improperly activated. Alternatively, CD43 may deliver a positive signal for proliferation of HPC if combined with a second, so far unknown, stimulus. If this additional stimulus is absent, CD43-mediated signaling may lead to apoptosis in these "improperly" activated cells, as with the T-cell receptor on thymocytes, CD4 on T-cells, the immunoglobulin receptor on B-cells etc.

As used herein, the term "antibody" refers to both singular and plural forms and includes any functional part of the antibody. Such functional portions are those which are able to bind to CD43 to effect cell death in susceptible cells and include, but are not limited to, the entire antibody and antibody fragments containing functional portions thereof. Functional portions may include, but are not limited to, any compound comprised of a sufficient portion of the light chain variable region and/or the heavy chain variable region to effect binding to the CD43 epitope and effect apoptosis-stimulating activity. The fragments may include the variable region of at least one heavy or light chain immunoglobulin polypeptide, and include, but are not limited to, Fab', Fab'2, Fab, Fab2, and Fv fragments. In addition, any of the methods of the present invention which utilize CD43 antibodies in order to stimulate cell death, including apoptosis, can be altered by substituting the CD43 antibody for any CD43 cross-linking agent in sufficient quantity, and under appropriate conditions to stimulate cell death.

The invention can be practiced using antibodies derived from any vertebrate host or the immunoglobulin genes of any vertebrate, including fish, reptiles, amphibians, birds, and mammals. Preferably the host is murine and most preferably a mouse. In addition, the antibodies may be produced by any recombinant means known in the art. Such recombinant antibodies include, but are not limited to, fragments produced in bacteria and antibodies in which the majority of the constant regions have been replaced by human antibody constant regions. For a discussion of transgenic animals expressing human Ig loci and producing humanized antibodies, see WIPO publication number WO 91/10741, 94/04679 and 94/04667. The antibodies obtained are tested for their ability to induce killing in $CD43^+$ non-HSCs.

Suitable apoptosis-inducing CD43 antibodies for use in

the present invention include antibodies produced and selected as described herein as well as the available apoptosis inducing CD43 antibodies described in the examples herein, e.g., MEM-59, G019, TPI/36, Leu-22 (Becton-Dickinson) or DF-TI (Dako), and antibodies having the same or substantially the same binding specificity as any of these antibodies.

As described in more detail below, themechanism of cell death appears to be apoptosis and may be related to the ability of the antibodies to induce multimerization of CD43 on the surface of the hematopoietic cells. Therefore, in determining the apoptosis-inducing activity of the antibodies they should be in a form that allows such multimerization. Preferably, the antibodies are affixed to a solid support at a sufficient concentration to allow efficient cross-linking of CD43. Suitable methods of determining the activity of the antibodies are known in the art and are described herein. These methods are within the skill of one in the art and do not require undue experimentation; for example an assay such as the one found in Example 4 could be used.

Methods of producing antibodies are known in the art. Any method known in the art of monoclonal or polyclonal antibody production can be used herein. Such methods include, but are not limited to, inoculating an animal with an immunogenic amount of CD43 or an antigenic portion thereof, separating plasma cells secreting antibodies of the desired specificity from the animal, cloning the DNA expressing the variable regions of the light and heavy chains and expressing the recombinant genes in a suitable host cell. Standard monoclonal antibody generation techniques can be used wherein the antibodies are obtained from immortalized antibody-producing hybridoma cells. These hybridomas can be produced by fusing plasma cells, preferably isolated from the immunized host spleen, with compatible immortalized cells, preferably a non-Ig secreting B cell myeloma.

The invention encompasses methods of purifying HSCs for use in conjunction with cancer therapy in a patient in need of HSC replacement therapy or augmentation. The enriched HSC composition may find use in either an allogeneic or autologous transplant setting. For autologous transplants, the methods comprise: removing a population of hematopoietic cells from the patient, depleting more mature hematopoietic cells to obtain a population of HPCs including HSCs and containing a reduced tumor cell burden, treating the population of HPCs according to the methods described herein to obtain a population of ΔR-CD43 cells; administering to the patient a myelosuppressive or myeloablative dose of a chemotherapeutic agent or radiation; and re-engrafting the ΔR-CD43 cells. Methods for administering chemotherapeutic agents and radiation, as well as methods of re-engrafting HSCs into a patient are well known in the art and are not described herein.

The invention further provides a composition highly enriched in HSCs and depleted in cells susceptible to CD43-mediated killing (designated ΔR-CD43 cells) obtained by the methods described herein. Such a composition has particular utility in reconstituting human hematopoietic systems. The cells of the composition are particularly useful as an autologous transplant provided to patients suffering from a myeloproliferative disorder in that tumor cells will be purged from the autograft while HSCs are spared.

The invention embodies methods of obtaining compositions enriched in HSCs from populations of hematopoietic cells. The methods comprise treating a population of hematopoietic cells with an amount of a CD43 antibody of sufficient amount and under conditions effective to induce cell death in the cell population while sparing HSCs. The methods of the invention include techniques which kill some HSCs as long as the ratio of HSCs killed to total HSCs is less than the ratio of non-HSCs killed to total non-HSCs.

A "HSC" may be defined as follows: (1) gives rise to progeny in all defined hematolymphoid lineages; and (2) limiting numbers of cells are capable of fully reconstituting a seriously immunocompromised host of all blood cell types and their progenitors, including the pluripotent hematopoietic stem cell by self renewal. Sources of hematopoietic cells for subsequent purification include, but are not limited to, bone marrow, both adult and fetal; mobilized peripheral blood (MPB); blood; umbilical cord blood; embryonic yolk sac; fetal liver; and spleen, both adult and fetal. Bone marrow cells may be obtained from any known source, including, but not limited to, ileum (e.g., from the hip bone via the iliac crest), sternum, tibiae, femora, spine and other bone cavities.

For isolation of bone marrow from fetal bone or other bone source, an appropriate solution may be used to flush the bone, including, but not limited to, salt solution, conveniently supplemented with fetal calf serum (FCS) or other naturally occurring factors, in conjunction with an acceptable buffer at low concentration, generally from about 5-25 mM. Convenient buffers include, but are not limited to, Hepes, phosphate buffers and lactate buffers. Otherwise, bone marrow may be aspirated from the bone in accordance with conventional techniques.

The enrichment of HPCs may be by negative selection (removal of other committed cells) or positive selection (isolation of cells), or a combination of both. Preferably, the more mature cells are removed from the starting population. This enriches for the HPCs that are susceptible to CD43-mediated cell killing. The susceptible HPCs are those which read out in standard BFU-E, CFU-GEMM, and, to a lesser extent, CFU-GM assays.

Various techniques may be employed to separate the cells by initially removing cells of dedicated lineage. Monoclonal antibodies may find use for identifying markers associated

with particular cell lineages and/or stages of differentiation. The antibodies may be attached to a solid support to allow for crude separation. The separation techniques employed should maximize the viability of the fraction to be collected. Various techniques of different efficacy may be employed to obtain "relatively crude" separations. Using such separation methods, up to 10%, usually not more than about 5%, preferably not more than about 1%, of the total cells are undesired cells that remain with the cell population to be retained. The particular technique employed will depend upon efficiency of separation, associated cytotoxicity, ease and speed of performance, and necessity for sophisticated equipment and/or technical skill.

Procedures for separation may include, but are not limited to, magnetic separation using antibody-coated magnetic beads; affinity chromatography; cytotoxic agents joined to a monoclonal antibody or used in conjunction with a monoclonal antibody, including, but not limited to, complement and cytotoxins; and "panning" with antibody attached to a solid matrix, e.g., plate, elutriation or any other convenient technique.

The use of separation techniques include, but are not limited to, those based on differences in physical (density gradient centrifugation and counter-flow centrifugal elutriation), cell surface (lectin and antibody affinity), and vital staining properties (mitochondria-binding dye rho123 and DNA-binding dye Hoechst 33342).

Techniques providing accurate separation include, but are not limited to, flow cytometry, which can have varying degrees of sophistication, e.g., a plurality of color channels, low angle and obtuse light scattering detecting channels, impedance channels, etc.

While it is believed that the particular order of separation is not critical to this invention, the order indicated is preferred. Preferably, cells are initially

separated by a coarse separation, followed by a fine separation to obtain the sensitive cell population which is then treated with a CD43 antibody or other CD43 cross-linking agent. The coarse separation methods used can be any known in the art, including but not limited to ficoll gradient separation to enrich the sample for mononuclear cells; ammonium chloride lysis or other comparable method for removing red blood cells; and elutriation. For a review of physical separation techniques applied to the separation of hematopoietic cells, particularly elutriation, refer to pending PCT application, Serial No. US94/10501.

Alternately, the methods of the invention may include one or more fine separation step with positive selection of a marker associated with HPCs and/or negative selection for markers associated with lineage committed cells. In general, the methods of the present invention encompass the use of the CD43 antibody, as described herein, in combination with standard cell sorting or antigen affinity-based positive and negative selection techniques known in the art, including but not limited to those specific for $CD34^+$, $CD34^+LIN^-$, $c\text{-}kit^+$, $CD38^-$, $HLA\text{-}DR^{lo/-}$ for adult, $HLA\text{-}DR^+$ for fetal, Thy^+ and Rho^{lo} alone or in combination, or any other markers shown to enrich for HSC and progenitors.

Lin^- cells generally refer to cells which lack markers associated with T cells (such as CD2, 3, 4 and 8), B cells (such as CD10, 19 and 20), myeloid cells (such as CD14, 15, 16 and 33), natural killer ("NK") cells (such as CD2, 16 and 56), RBC (such as glycophorin A), megakaryocytes, mast cells, eosinophils or basophils. The absence or low expression of such lineage specific markers is identified by the lack of binding of antibodies specific to the cell specific markers, useful in so-called "negative selection". Analyses for hematopoietic progenitors have been reported by Whitlock and Witte (1982) *Proc. Natl. Acad. Sci. USA* *79*:3608-3612; and Whitlock et al. (1987) *Cell* *48*:1009-1021.

Table 1 summarizes probable phenotypes of HSCs in fetal, adult, and mobilized peripheral blood. In Table 1 myelomonocytic stands for myelomonocytic associated markers, NK stands for natural killer cells and AMPB stands for adult mobilized peripheral blood. As used herein both infra, supra and in Table 1, the negative sign or, uppercase negative sign, ($^-$) means that the level of the specified marker is undetectable above Ig isotype controls by FACS analysis, and includes cells with very low expression of the specified marker.

Table 1

Probable Stem Cell Phenotypes

	NK and T cell markers			B cell markers			Myelomonocytic			Other						P-gp Activity
	CD2	CD3	CD8	CD10	CD19	CD20	CD14	CD15	CD16	CD33	CD34	CD38	HLA-DR	C-Kit	Thy	Rho	
FBM	-	-	-	-	-	-	-	-	-	?	+	-	+	+	+	lo	+
ABM	-	-	-	-	-	-	-	-	-	-	+	-	lo/-	+	+	lo	+
AMPB	-	-	-	-	-	-	-	-	-	lo/-?	+	?	lo/-	?	+	lo	+

For the isolation of HSCs, the methods of the present invention are superior to the prior art in that they require less manipulation of the hematopoietic cells and, in many cases, will provide a more pure population of HSCs. Typically, the methods of isolating HSCs known in the art require successive purification steps each relying on antibody affinity to a particular cell surface antigen or lineage marker.

Further, with each successive manipulation or separation procedure, the HSCs are subjected to the risk of contamination with bacteria, viruses, fungi, mycoplasma etc. In addition, each separation procedure kills, injures, and eliminates by missorting a fraction of the HSCs. Thus, the HSC isolation methods of the invention result in a lower risk of contamination with bacteria, viruses, fungi, etc., as well as potentially higher yields of HSCs.

The compositions comprising ΔR-CD43 cells are found to provide for production of myeloid cells and lymphoid cells in appropriate cultures, cultures providing hydrocortisone for production of myeloid cells (associated with Dexter-type cultures) and B lymphocytes in cultures lacking hydrocortisone (associated with Whitlock-Witte-type cultures). In each of the cultures, mouse or human stromal cells may be provided, which may come from various strains, including, but not limited to, AC3 or AC6 (otherwise referred to herein as "SyS1" or "AC6.21"), stromal cells derived from mammalian bone marrow by selection for the ability to maintain human HSCs, and the like. Suitable bone marrow sources include, but are not limited to, human, murine and porcine.

Culture conditions of the cells may vary both before and after CD43 selection. Preferably, during CD43 selection, the growth medium contains suitable amounts of stem cell factor ("SCF") and IL3. Suitable cytokines may also include, but are not limited to, G-CSF, GM-CSF, IL6, FLK2, IL11, IL1, IL7,

leukemia inhibiting factor (LIF) and MIP-1α. Suitable amounts of these cytokines are those which are effective to enhance CD43-induced apoptosis in HPC, compared to apoptosis in the absence of cytokines, but allow the highest yield of HSCs by maintaining viability and minimizing differentiation. Since cell cycling appears to be important in CD43-mediated cell killing, the HPC may be cultured with cytokines prior to CD43 selection to induce more HPCs to enter the cell cycle.

The cells obtained as described above and in the Examples may be used immediately or frozen at liquid nitrogen temperatures and stored for long periods of time, being thawed and capable of being reused. The cells will usually be stored in 10% DMSO, 50% FCS, 40% RPMI 1640 medium. Once thawed, the cells may be expanded by use of growth factors and/or stromal cells associated with proliferation and differentiation.

To demonstrate differentiation to T cells, fetal thymus is isolated and cultured for from 4-7 days at about 25°C and irradiated, so as to deplete substantially the lymphoid population. The cells to be tested for T cell activity are then microinjected into the thymus tissue, where the HLA of the population which is injected is mismatched with the HLA of the thymus cells. The thymus tissue may then be transplanted into a scid/scid mouse as described in U.S. Patent No. 5,147,784, particularly transplanting under the kidney capsule.

Specifically, the population enriched for HSCs by CD43 depletion (or the untreated control) can be microinjected into HLA mismatched thymus fragments. After 6-10 weeks, assays of the thymus fragments injected with the ΔR-CD43 cells can be performed and assessed for donor derived T cells. Thymus fragments injected with the ΔR-CD43 cells will generate and sustain $CD3^+$, $CD4^+$, and $CD8^+$ T cells along with their progenitors.

Further demonstration of the sustained ability of the

various cell populations may be accomplished by the detection of continued myeloid and B-lymphoid cell production in the SCID-hu bone model. Kyoizumi et al. (1992) *Blood 79*:1704; and Galy et al. (1994) *Blood 84*:104. To analyze this, one may isolate human fetal bone and transfer a longitudinally sliced portion of this bone under the skin of a scid/scid animal: the bone cavity is diminished of endogenous cells by whole body irradiation of the mouse host prior to injection of the test donor population. The HLA of the population which is injected is mismatched with the HLA of the bone cells. ΔR-CD43 cells from human hematopoietic sources will be expected to sustain B lymphopoiesis and myelopoiesis in the SCID-hu bone model.

For red blood cells, one may use conventional techniques to identify BFU-E units, for example methylcellulose culture demonstrating that the cells are capable of developing the erythroid lineage. Metcalf (1977) In: Recent Results in Cancer Research 61. Springer-Verlag, Berlin, pp. 1-227

Once the ΔR-CD43 cells have been isolated, they may be propagated by growth in conditioned medium from stromal cells, such as stromal cells that can be obtained from bone marrow, fetal thymus or fetal liver, and are shown to provide for the secretion of growth factors associated with HPC maintenance, or co-culturing with such stromal cells, where the stromal cells may be allogeneic or xenogeneic. Before using in the co-culture, the mixed stromal cell preparations may be freed of hematopoietic cells employing appropriate monoclonal antibodies for removal of the undesired cells, e.g., with antibody-toxin conjugates, antibody and complement, etc. Alternatively, cloned stromal cell lines may be used where the stromal lines may be allogeneic or xenogeneic.

The invention also encompasses methods of use of the ΔR-CD43 cell populations. The subject cell compositions may find use in a variety of ways. Since the cells are naive, they can be used to fully reconstitute an irradiated host and/or a host subject to chemotherapy; or as a source of cells for specific lineages, by providing for their maturation, proliferation and differentiation into one or more selected lineages by employing a variety of factors, including, but not limited to, erythropoietin, colony stimulating factors, e.g., GM-CSF, G-CSF, or M-CSF, interleukins, e.g., IL-1, -2, -3, -4, -5, -6, -7, -8, etc., or the like, or stromal cells associated with the HSCs becoming committed to a particular lineage, or with their proliferation, maturation and differentiation. The ΔR-CD43 cells may also be used in the isolation and evaluation of factors associated with the self renewal, differentiation, and maturation of hematopoietic cells. Thus, the ΔR-CD43 cells may be used in assays to determine the activity of media, such as conditioned media, evaluate fluids for cell growth activity, involvement with dedication of particular lineages, or the like.

The ΔR-CD43 cells may be used as targets for gene therapy for the treatment of various diseases, particularly genetic diseases. Genetic diseases associated with hematopoietic cells may be treated by genetic modification of autologous or allogeneic HSCs to provide cells which do not contain the genetic defect. For example, diseases including, but not limited to, β-thalassemia, sickle cell anemia, adenosine deaminase deficiency, recombinase deficiency, recombinase regulatory gene deficiency, etc. may be corrected by introduction of a wild-type gene into the ΔR-CD43 cells, either by homologous or random recombination. Other indications of gene therapy are introduction of drug resistance genes to enable normal HSCs to have an advantage and be subject to selective pressure during chemotherapy.

Suitable drug resistance genes include, but are not limited to, the gene encoding the multidrug resistance (MDR) protein.

Diseases other than those associated with hematopoietic cells may also be treated by genetic modification, where the disease is related to the lack of a particular secreted product including, but not limited to, hormones, enzymes, interferons, growth factors, or the like. By employing an appropriate regulatory initiation region, inducible production of the deficient protein may be achieved, so that production of the protein will parallel natural production, even though production will be in a different cell type from the cell type that normally produces such protein. It is also possible to insert a ribozyme, antisense or other message to inhibit particular gene products or susceptibility to diseases, particularly hematolymphotropic diseases.

Alternatively, one may wish to remove a particular variable region of a T-cell receptor from the T-cell repertoire. By employing homologous recombination, or antisense or ribozyme sequence which prevents expression, the expression of the particular T-cell receptor may be inhibited. For hematotrophic pathogens, such as HIV, HTLV-I and II, etc. the HSCs could be genetically modified to introduce an antisense sequence or ribozyme which would prevent the proliferation of the pathogen in the HSC or cells differentiated from the HSCs. Methods for recombination in mammalian cells may be found in Molecular Cloning, A Laboratory Manual (1989) Sambrook, Fritsch and Maniatis, Cold Spring Harbor, NY.

The present invention further encompasses methods for obtaining and compositions of cells which are highly enriched in HSCs. The method comprises incubating the compositions described above under conditions suitable for regeneration and/or proliferation and differentiation of HSCs. Compositions comprising the original HSCs and/or the regenerated HSCs are obtained thereby. By appropriate

selection with particular factors and the development of bioassays which allow for self regeneration of HSCs and screening of the HSCs as to their markers, a highly enriched, viable, HSC composition may be produced for a variety of purposes.

In addition, the present invention encompasses compositions comprising a CD43 apoptosis-stimulating antibody polypeptide. Methods of making such antibodies are known in the art and are not described in detail herein.

The present invention further encompasses methods of screening compounds to identify a potential apoptosis-stimulating CD43 ligand. The method includes the steps of obtaining a composition comprising CD43 or a functional portion thereof; contacting the CD43 to a test compound under conditions sufficient to allow binding of the test compound to CD43; measuring binding of the test compound to CD43; administering a test compound which specifically binds to CD43 to a population of HPC cells; and measuring the rate of apoptosis in the HPC cells. Functional portions of CD43 include any CD43 amino acid sequence or structural epitope which is present on the surface of $CD43^+$ cells. Conditions sufficient to allow binding of the test compound to CD43, include any set of conditions known in the art to be used in a pharmaceutical drug screening assay, preferably these conditions include buffer pH and osmolarity which approximate physiological conditions, and preferably temperatures between about 0°C and about 37°C.

The measuring of the binding of the test compound to CD43 may be accomplished by any method known in the art including, but not limited to, methods that rely on binding of the test compound to labeled CD43. Such antibody-based methods of measuring would include ELISAs and RIAs which are known in the art. Preferably, the test compound is attached to a solid support and the CD43 (or functional portion thereof) is labeled with a radionuclide, for example ^{125}I.

The radiolabeling of proteins is known in the art and not described herein. A solution of the radiolabeled-CD43 is contacted to the test compound attached to the solid support. Optionally the solid support is then washed and the radionuclide bound to the support is measured using for example a scintillation counter.

Alternatively, CD43 may be bound to a biosensor which is then treated with the test compound. The biosensor will detect an increase in weight due to binding of the test compound to CD43. In another alternative, a recombinant CD43-F_c fusion protein could be prepared and expressed in an appropriate expression system. The fusion protein could be incubated with a test sample, e.g., a cell line that may express a putative CD43 ligand, and the binding of the fusion protein to the putative ligand detected by an anti-F_c antibody.

A test compound that specifically binds to CD43 is administered to a population of HPCs and the subsequent measuring of the rate of apoptosis in the HPC may be performed by any method known in the art, including, but not limited to, the methods described in the Examples section below.

The following examples are provided to illustrate, but not to limit the invention.

Example 1

Surface Expression of CD43

Surface expression of CD43 on primitive human bone marrow HPCs was analyzed by multi-parameter flow cytometry using the anti-CD43 monoclonal antibody, MEM-59 Horejsi et al. (1988) *Folia Biologica (Praha)* 34:23. $CD34^+$ cells were isolated from normal adult bone marrow aspirates enriched for mononuclear cells by Ficoll-Paque density sedimentation, and enriched for $CD34^+$ cells using the Ceprate LC34-biotin kit according to the manufacturer's instructions. To measure

CD43 expression, $CD34^+$ cells were stained with monoclonal antibodies Tuk 3 (anti-CD34; IgG3) labeled with sulforhodamine, and MEM-59 (anti-CD43; IgG1), followed by goat anti-mouse IgG1 labeled with phycoerythrin. Next, the cells were incubated with mouse IgG1 to block the cell-bound goat antibody, stained with anti-Lineage (LIN) monoclonal antibodies labeled with fluorescein and analyzed by multi-parameter flow cytometry. The lineage (LIN) monoclonal antibody panel included Leu-5b (CD2), Leu-12 (CD19), Leu-M3 (CD14), Leu-M1 (CD15), Leu-11a (CD16), and JC159 (glycophorin). Primitive HPC were isolated from $CD34^+$ cells stained with anti-CD34 and anti-LIN monoclonal antibodies by cell sorting. The gated population of $CD34^{hi}LIN^-$ cells was collected. The results are shown in Figure 1. Primitive HPC were defined as cells with high expression of CD34 ($CD34^{hi}$) which are LIN^-. $CD34^{hi}$ cells are defined as those cells which have at least 100 times CD34 antigen density compared to isotype controls. As shown in Figure 1, although all $CD34^+$ cells expressed CD43, this population was divided on the basis of CD43 expression into two subpopulations with $CD34^{hi}CD43^{hi}$ and $CD34^{lo}CD43^{lo}$ phenotypes. Figure 1A. The $CD34^{hi}CD43^{hi}$ subpopulation included primitive $CD34^{hi}LIN^-$ cells, whereas the $CD34^{lo}CD43^{lo}$ subpopulation was represented by the more differentiated $CD34^{lo}LIN^{lo}$ cells.

Example 2

Anti-CD43 Antibody Reduces $CD34^{hi}LIN^-$ Cell Population Growth

To examine the role of CD43 in the survival and proliferation of HPC, the effect of immobilized MEM-59 on the growth of $CD34^{hi}LIN^-$ cells cultured in the presence of IL-3 and stem cell factor (SCF) was tested.

$CD34^{hi}LIN^-$ cells (1.5×10^4) were cultured in IMDM medium supplemented with FCS (10%), IL-3 (10 ng/ml), and SCF (50 ng/ml) in the presence of MEM-59 immobilized to a tissue

culture plate (the concentrations of the MEM-59 solutions used for coating the plate at 37°C for 1h are indicated are indicated in Figure 2). After 12 days of culture, the cells bound to immobilized MEM-59 were treated with O-sialoglycoprotein endopeptidase (glycoprotease) (Accurate Chemicals and Scientific Corporation, Westbury, NY) for 45 min at 37°C (the enzyme was reconstituted according to the manufacturer's instruction by diluting 1:10 in RPMI medium), harvested by pipetting, and counted (dead cells identified by the trypan blue staining were excluded). O-sialoglycoprotein endopeptidase cleaves CD43. Sutherland et al. (1992) *J. Immunol. 148*:1458. The data obtained showed that this glycoprotease treatment releases the $CD43^+$ cells from immobilized MEM-59 antibody apparently by cleaving the MEM-59 epitope since $CD43^+$ cells treated with glycoprotease do not bind the MEM-59 antibody. The viability of cells was not affected by the enzyme treatment. The number of control cells treated with AFP-02 negative control MAb (anti-human α-fetoprotein) was 1.2×10^6.

The results are depicted in Figure 2 and indicate that number of cells which were harvested after 12 days of culture with MEM-59 was significantly smaller when compared with control cells and was inversely proportional to the concentration of immobilized MEM-59.

Example 3

MEM-59 (αCD43) antibody specifically induces cell death

$CD34^{hi}LIN^-$ cells (10^4) were cultured in triplicates with IL-3 (10 ng/ml) and SCF (10 ng/ml) in the presence of immobilized MAbs. After 7 days of culture, cell growth in the individual wells was analyzed by the MTT assay according to the method described by Mosmann (1983) *J. Immunol. Meth. 65*:55. In brief, the Colorimetric MTT (tetrazolium) assay was performed as follows: MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma

catalog no. M2128) was dissolved in PBS at 5 mg/ml and filtered to sterilize and remove a small amount of insoluble residue present in some batches of MTT. At the times indicated below, stock MTT solution (10 µl per 100 µl medium) was added to all wells of an assay, and plates were incubated at 37°C for 4 h. Acid-isopropanol (100 µl of 0.04 N HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read on a Vmax microplate reader, using a wavelength of 570 nm and a reference wavelength of 650 nm. Plates were normally read within 1 h of adding the isopropanol.

$CD34^{hi}LIN^-$ cells cultured in parallel in the absence of MAbs were used as a standard. After the culture was terminated, the standard cells were serially diluted and analyzed side by side with the MAb-treated cells. Optical densities (OD) of the serially diluted standard cells obtained were: OD_{2N}=0.764±0.060; OD_N=0.470±0.040; $OD_{N/2}$=0.348±0.006; $OD_{N/4}$=0.233±0.006; $OD_{N/8}$=0.167±0.003 and $OD_{N/16}$=0.111±0.004 (N represents the growth of the standard cells harvested from one well; OD_{2N} is an optical density of two pooled wells of the standard cells). The following MAbs which were all of the IgG1 isotype were used: AFP-02 (negative control), MEM-12 (HLA-DR), MEM-83 (CD11a), HP2.1 (CD49d), and MEM-59 (CD43).

As shown in Table 2, where cell growth is expressed as the percent of the growth of standard cells(N), anti-CD43 Mab significantly inhibited cell growth whereas monoclonal antibodies recognizing other surface molecules expressed on $CD34^{hi}LIN^-$ cells did not.

Table 2

Effect of immobilized monoclonal antibodies recognizing different surface molecules in the growth of $CD34^{hi}LIN^{-}$ cells.

MAb	Optical density (means ± SD)	Cell growth (%)(means)
Neg. control	0.613±0.124	130
anti-CD59	0.473±0.099	100
anti-HLA-DR	0.507±0.145	108
anti-CD11a/CD18	0.410±0.113	77
anti-CD49d/CD29	0.505±0.114	108
anti-CD43	0.170±0.028	15

Example 4

Anti-CD43 Antibody Stimulates Cell Death

The results presented in Examples 1-3 do not distinguish between suppression of the growth of $CD34^{hi}LIN^-$ cells induced by MEM-59 by initiation of cell death from inhibition of cell proliferation (e.g., by preventing cells from entering the cell cycle). To test whether MEM-59 induces cell death, the short term effect of immobilized MEM-59 on the viability of $CD34^{hi}LIN^-$ cells was analyzed. $CD34^{hi}LIN^-$ cells were cultured in the presence of IL-3 (10 ng/ml), SCF (50 ng/ml) and immobilized MEM-59 or the negative control monoclonal antibodies (the monoclonal antibody concentrations used for coating the plate are indicated in Figure 3). After 48 hours of culture, the cells were treated with O-sialoglycoprotein endopeptidase, harvested, stained with propidium iodide (1 µg/ml; 5 minutes at room temperature) and analyzed by flow cytometry. The results obtained are shown in Figure 3 where the X-axis shows log red fluorescence, the Y-axis shows forward light scatter and the percentage of live cells (gated) are indicated. Based on these results, the 50 µg/ml concentration of MEM-59 was used for coating plates in each of the following Examples.

The results presented in Figure 3 indicate that, in contrast to control cells, the viability of cells cultured with various concentrations of MEM-59 for 48 hours was markedly decreased in a dose-dependent manner indicating that immobilized MEM-59 induced cell death. No such effect was observed in $CD34^{hi}LIN^-$ cell cultures treated with MAbs recognizing CD59, HLA-DR, CD11a/CD18 or CD49d/CD29. Likewise, soluble MEM-59 did not induce death of these cells, indicating that crosslinking of CD43 on the cell surface may be important for initiation of this process.

Example 5

Anti-CD43 Antibody Stimulates Apoptosis

To examine whether apoptosis is the mechanism responsible for MEM-59-induced cell death, $CD34^{hi}LIN^-$ cells were cultured for 48 hours with immobilized MEM-59 and analyzed for the presence of intracellular DNA fragments using the terminal deoxynucleotidyl transferase assay.

Briefly, $CD34^+LIN^-$ cells were cultured in the presence of IL-3 (10 ng/ml) and SCF (10 ng/ml) with immobilized MEM-59 or the negative control monoclonal antibody. After 48 hours of culture, the cells were photographed. Figure 4A. Next, the cells were treated with O-sialoglycoprotein endopeptidase, harvested and analyzed by the terminal deoxynucleotidyl transferase assay according to the methods described by Gorczyca et al. (1993) *Leukemia* 7:659; and Gavrieli et al. (1992) *J. Cell. Biol. 119*:493, followed by flow cytometry. The percentage of apoptotic cells containing DNA fragments within "live" gated populations are indicated in Figure 4B.

In order to perform the apoptosis assay, cells were fixed in PBS containing 1% formaldehyde, washed in PBS and resuspended in cold 75% ethanol (-20°C). After washing with deionized water, cells were resuspended in 50 µl of a solution containing 5 units of terminal deoxynucleotidyl transferase, 2.5 mM $CoCl_2$, 0.2 M potassium cacodylate, 25 mM Tris-HCl, BSA (0.25 mg/ml), and 0.5 nM biotin-16-dUTP (all chemicals and nucleotides were purchased from Boehringer Mannheim Biochemical, Indianapolis, IN). The cells were incubated for 60 minutes at 37°C, washed in PBS and resuspended in 100 µl PBS containing 40 mM NaCl, 30 mM sodium citrate, streptavidin-FITC (2.5 µg/ml), and 0.1% Triton X-100. Cells were incubated in this solution for 30 minutes at room temperature in the dark. After washing in PBS, cells were resuspended in PBS containing 1% formaldehyde and analyzed by flow cytometry on a FACScan (Becton Dickinson, Mountain View, CA).

The results obtained are depicted in Figure 4 and

indicate that a significant portion of the MEM-59-treated cells (19% of the "live" gated population as measured by flow cytometry) was found to be in a progressive stage of apoptosis, whereas virtually no apoptotic cells were detected in the negative control MAb-treated cell population (Figure 4B). In addition, many of these MEM-59-treated cells had features characteristic for apoptotic cells (segmented nucleus, shrinking body, membrane blebbing resulting in a formation of vesicles – Figure 4A).

Example 6
Anti-CD43 Antibody Stimulates Cell Death without Accessory Cells

To distinguish whether MEM-59-induced death of primitive HPC directly or whether this process was initiated by MEM-59-activated accessory cells (e.g., via a secreted factor), single $CD34^{hi}LIN^-$ cells were deposited using the automatic cell deposition unit into individual wells of Terasaki plates coated with MEM-59 or the negative control MAb. The cells were cultured in the presence of IL-3 (10 ng/ml) and SCF (50 ng/ml). The plates were visually scored under the microscope for the presence of proliferating cells daily, starting the third day of culture. Proliferating cells could be identified in the wells containing more than two cells – these wells were scored positive. The numbers of positive wells indicated were obtained after five days of culture; at this time, all of the positive wells contained more than five cells). The number of wells containing proliferating cells was markedly lower in the MEM-59-coated plates, as compared to those treated with the negative control MAb (Table 3), indicating that MEM-59 directly affects single $CD34^{hi}LIN^-$ cells.

Table 3

Effect of MEM-59 on the growth of single cells.

	Number of positive wells (out of 72)		
	Experiment 1	Experiment 2	Experiment 3
Neg. control MAb	35	24	26
MEM-59	7	8	4

The results depicted in Figure 2 and Table 3 indicate that MEM-59 suppressed the proliferation of single $CD34^{hi}LIN^-$ cells. Thus, induction of apoptosis by MEM-59 operates directly and does not require accessory cells.

Example 7

Sensitivity of Different Classes of HPCs to the MEM-59-Induced Cell Death

To examine whether there are differences in the sensitivity of HPCs committed to individual hemopoietic lineages to the MEM-59-induced cell death, the effect of immobilized MEM-59 on clonogenic HPCs present in the $CD34^{hi}LIN^-$ cell population was analyzed.

$CD34^{hi}LIN^-$cells (4×10^4) were cultured for 3 days in the presence of SCF, IL-3, and immobilized MEM-59 or AFP-01 (control). The cells were then harvested and the content of clonogenic cells in the harvested population was analyzed in the HPC assay. 1/20 of the harvested cell population, corresponding to 2×10^3 input cells, was assayed. Each data point represents the mean of duplicate assays. The results are presented in Table 4. The results obtained indicate that the number of all types of HPCs giving rise to CFU-GM, BFU-E and CFU-GEMM was smaller in the MEM-59-treated cell population in comparison with the control. However, the HPCs

giving rise to the BFU-E and CFU-GEMM colonies were affected by the MEM-59-induced cell death more dramatically in comparison with those forming the CFU-GM colonies.

Table 4

Effect of Anti-CD43 mAb on $CD34^{hi}LIN^{-}$ Hematopoietic Progenitor Cells

	Number of colonies					
	Control			MEM-59		
Experiment	CFU-GM	BFU-E	CFU-GEMM	CFU-GM	BFU-E	CFU-GEMM
1	269	41	17	158	5	0
2	201	251	39	52	22	3
3	254	24	12	148	5	0

Example 8

Effect of Immobilized MEM-59 on Resting $CD34^{hi}LIN^-$ cells

To determine whether the capacity of MEM-59 to induce the death of HPC is dependent on the cytokines initiating their proliferation, $CD34^{hi}LIN^-$ cells were cultured for 48 hours with immobilized MEM-59 either in the presence or absence of SCF (50 ng/ml), IL-3 (10 ng/ml) and IL-6 (10 ng/ml). $CD34^{hi}LIN^-$ cells (4×10^4) were cultured with immobilized MEM-59 (or the AFP-01 negative control MAb) in the presence or absence of SCF, IL-3 and IL-6. After 48 hours of culture, the cells were harvested and their clonogenic potential was analyzed in the HPC assay (1/20 of the harvested cell population was assayed). The results of a representative experiment performed in duplicate are presented in Table 5.

Table 5

Effect of Immobilized MEM-59 on Resting $CD34^{hi}LIN^-$ cells

Antibody	Number of CFU-GM/BFU-E/CFU-GEMM	
	No cytokines	SCF+IL-3+IL-6
AFP-01	324/59/24	203/117/24
	299/59/27	264/151/28
MEM-59	286/39/22	139/24/7
	276/43/19	117/29/8

The viability of the MAb-treated cells, as well as presence of clonogenic HPCs in this cell population were analyzed. In contrast to the cells cultured in the presence of the cytokines, the viability of the cells cultured in their absence was not significantly affected by immobilized

MEM-59 (Fig. 5, Table 5). The number of clonogenic HPCs in the MEM-59-treated cell population cultured in the absence of the cytokines was slightly lower in comparison to that treated with the negative control MAb (Table 5). However, the killing of clonogenic HPCs in the MEM-59-treated $CD34^{hi}LIN^-$ cell population was significantly less dramatic in the absence of cytokines (Table 5).

Example 9
Effect of MEM-59 on the Survival of Differentiated Myeloid Cells

To examine the relationship between the differentiation of $CD34^{hi}LIN^-$ cells and their sensitivity to MEM-59-induced cell death, these cells were cultured in the presence of SCF, IL-3 and IL-6. These cytokines induce differentiation of $CD34^{hi}LIN^-$ cells to myeloid cells, expressing CD15 and CD14 antigen. At the time points indicated in Table 6, aliquots of the cells were collected. The collected cells (4×10^4 per well) were then cultured for 48 hours in the presence of the same mixture of cytokines and immobilized MEM-59 or AFP-01 (negative control) and then analyzed by the MTT assay (optical density is proportional to the number of viable cells). The effect of immobilized MEM-59 on the survival of freshly isolated $CD34^{hi}LIN^-$ cells cultured for 48 hours either in the presence or the absence of the cytokines were analyzed in parallel (0 days). The results obtained are presented in Table 6. In Table 6, the percent survival was calculated as optical density of the MEM-59-treated sample solution x 100/optical density of the AFP-01-treated cell sample solution.

Table 6

Effect of Anti-CD43 mAb on the Survival of Differentiated Cells Derived from the $CD34^{hi}LIN^{-}$ Cell Population

	Optical Density (means)					
	No Addition	Cytokines				
	0 days	0 days	3 days	7 days	14 days	28 days
Control	0.062	0.332	0.438	0.349	0.408	0.383
MEM-59	0.073	0.165	0.244	0.355	0.495	0.424
% Survival	118	50	56	102	121	111

The results presented in Table 6 indicate that only cells cultured in the presence of cytokines for less than one week underwent apoptosis in response to the MEM-59 mediated stimulation. In contrast, cells cultured under these conditions for seven or more days lost their sensitivity to MEM-59 induced cell death.

Example 10

Effect of MEM-59 on the Survival of Lymphocytes

To test whether immobilized MEM-59 induces directly apoptosis in peripheral lymphocytes, resting and PHA- or IL-7-activated peripheral mononuclear cells depleted of monocytes were cultured in the presence of immobilized MEM-59 or the negative control MAb (monocytes were removed to eliminate any effect of these cells which might be stimulated by MEM-59). Peripheral mononuclear leukocytes were isolated from the blood of healthy donors by Ficoll-Paque density gradient centrifugation. The isolated cells were stained with anti-CD14 MAb and propidium iodide to exclude dead cells and sorted on the FACStar. $CD14^-$ cells were collected.

After 2 or 5 days of culture, the viability of the MAb-treated cells was analyzed. The viability of resting or IL-7-activated cells treated with the negative control MAb remained high (~80-90%) during the first 48 hours of culture (Fig. 6). After 5 days, the viability of the resting control cells slightly dropped, while the viability of IL-7-activated cells remained virtually constant. In contrast, the viability of cells cultured with the negative control MAb in the presence of PHA was relatively low (~40%) after 48 hours of culture, but it increased after 5 days. However, in all instances, immobilized MEM-59 did not have any significant effect on the viability of lymphocytes, as compared to the negative control MAb.

Example 11

Effect of Various anti-CD43 MAbs on the Proliferation of $CD34^{hi}LIN^{-}$ Cells

In order to determine the efficacy of a variety of CD43 antibodies, $CD34^{hi}LIN^{-}$ cells (3×10^3) were cultured in duplicate for 14 days in the presence of SCF, IL-3, IL-6 and MAbs immobilized to the bottom of a 96 well-tissue culture plate. The immobilization procedure was as follows. Plates were incubated with a solution of goat α-mouse Ig (100 µg/ml in PBS) for 2 hours at 37°C. Plates were washed and incubated for 4 hours at 4°C with solutions of MAbs. Control (AFP-01), MEM-59, G019, TPI/36 were used as ascites fluids (diluted 1:100 in PBS). Leu-22 (Becton-Dickinson) and DF-TI (Dako) were used as purified Abs at final concentrations of 10 µg/ml. Cell proliferation in the individual wells was then measured by MTT assay. The relative proliferation expressed of cells treated with an anti-CD43 MAb was calculated; optical density of the cell sample treated with anti-CD43 MAb x 100/ optical density of the sample treated with an irrelevant control MAb. Note that MEM-59 immobilized via goat anti-mouse Ig induces cell death less effectively then MEM-59 immobilized directly to the plate. The results obtained are presented in Table 7.

Table 7

MAb	Optical density (means)	Relative Proliferation(%)
Control	0.436	100
MEM-59	0.222	51
G-19	0.352	81
TP1/36	0.261	60
Leu-22	0.053	12
DF-T1	0.270	62

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

Claims

We claim:

1. A method of enriching, *in vitro*, a population of hematopoietic cells for stem cells, comprising the steps of

a) obtaining the population of hematopoietic cells; and

b) treating the population of hematopoietic cells with an amount of a CD43 cross-linking agent sufficient to induce cell death of susceptible $CD43^+$ cells, while allowing stem cells to survive.

2. A composition comprising an enriched population of stem cells obtained by the method according to claim 1.

3. A method of treating a patient with a hematopoietic cell malignancy, comprising the steps of:

(a) removing a population of hematopoietic cells from a patient with a malignancy to obtain an *in vitro* population of hematopoietic cells;

(b) enriching the *in vitro* population of hematopoietic cells for stem cells according to the method of claim 1 to obtain a population of stem cells;

(c) administering to the patient with a malignancy a sufficient dose of a chemotherapeutic agent or radiation to significantly reduce the level of malignancy; and

(d) re-engrafting the population of stem cells into the patient.

4. A kit or composition comprising a CD43 apoptosis-stimulating antibody for use in the enrichment of an *in vitro* population of hematopoietic cells for stem cells.

5. A method of screening compounds to identify a potential apoptosis-stimulating CD43 ligand, which method comprises:

(a) obtaining a composition comprising CD43, or a functional portion thereof;

(b) contacting the CD43 to a test compound under conditions sufficient to allow binding of the test compound to CD43;

(c) measuring binding of the test compound to CD43;

(d) administering a test compound which specifically binds to CD43 to a population of $CD43^+$ progenitor cells; and

(e) measuring the rate of apoptosis in the $CD43^+$ progenitor cells.

6. A method of screening for hematopoietic cytokines, comprising the steps of

a) obtaining a composition according to claim 2;

b) adding a putative hematopoietic cytokine to an aliquot of the composition to obtain a test sample; and

c) comparing the effects of the putative cytokine on the physical parameters of the cells to a control, untreated, aliquot of cells, wherein the putative cytokine is determined to possess hematopoietic-specific activity if it changes the physical parameters of the test sample as compared to the control.

7. Use of a CD43 apoptosis-stimulating antibody in a method of enriching, *in vitro*, a population of hematopoietic cells for stem cells, e.g., a method according to claim 1.

Figure 1

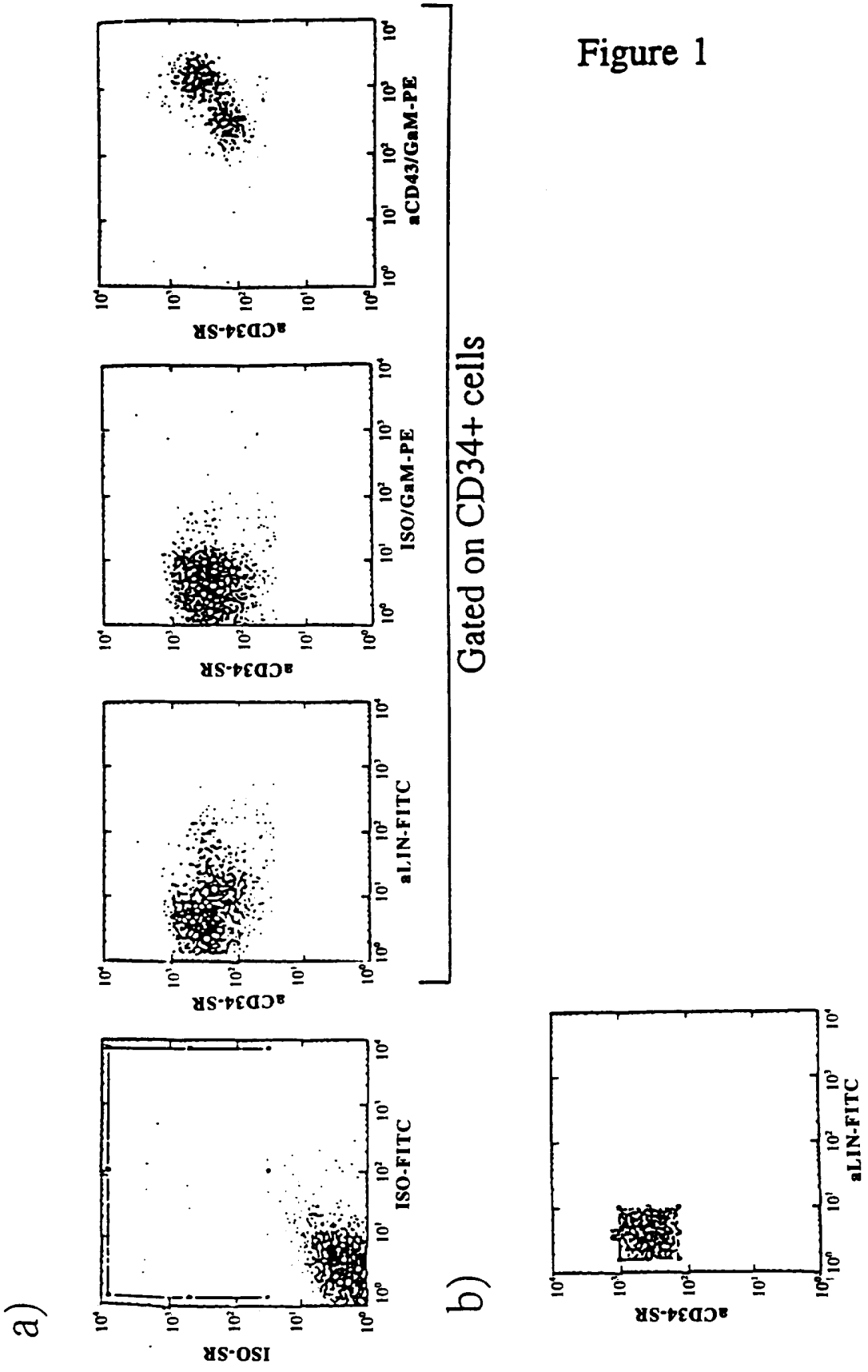

Gated on CD34+ cells
aCD34-SR
aCD43/GaM-PE
ISO/GaM-PE
aLIN-FITC
ISO-FITC
a)
b)

Figure 2

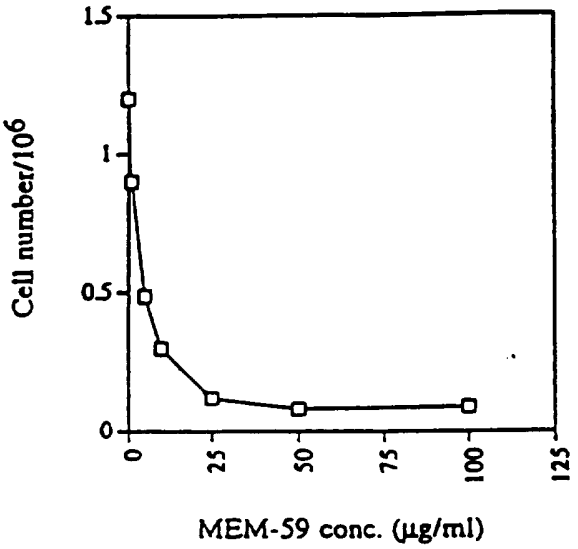


Figure 3

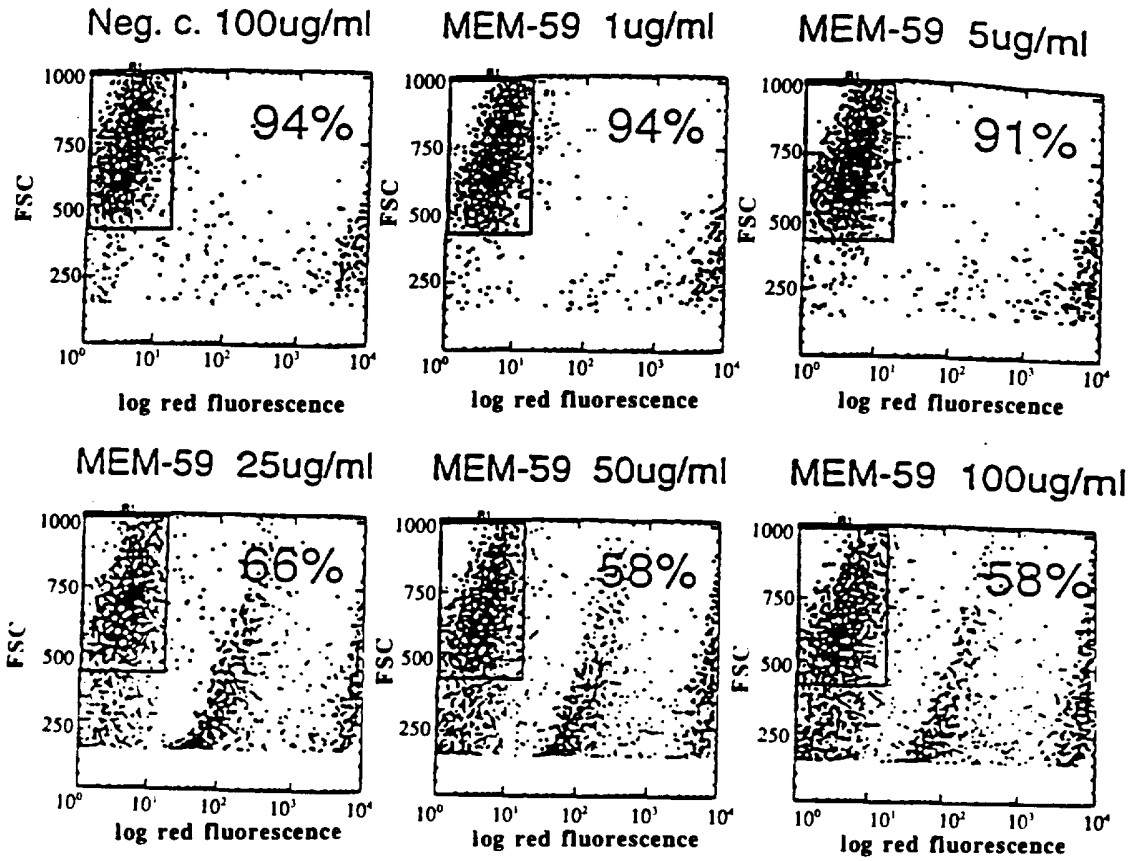

Neg. c. 100ug/ml
MEM-59 1ug/ml
MEM-59 5ug/ml
MEM-59 25ug/ml
MEM-59 50ug/ml
MEM-59 100ug/ml
94%
94%
91%
66%
58%
58%
FSC
log red fluorescence
1000
750
500
250
10^0
10^1
10^2
10^3
10^4

Figure 4

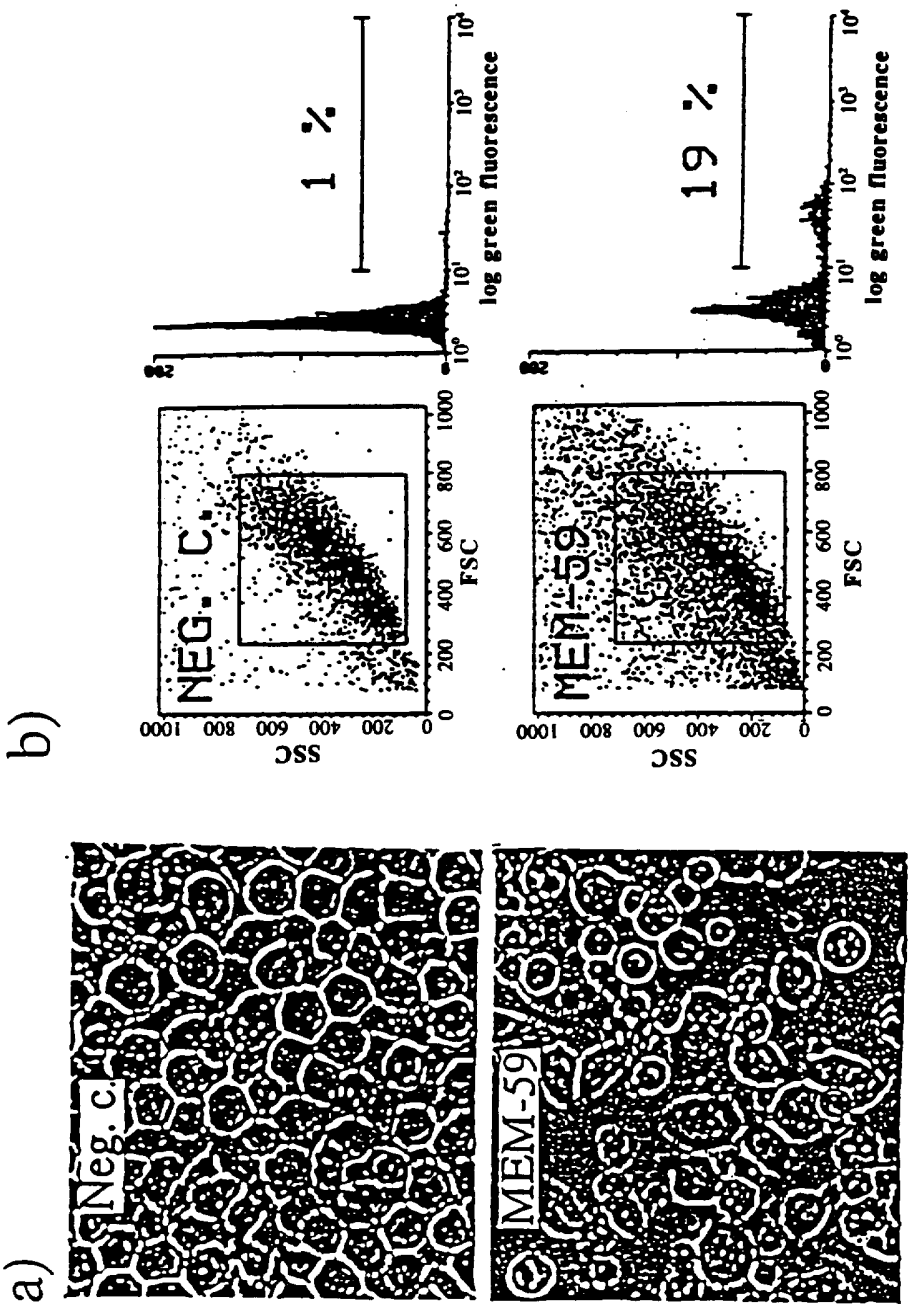


Figure 5

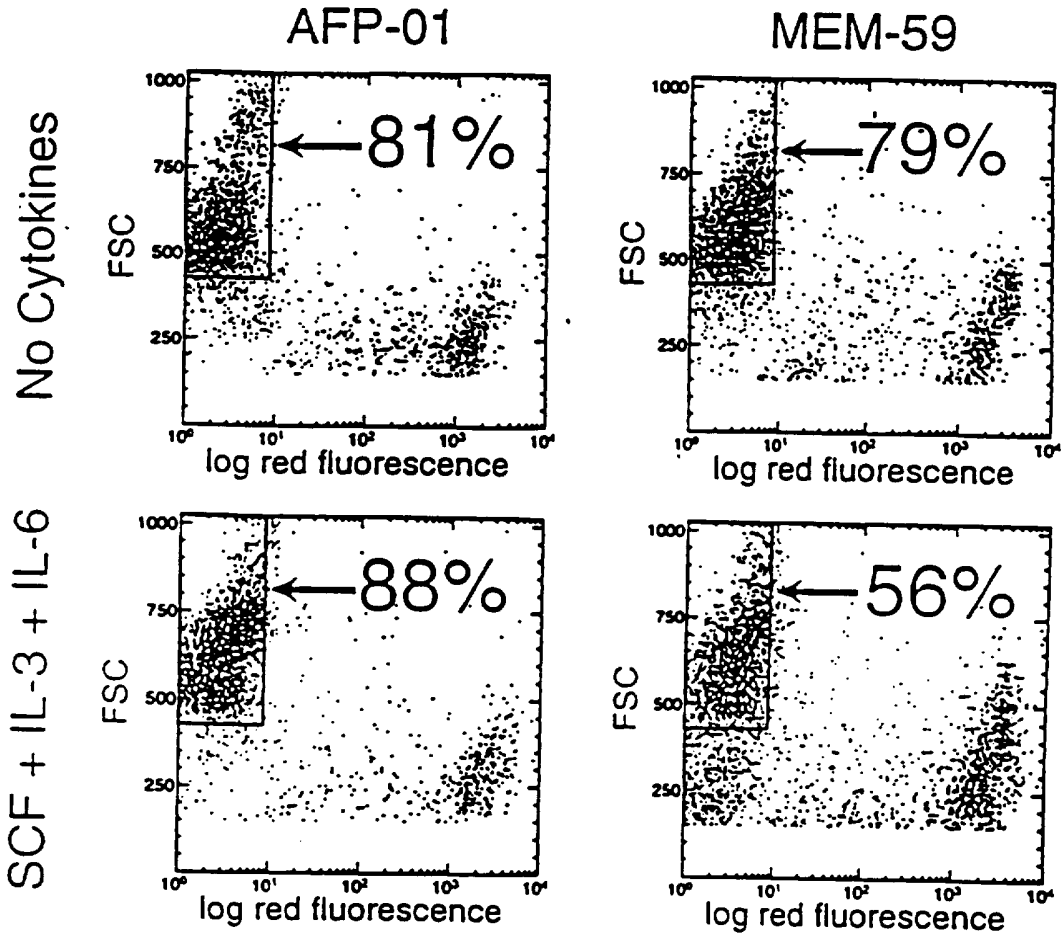

AFP-01
MEM-59
No Cytokines
SCF + IL-3 + IL-6
81%
79%
88%
56%
FSC
log red fluorescence

Figure 6

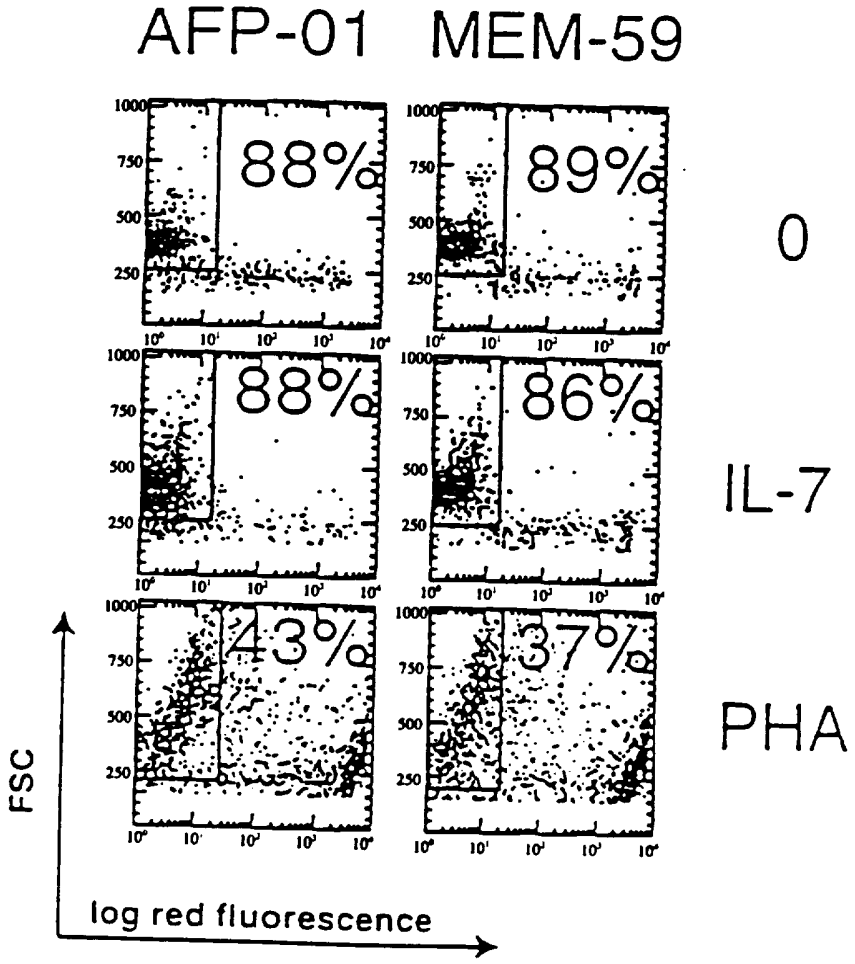
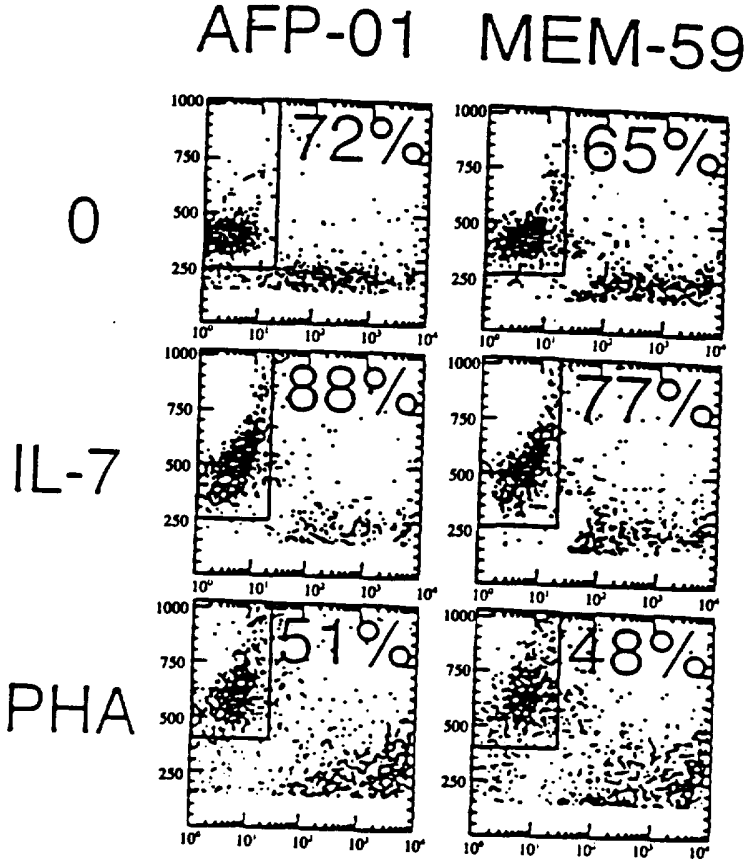

AFP-01 MEM-59
88%
89%
88%
86%
43%
37%
0
IL-7
PHA
FSC
log red fluorescence
48 Hours

Figure 6 (continued)



INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/EP 95/04479

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/08 A61K35/28 C07K16/28 G01N33/68

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K G01N

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 01534 (THE UNIVERSITY OF PITTSBURGH) 20 January 1994	2
A	see the whole document	1,3-7

A	EP,A,0 451 611 (SYSTEMIX,INC.) 16 October 1991	1-7
	cited in the application	
	see the whole document	

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
20 March 1996	27.03.96
Name and mailing address of the ISA	**Authorized officer**
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Moreau, J

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NATURE, vol. 337, no. 6203, 12 January 1989 LONDON GB, pages 181-184, SMITH C.A. ET AL. 'Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures' cited in the application see the whole document ---	1-7
A	JOURNAL OF IMMUNOLOGY, vol. 148, no. 5, 1 March 1992 BALTIMORE US, pages 1458-1464, SUHERLAND D.R. ET AL. 'Cleavage of the cell-surface O-sialoglycoproteins CD34, CD43, CD44, and CD45 by a novel glycoprotease from Pasteurella haemolytica' cited in the application see the whole document ---	1-7
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 89, no. 7, 1 April 1992 WASHINGTON US, pages 2804-2808, BAUM C.M. ET AL. 'Isolation of a candidate human hematopoietic stem-cell population' cited in the application see the whole document ---	1-7
X	NATURE, vol. 350, no. 6320, 25 April 1991 LONDON GB, pages 706-709, PARK J.K. ET AL. 'Enhancement of T-cell activation by the CD43 molecule whose expression is defective in Wiskott-Aldrich syndrome' cited in the application see the whole document --- -/--	4

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 109, no. 25, 19 December 1988 Columbus, Ohio, US; abstract no. 228128, STEFANOVA, IRENA ET AL 'Characterization of a 95 kDa human leukocyte sialoglycoprotein: its identity with CD43, gpL115, leukosialin and sialophorin' see abstract & FOLIA BIOL. (PRAGUE) (1988), 34(4), 255-65, 5 PLATES CODEN: FOBLAN;ISSN: 0015-5500, 1988	4

P,X	BLOOD 86 (2). 1995. 502-511, BAZIL V ET AL 'Apoptosis of human hematopoietic progenitor cells induced by crosslinking of surface CD43, the major sialoglycoprotein of leukocytes.' see the whole document	1-7

P,X	24TH ANNUAL MEETING OF THE INTERNATIONAL SOCIETY FOR EXPERIMENTAL HEMATOLOGY, DUESSELDORF, GERMANY, AUGUST 27-31, 1995. EXPERIMENTAL HEMATOLOGY (CHARLOTTESVILLE) 23 (8). 1995. 832, BAZIL V ET AL 'Crosslinking of surface CD43 (leukosialin) initiates apoptosis of early hemopoietic progenitor cells, but not stem cells.' see abstract 317	1-7

P,X	9TH INTERNATIONAL CONGRESS OF IMMUNOLOGY. THE 9TH INTERNATIONAL CONGRESS OF IMMUNOLOGY;MEETING SPONSORED BY THE AMERICAN ASSOCIATION OF IMMUNOLOGISTS AND THE INTERNATIONAL UNION OF IMMUNOLOGICAL SOCIETIES, SAN FRANCISCO, CALIFORNIA, USA, JULY 23-29, 1995 BAZIL V ET AL 'CD43 (leukosialin) transduces a signal initiating apoptosis of human hemopoietic progenitor cells.' see page 492, abstract 2915	1-7

2

·national application No.

PCT/EP 95/04479

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

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

1. [X] Claims Nos.: 3
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim 3 is directed to a mehtod of treatment of the human/ animal body, the search has been carried out and based on the alleged effects of the compound/composition.

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

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

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

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

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

2. [ ] As all searchable claims could be searches without effort jusufying an additional fee. this Authority did not invite payment of any additional fee.

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

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

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 95/04479

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9401534	20-01-94	AU-B-	4771793	31-01-94
		CA-A-	2139877	20-01-94
		EP-A-	0652943	17-05-95
		JP-T-	8500009	09-01-96
		CN-A-	1091955	14-09-94
EP-A-451611	16-10-91	US-A-	5061620	29-10-91
		AU-B-	641488	23-09-93
		AU-B-	7398691	03-10-91
		CA-A-	2039315	01-10-91
		JP-A-	7313150	05-12-95