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(54) **PROGENITOR CELL PRESERVATION FACTORS AND RELATED METHODS AND PRODUCTS**  
STAMMZELLEN SCHUTZFAKTOR UND VERWANDTE METHODEN UND PRODUKTE  
FACTEURS DE CONSERVATION DE CELLULES SOUCHES, PROCEDES ET PRODUITS S'Y  
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- **MOORE J.G. ET AL.:** "Preservation of hematopoietic progenitors for prolonged periods in suspension cultures by Flk2/flt3 receptor-interacting lectin (FRIL), a new lectin identified in red kidney beans" **BLOOD**, vol. 90, 15 November 1997 (1997-11-15), page 428A **XP000938895**
- **COLUCCI G. ET AL.:** "cDNA cloning of FRIL, a lectin from Dolichos lablab, that preserves hematopoietic progenitors in suspension culture." **PROC. NATL. ACAD. SCI. USA**, vol. 96, January 1999 (1999-01), pages 646-650, **XP002145996**
- **GOWDA L.R. ET AL.:** "The complete primary structure of a unique Mannose/Glucose-specific lectin from field bean (Dolichos lab lab)" **J. BIOL. CHEM.**, vol. 269, no. 29, 22 July 1994 (1994-07-22), pages 18789-18793, **XP002145997** cited in the application
- **LENFANT M ET AL:** "INHIBITOR OF HEMATOPOIETIC PLURIPOTENT STEM CELL PROLIFERATION: PURIFICATION AND DETERMINATION OF ITS STRUCTURE" **PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,US,NATIONAL ACADEMY OF SCIENCE. WASHINGTON**, vol. 86, 1 February 1989 (1989-02-01), pages 779-782, **XP002032459 ISSN: 0027-8424**

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| <ul style="list-style-type: none"><li>• KEMSHEAD J T: "IMMUNOMAGNETIC MANIPULATION OF HEMATOPOIETIC CELLS: A REVIEW OF CURRENT TECHNOLOGY" INTERNATIONAL CONFERENCE ON METERING APPARATUS AND TARIFFS FOR ELECTRICITY SUPPLY,XX,XX, vol. 1, no. 1, 1992, pages 35-44, XP000613792</li></ul> | <ul style="list-style-type: none"><li>• MOORE J. G. ET AL.: "A new lectin in red kidney beans specifically stimulates proliferation of 3T3 fibroblasts transfected with the Flk2/flt3 receptor" BLOOD, vol. 90, no. 10, 15 November 1997 (1997-11-15), page 308A, XP001147834</li></ul> |
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**Description**

[0001] This application is a continuation in part of U.S. Ser. No. 08/881,189, filed June 24, 1997.

**BACKGROUND OF THE INVENTION**Field of the Invention

[0002] The invention relates to the preservation of progenitor cells. More specifically, the invention relates to the *in vivo* preservation of progenitor cells, such as hematopoietic progenitor cells.

Summary of the Related Art

[0003] The wide variety of functionally and phenotypically different types of cells in a multi-cellular eukaryotic organism results in part from the proliferation and differentiation of rare and mostly quiescent populations of progenitor cells. For example, hematopoiesis involves the process of producing a balanced supply of different blood cells from such progenitor cells found in the adult bone marrow. The development of other cell types also depends upon production of the differentiated cells from such progenitor cells.

[0004] Progenitor cells are activated by signals, such as cell-cell contact or soluble regulators, to generate daughter cells that are identical to the parent (*i.e.*, self-renewal of the parent) and/or to generate daughter cells that are more differentiated than the parent, thus beginning an irreversible process that ends with the production of differentiated, functional cells. In the process of hematopoiesis, differentiation is coupled to proliferation as a progenitor cell gives rise to more differentiated daughter cells that progressively become committed to producing only one blood cell type. The enormous activation of hematopoietic progenitor cells needed to meet the body's daily requirement for hundreds of billions of new mature blood cells is directed by potent soluble regulators (*e.g.*, colony stimulating factors and cytokines) acting upon the hematopoietic progenitor cells themselves, and their more differentiated daughter cells.

[0005] Although progenitor cells eventually produce so many of the mature cells of the body, they occur only rarely. Moreover, typically the more primitive (*i.e.*, undifferentiated) the progenitor cell, the more rare the progenitor cell. For example, the currently believed most primitive of the hematopoietic progenitor cells, which are called hematopoietic stem cells, occur at a frequency of only from about 1 in 10,000 to about 1 in 100,000 of the cells in the bone marrow. Hematopoietic stem cells have the capacity to generate more than  $10^{13}$  mature blood cells of all lineages, including other progenitor cells which, although more differentiated than hematopoietic stem cells, are themselves capable of giving rise to several different types of mature blood cells.

[0006] Hematopoietic stem cells are responsible for sustaining blood cell production over the life of an animal. The small population of hematopoietic stem cells is sufficient to produce all the mature blood cells in a healthy individual; however, some unhealthy individuals suffer from a lack of a sufficient number of progenitor cells and/or mature blood cells. For example, cancer patients receiving chemotherapeutic or radiotherapy treatments designed to kill the rapidly dividing cancer cells also suffer from the depletion of white blood cells and platelets, thus exposing these patients to life-threatening opportunistic infections and bleeding episodes. Indeed, this hematopoietic progenitor cell-depleting activity is the dose-limiting factor for most of these chemotherapeutic and radiotherapeutic agents.

[0007] Many cancer patients are routinely treated with cytokines, including G-CSF, GM-CSF, SCF, Erythropoietin, and IL-11, to accelerate restoration of hematopoiesis following chemotherapy (Moore, M.A., *Blood* 78: 1-19, 1991). However, these cytokines lead to the irreversible differentiation of hematopoietic progenitor cells, including hematopoietic stem cells, into more differentiated daughter cells. Thus, better protection of hematopoietic progenitor cells is needed during chemotherapy.

[0008] Workers in the field have attempted to use cytokines in mice to protect progenitors from the toxicity of chemotherapy (Neta et al., *J. Immunol.* 136: 2483-2485, 1986; Neta et al., *J. Immunol.* 140: 108-111, 1988; Neta et al., *J. Exp. Med.* 173: 1177-1182, 1991; de Haan et al., *Blood* 87: 4581-4583, 1996; Lyman and Jacobsen, *Blood* 91: 1101-1134, 1998; Dalmau et al., *Bone Marrow Transplant.* 12: 551-563, 1993; Grzegorzewski et al., *J. Exp. Med.* 180: 1046-1057, 1994; Grzegorzewski et al., *Blood* 94:1066a (Abstr.)1999). Marshall et al. (*Euro. J. Cancer* 34: 1023-1029, 1998) and Gilmore et al. (*Exp. Hematol.* 27: 195-202, 1999) describe the use in clinical trials of a chemokine that allegedly inhibits progenitor cell proliferation, MIP1- $\alpha$ , as a chemoprotectant. Marshall (Marshall, A., *Nat. Biotechnol.* 16: 129, 1999) describes the use of MTIF-1, a chemokine that allegedly inhibits progenitor cell proliferation, in clinical trials as a chemoprotectant.

[0009] There are several drawbacks to using chemokines, cytokines, and other immunoregulators as chemoprotectants during the chemotherapeutic or radiotherapeutic treatment of cancer patient. These drawbacks include the cost of production and toxicity to the patient.

[0010] Therefore, there is a need for improved reagents that are non-toxic and inexpensive to produce for use in

preserving progenitor cells.

## **BRIEF SUMMARY OF THE INVENTION**

**[0011]** The invention relates to the use of a therapeutically effective amount of a composition containing a FRIL protein comprising the amino acid sequence set forth in SEQ ID NO: 6 for the preparation of a medicament for treatment of a condition characterized by hematopoietic progenitor cell-depleting activity of a radiotherapeutic or a chemotherapeutic treatment or a combination of a radiotherapeutic and a chemotherapeutic treatment, wherein the therapeutically effective amount of said composition alleviates or reduces the hematopoietic progenitor cell-depleting activity of said therapeutic treatment in the patient, and wherein the medicament is to be administered prior to the radio therapeutic or chemotherapeutic treatment; wherein the medicament is to be administered about 5 days to about 2 hours before the therapeutic treatment and is to be administered daily.

**[0012]** The FRIL family member is from the legume *Phaseolus vulgaris*.

**[0013]** The invention provides a recombinant nucleic acid molecule encoding a composition of a member of the FRIL family of progenitor cell preservation factors comprising the amino acid sequence SEQ ID NO:6 for the claimed use.

**[0014]** The invention provides a pharmaceutical formulation comprising an essentially pure composition of the FRIL family of progenitor cell preservation factor identified above and a pharmaceutically acceptable carrier.

**[0015]** The invention relates to administration of a therapeutically effective amount of the formulation to a patient suffering from a condition whereby the patient's hematopoietic progenitor cells are depleted, and wherein the pharmaceutical formulation alleviates and/or reduces the condition in the patient.

**[0016]** The invention relates to the administration of a therapeutically effective amount of the formulation to a patient prior to treatment of the patient with a therapeutic treatment having a hematopoietic progenitor cell-depleting activity alleviates and/or reduces the hematopoietic progenitor cell-depleting activity of the therapeutic treatment in the patient. In certain embodiments, the patient is a human or is a domesticated mammal. In some embodiments, the patient has cancer. In some embodiments, the therapeutic treatment is a radiotherapeutic or a chemotherapeutic treatment, including, without limitation, cytarabine (Ara-C), doxorubicin (Dox), or 5-fluorouracil (5-FU), or a combination of a radiotherapeutic and a chemotherapeutic.

**[0017]** The invention provides a method for preserving progenitor cells *in vivo*, comprising administering to a patient an effective amount of a composition of a FRIL family member for an effective period of time, wherein the progenitor cells in the patient are rendered quiescent. Preferably, the patient is a human or a domesticated animal.

**[0018]** In certain embodiments of the invention, the patient is a cancer patient. The effective amount of the composition of a FRIL family member is administered prior to the treatment of the patient with a therapeutic treatment having a hematopoietic progenitor cell-depleting activity wherein the medicament is to be administered about 5 days to about 2 hours before the therapeutic treatment and is to be administered daily. In some embodiments, the therapeutic treatment is a radiotherapeutic or a chemotherapeutic treatment, including, without limitation, cytarabine (Ara-C), doxorubicin (Dox), or 5-fluorouracil (5-FU), or a combination of a radiotherapeutic and a chemotherapeutic.

**[0019]** According to the invention, compositions of a FRIL family member may be used as therapeutic agents to preserve progenitor cells in patients, such as cancer patients receiving chemotherapy, who suffer from a condition that diminishes their progenitor cells. For example, compositions of a FRIL family member according to claim 1 may be administered with a pharmaceutically-acceptable carrier (e.g., physiological sterile saline solution) via any route of administration to a cancer patient receiving chemotherapy in an attempt to reduce the progenitor cell-depleting effects of the chemotherapeutic so that the patient can receive a higher dose of the chemotherapeutic and, preferably, recover from cancer. Pharmaceutically-acceptable carriers and their formulations are well-known and generally described in, for example, Remington's Pharmaceutical Sciences (18th Edition, ed. A. Gennaro, Mack Publishing Co., Easton, PA, 1990).

## **BRIEF DESCRIPTION OF THE DRAWINGS**

**[0020]** Figures which are not within the scope of the claims are only for illustrative purposes.

Figure 1 is a map of a cloning vector pCR2.1-DLA manufactured by ligating a cDNA according to the invention in the *EcoRI* site of the cloning vector pCR2.1.

Figure 2 shows a direct amino acid sequence comparison of the  $\beta$  subunit top strand, (SEQ ID NO: 11); and  $\alpha$  subunit top strand (SEQ ID NO 12:) of the mannose lectin described by Gowda et al. (*J Biol Chem* 269: 18789-18793, 1994) and the derived amino acid sequence of DI-FRIL, a representative, non-limiting FRIL family member of the invention, encoded by a representative, non-limiting nucleic acid of the invention.

Figure 3 is a map of a cloning vector pCR2.1-DLA(D) manufactured by ligating a mutated cDNA in the *EcoRI* site of the cloning vector pCR2.1.

Figure 4 is a map of a cloning vector pBS-SpDLA manufactured by ligating a recombinant fragment in the *EcoRI* site of the cloning vector pBluescript SK+.

Figure 5 is a map of a cloning vector pCR2.1-SpM1(D) manufactured by ligating a mutated recombinant clone in the *EcoRI* site of the cloning vector pCR2.1.

Figure 6 is a map of a recombinant expression vector pBIN-VicPro manufactured by subcloning the vicilin promoter obtained from the pCW66 vector in the *EcoRI/ClaI* site of the plant binary vector pBIN19 for *Agrobacterium*-mediated transformation.

Figure 7 is a map of a recombinant expression vector pBINVicPro-SpDLA manufactured by ligating a recombinant fragment in the *EcoRI/SacI* site of the pBINVicPro vector.

Figure 8 is a map of a recombinant expression vector pBINVicPro-SpDLA(D) manufactured by ligating a mutated recombinant clone in the *EcoRI* site of the pBINVicPro vector.

Figure 9 is a map of a recombinant expression vector pGEX4T-1-DLA manufactured by ligating a wild-type cDNA clone in the *EcoRI/SalI* site of the *E. coli* expression vector pGEX4T-1.

Figure 10 is a map of a recombinant expression vector pGEX4T-1-DLA(D) manufactured by ligating a mutant cDNA clone in the *EcoRI/XhoI* site of the *E. coli* expression vector pGEX4T-1.

Figure 11 is a representation of an electrophoretogram of a Southern blot of total protein extracts of *E. coli* cells transformed with the recombinant expression vectors pGEX4T-1-DLA and pGEX4T-1-DLA(D).

Figure 12 is a representation of an electrophoretogram of a Western blot of purified GST-fusion proteins with and without cleavage by thrombin.

Figure 13A is a representation of a graph showing that a crude extract of an *E. coli* culture containing expressed DI-FRIL, a representative, non-limiting FRIL family member of the invention, specifically stimulates hFLT3 3T3 cells; Figure 13B is a graph showing that the same extract does not stimulate untransfected 3T3 cells.

Figure 14A is a representation of a histogram showing that purified DI-FRIL, a representative, non-limiting FRIL family member of the invention, preserves cord blood mononuclear cells in a dose-responsive manner.

Figure 14B is a representation of a histogram showing that purified DI-FRIL, a representative, non-limiting FIZIL family member of the invention, preserves hematopoietic progenitors in a dose-responsive manner.

Figure 15A is a representation of a photograph of colonies derived from human cord blood mononuclear cells cultured in 40 ng/mL DI-FRIL, a representative, non-limiting FRIL family member of the invention, for 3 weeks, and then replated in methylcellulose colony assay medium.

Figure 15B is a representation of a photograph of colonies derived from human cord blood mononuclear cells cultured in 40 ng/mL DI-FRIL, a representative, non-limiting FRIL family member of the invention, for 4 weeks, and then replated into methylcellulose colony assay medium.

Figure 16 is a schematic diagram showing the serial replating of progenitor cells cultured in DI-FRIL, a representative, non-limiting FRIL family member of the invention, or DI-FRIL+recFL (*i.e.*, recombinant FLT3-Ligand). The human cord mononuclear cells were first cultured in suspension in 40 ng/mL DI-FRIL or 40 ng/mL DI-FRIL + 40 ng/mL recFL (solid black box). The cells were then harvested and assessed for progenitor activity by being replated into methylcellulose colony assay medium for 6 weeks (middle striped box). Then the cells were harvested from the colony assay and again replated into methylcellulose colony assay medium for an additional 4 weeks (far right striped box). Progenitor frequencies were determined for cells after 3 weeks of suspension culture in DI-FRIL or DI-FRIL+recFL, and after an additional 6 weeks of methylcellulose culture (absent-DI-FRIL and/or recFL).

Figure 17 is a representation of a photograph of colonies derived from human cord blood mononuclear cells initially cultured in 40 ng/mL DI-FRIL, a representative, non-limiting FRIL family member of the invention, for 3 weeks, then replated into methylcellulose colony assay medium for 6 weeks, and then replated again into methylcellulose colony assay medium for an additional 4 weeks.

Figure 18 is a representation of a bar graph showing that a representative, non-limiting FRIL family member of the invention, DI-FRIL, encoded by a representative, non-limiting nucleic acid of the invention is sufficient to preserve progenitor cells *ex vivo*, whereas a cytokine cocktail fails to preserve such cells.

Figures 19A and 19B are representations of line graphs showing the biological specificity of receptor-transfected 3T3 cells. Figure 19A shows that rhM-CSF specifically stimulated Fms 3T3 (solid circles) but neither mFlt3/Fms 3T3 (open circles) nor parent 3T3 cells (solid squares) in biological screening assay in a dose-dependent manner. Figure 19B shows that PHA-LCM (reciprocal dilution) stimulated mFlt3/Fms 3T3 (solid circles) and Stk 3T3 (open circles) but not parent untransfected 3T3 cells (solid squares).

Figures 20A-20D are representations of the detected biological activities of PHA-LCM fractionated by anion exchange chromatography. Figure 20A shows the number of viable cells cord blood cells observed microscopically.

Figure 20B shows the stimulation of mFlt3/Fms 3T3 cells by anion exchange column fractions. Figure 20C shows the stimulation of Stk 3T3 cells by anion exchange column fractions. Figure 20D shows the stimulation of Fms 3T3 cells by anion exchange column fractions.

Figures 21A and 21B are representations of line graphs showing the co-factors required in the Flt3 3T3 assay

during purification of Pv-FRIL, a representative, non-limiting FRIL family member of the invention. Figure 21A shows that the plateau stimulation of Flt3 3T3 cells decreased during purification from crude, 10-fold concentrated PHA-LCM (solid circles) to partially purified (open circles), and highly purified (solid squares). Medium control is shown as open squares. Figure 21B shows that the decreased plateau stimulation of Flt3 3T3 cells (solid circles) was restored by addition of sub-optimal concentrations (1:200) of crude PHA-LCM (solid squares). Corresponding medium controls are shown in open symbols.

Figures 22A-22D are representations of a series of flow cytometry histograms showing the re-analysis of CD34 expression of cord blood CD34<sup>+</sup> cells after two weeks in suspension culture with pooled AQS affinity column fractions. CD34 expression was re-analyzed by flow cytometry on pooled AQS affinity column fractions 1-5 (Figure 22A), fractions 6-10 (Figure 22B), fractions 11-15 (Figure 22C), and fractions 16-20 (Figure 22D). Fluorescence intensity on the abscissa and frequency of events on the ordinate in Figures 22A-22D.

Figure 23 is a representation of a line graph showing the IL1-dependent response of mFlt3/Fms 3T3 cells to FRIL isolated from commercial red kidney bean extract. mFlt3/Fms 3T3 cells respond to FRIL in the presence of rhIL1 (solid circles) but not the absence of IL1 (solid squares). Corresponding medium only controls are shown with open symbols.

Figure 24A is a map of a cloning vector pCR2.1-Pv-FRIL manufactured by ligating a cDNA according to the invention in the EcoRI site of the cloning vector pCR2.1.

Figure 24B shows a direct amino acid sequence comparison of Pv-FRIL, a representative, non-limiting FRIL family member of the invention, with DLL, the, mannose-binding lectin of Gowda et al., and the PHA lectin, PHA-E.

Figure 25 is a map of a cloning vector pCR2.1-SpPv-FRIL manufactured by ligating a cDNA according to the invention in the XhoI site of the cloning vector pCR2.1.

Figure 26 is a map of a cloning vector pM-SpPv-FRIL manufactured by ligating a cDNA according to the invention in the BglII/XhoI sites of the cloning vector SpPv-FRIL.

Figures 27A-27B are representations of line graphs showing the total cell numbers and progenitor levels in the presence of DI-FRIL, a representative, non-limiting FRIL family member of the invention, or cytokines. Enriched CB CD34<sup>+</sup> cells were cultured for 3, 6, 10, or 13 days in the presence of DI-FRIL (solid symbols) or cytokines (open symbols). Colonies were scored on day 14 and progenitor levels were calculated based on total cell numbers. Values shown represent the mean $\pm$ SEM of data from up to 10 experiments. Figure 27A shows the total cell numbers over time. Figure 27B shows the progenitor levels in cultures over time.

Figures 28A-28D are representations of line and bar graphs showing the total cell numbers and progenitor levels first in the presence of DI-FRIL, a representative, non-limiting FRIL family member of the invention, and second in the presence of cytokines. Figure 28A shows the total numbers of cells cultured with DI-FRIL for the entire 10 days (solid symbols) or for 6 days followed by 4 days of cytokine stimulation (open symbols). Figure 28B shows the progenitor levels in cells cultured with DI-FRIL for the entire 10 days (solid symbols) or for 6 days followed by 4 days of cytokine stimulation (open symbols). Figure 28C shows the total numbers of cells cultured with DI-FRIL for 13 days (solid symbols) or for 10 days followed by 3 days of cytokine stimulation (open symbols). Figure 28D shows the progenitor levels in cells cultured with DI-FRIL for 13 days (solid symbols) or for 10 days followed by 3 days of cytokine stimulation (open symbols).

Figures 29A-29D are representations of representative Southern blot analyses showing the quantitative analysis of SRC after *ex vivo* cultures with DI-FRIL, a representative, non-limiting FRIL family member of the invention, or cytokines and after transplantation into mice. Figure 29A is a representation of a representative Southern blot showing human DNA in the marrow of mice transplanted with cells that were cultured with DI-FRIL for 6 days (lane 1), DI-FRIL for 10 days (lane 2), or with DI-FRIL for 6 days followed by 4 days with cytokine stimulation (lane 3). Figure 29B is a representation of a representative Southern blot showing human DNA in the marrow of mice transplanted with cells cultured with DI-FRIL for 10 days (lanes 1-2), or with DI-FRIL for 6 days followed by 4 days with cytokine stimulation (lanes 3-4). Figure 29C is a representation of a representative Southern blot showing human DNA in the marrow of mice transplanted with the original cells prior to seeding (lane 1), or with cells cultured with DI-FRIL for 13 days (lane 2). Figure 29D is a representation of a representative Southern blot showing human DNA in the marrow of mice transplanted with cells cultured with FRIL for 10 days (lane 1), DI-FRIL for 6 days followed by 4 days of cytokine stimulation (lane 2), DI-FRIL for 13 days (lane 3), or with DI-FRIL for 10 days followed by 3 days of cytokine stimulation (lane 4).

Figure 29E is a representation of a Southern blot analysis showing the detection of a 0%, 0.1%, 1%, and 10% human DNA per murine DNA.

Figure 30 is a representation of a survival chart summarizing the levels of human cell engraftment in the marrow of mice transplanted with CD34<sup>+</sup> cells cultured in either DI-FRIL, a representative, non-limiting FRIL family member of the invention, for ten days (left panel), or in the presence of DI-FRIL for 6 days followed by culture in the presence of cytokines for four days.

Figure 31 is a representation of a representative Southern blotting analysis showing the levels of human cell en-

graftment in the BM of NOD/SCID B2M<sup>null</sup> transplanted with CD34<sup>+</sup>CD38<sup>-low</sup> cells cultured in the presence of DI-FRIL, a representative, non-limiting FRIL family member of the invention. Sorted cells (2x10<sup>5</sup> initial cells /treatment) were cultured in the presence of DI-FRIL for 6 days followed by additional 4 days exposure to cytokines, or with DI-FRIL alone for 10 days. After 10 days, 3.6x10<sup>5</sup> cells harvested from cytokine culture were divided and transplanted into 3 mice (lanes 1-3), while 3.5x10<sup>4</sup> cells harvested from DI-FRIL alone culture were transplanted to one mouse (lane 4). DNA was harvested from the bone marrow of transplanted mice and subjected to Southern blotting analysis with radiolabeled human chromosome 17-specific  $\alpha$ -satellite probe (p17H8). A representative experiment out of 4 is shown.

Figure 32 is a representation of a bar graph showing the average fold increase of engraftment levels obtained with CD34<sup>+</sup>CD38<sup>-low</sup> cells that were cultured with DI-FRIL, a representative, non-limiting FRIL family member of the invention, for 6 days and with cytokines for 4 days (right bar), as compared to 10 days with DI-FRIL alone (left bar). Data represent mean  $\pm$  SE from 4 experiments.

Figures 33A-33F are representations of a bar graph (Figure 33A) and representative flow cytometry histograms (Figure 33B-33F) showing the multilineage differentiation of SRC cultured with DI-FRIL, a representative, non-limiting FRIL family member of the invention, in the BM of transplanted mice. Figure 33A shows the number of colonies per 2 x 10<sup>5</sup> cells, where the cells treated as indicated prior to transplantation into mice were recovered from the murine BM, and seeded into semisolid media selective for human colonies. Progenitor levels were calculated based on the total human cell numbers (2 x 10<sup>5</sup> cells) in the marrow of transplanted mice. Values shown represent the mean  $\pm$  SEM of data from 3 experiments, 9 mice/treatment. Figure 33B shows a representative flow cytometry analysis showing nonspecific labeling where mouse IgG was used as isotype control. Figures 33C and 33D show representative flow cytometry analyses of BM cells from mice that were transplanted with CD34<sup>+</sup> cells (Figure 33C) or CD34<sup>+</sup>CD38<sup>-low</sup> cells (Figure 33D) precultured with DI-FRIL for 10 days, where the harvested BM cells were stained with anti human CD45 and anti-human CD19. Figures 33E and 33F show representative flow cytometry analyses of BM cells from mice that were engrafted by CD34<sup>+</sup> cells (Figure 33E) or CD34<sup>+</sup>CD38<sup>-low</sup> cells (Figure 33F) precultured with DI-FRIL for 10 days, where the harvested BM cells were subsequently cultured with SCF + IL-15 for 10 days, and then stained with human specific monoclonal anti-CD45 and anti-CD56.

Figures 34A-34B are representations of bar graphs showing the growth effect of DI-FRIL, a representative, non-limiting FRIL family member of the invention, on CD34<sup>+</sup> cells and progenitors compared to Flt3 ligand and cytokine combinations. (+) indicates co-culture of factors for the entire 10 days while (->) indicates substitution of the first factor after 6 days with cytokines, as indicated under the x axis. Figure 34A shows the total cell numbers. Figure 34B shows the percentage of CFU-GEMM out of total colonies. Values shown are per 2x10<sup>5</sup> seeded cells and represent the mean  $\pm$  SEM of data from 5 experiments.

Figures 35A-35G are representatives of flow cytometry histograms showing the cell cycle analyses of CD34<sup>+</sup> cells cultured with DI-FRIL, a representative, non-limiting FRIL family member of the invention, or various cytokines, or with combinations thereof. DNA content was determined by flow cytometry with propidium iodide staining. Enriched CD34<sup>+</sup> cells were cultured for 3 days with no treatment (Fig. 35A), DI-FRIL (Fig. 35B), Flt3-L (Fig. 35C), DI-FRIL+Flt3-L (Fig. 35D), SCF+G-CSF+IL-3+IL-6 (SG36) (Fig. 35E), SG36+DI-FRIL (Fig. 35F), and SG36+Flt3-L (Fig. 35G).

Figures 36A-36C are representations of line graphs showing the dose response of CB mnc chemotherapeutic agents in the presence and absence of DI-FRIL, a representative, non-limiting FRIL family member of the invention. Chemotherapy agents were assayed over a 5-log dose range on CB MNC (2 x 10<sup>5</sup> cells /0.1 mL) in AIMV (Life Technologies) containing Agar-SCM (StemCell Technologies). Viable cells were determined after 5 days of culture by XTT. Solid squares indicate chemotherapy drug with no DI-FRIL; solid triangles indicate cultures containing DI-FRIL at 10 ng/ml in all wells; and open circles indicate DI-FRIL in all wells at 100 ng/ml. Figure 36A shows the dose response to Ara-C; Figure 36B shows the dose response to doxorubicin; and Figure 36C shows the dose response to 5-FU.

Figure 37 is a representation of a photograph of purified DI-FRIL, a representative, non-limiting FRIL family member of the invention, resolved by SDS-PAGE analysis. Five discrete bands appeared which corresponded to the  $\alpha$  and  $\beta$  subunits, each of which was subjected to amino-terminal sequencing. The amino acid sequences of the five bands are as indicated.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

**[0021]** The invention relates to the use of a therapeutically effective amount of a composition containing a FRIL protein comprising the amino acid sequence set forth in SEQ ID NO: 6 for the preparation of a medicament for treatment of a condition characterized by hematopoietic progenitor cell-depleting activity of a radiotherapeutic or a chemotherapeutic treatment or a combination of a radiotherapeutic and a chemotherapeutic treatment, wherein the therapeutically effective amount of said composition alleviates or reduces the hematopoietic progenitor cell-depleting activity of said

therapeutic treatment in the patient, and wherein the medicament is to be administered prior to the radio therapeutic or chemotherapeutic treatment; wherein the medicament is to be administered about 5 days to about 2 hours before the therapeutic treatment and is to be administered daily.

**[0022]** The invention describes an essentially pure composition of a member of the FRIL family of progenitor cell preservation factors comprising the amino acid sequence SEQ ID NO:6 for the claimed use. The term, "FRIL family of progenitor cell preservation factors" is used to mean a family of lectins, wherein each FRIL family member molecule binds to a normally glycosylated FLT3 receptor, wherein each FRIL family member molecule preserves progenitor cells, and wherein one FRIL family member molecule that is isolated from a hyacinth bean (*i.e.*, *Dolichos lab lab*) has an amino acid sequence which comprises the following eight amino acid sequence: TNNVLQXT (SEQ ID NO: 24). By "FRIL family member" or "FRIL family member molecule" is meant one or more molecules of the FRIL family of progenitor cell preservation factors.

**[0023]** Both amino acid sequence identity and nucleic acid sequence identity between two proteins or two nucleic acid molecules can be measured according to standard methods. For example, in order to compare a first amino acid sequence to a second amino acid sequence or a first nucleic acid sequence to a second nucleic acid sequence for the purpose of determining percentage identity between the two sequences, the sequences are aligned so as to maximize the number of identical amino acid or nucleic acid residues. The sequences of proteins sharing at least 50% amino acid sequence identity or the sequences of nucleic acids sharing at least 45% nucleic acid sequence identity can usually be aligned by visual inspection. If visual inspection is insufficient, the proteins or nucleic acids may be aligned in accordance with the FASTA method in accordance with Pearson and Lipman (*Proc. Natl. Acad. Sci. USA* 85: 2444-2448, 1988), or, preferably, any of the methods described by George, D.G. et al., in *Macromolecular Sequencing and Synthesis, Selected Methods and Applications*, pages 127-149, Alan R Liss, Inc. (1988), such as formula 4 at page 137 using a match score of 1, a mismatch score of 0, and a gap penalty of -1. From this method, percentage of sequence identity between the first and second amino acid sequences or between the first and second nucleic acid can be determined.

**[0024]** Other methods for determining amino acid or nucleic acid sequence identity are described in Feng and Doolittle (*Journal of Molecular Evolution* 25: 351-360, 1987) and Higgins and Sharp (*CABIOS* 5:151-153,1989).

**[0025]** Another method for determining amino acid or nucleic acid sequence identity between two proteins or nucleic acids is by using sequence analysis software with the default parameters specified therein. Various software packages exist including Sequence Analysis Software Package of the Genetics Computer Group (University of Wisconsin Biotechnology Center, Madison, WI), and the various BLAST programs of the National Center for Biotechnology (National Library of Medicine, Bethesda, MD).

**[0026]** Unless otherwise specified, percentage of amino acid sequence identity or percentage of nucleic acid sequence identity is determined using the basic BLAST program of the National Center for Biotechnology (National Library of Medicine, Bethesda, MD), using the default settings defined therein.

**[0027]** Another test for percentage identity of two nucleic acid sequences is whether they hybridize under normal hybridization conditions, preferably under stringent hybridization conditions. Thus, also included in the invention are proteins that are encoded by nucleic acid molecules that hybridize under high stringent conditions to a sequence complementary to SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, and/or SEQ ID NO: 7. The term "stringent conditions," as used herein, is equivalent to "high stringent conditions" and "high stringency." These terms are used interchangeably in the art.

**[0028]** Stringent conditions are defined in a number of ways. In one definition, stringent conditions are selected to be about 50°C lower than the thermal melting point ( $T_m$ ) for a specific sequence at a defined ionic strength and pH. The  $T_m$  is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched sequence. Typical stringent conditions are those in which the salt concentration is at least about 0.02 M at pH 7 and the temperature is at least about 60°C. "Stringent conditions," in referring to percentage identity (e.g., homology) or substantial similarity in the hybridization context, can be combined conditions of salt, temperature, organic solvents or other parameters that are typically known to control hybridization reactions. The combination of parameters is more important than the measure of any single parameter. If incompletely complementary sequences recognize each other under high stringency conditions, then these sequences hybridize under conditions of high stringency (see U.S. Pat No. 5,786,210; Wetmur and Davidson, *J. Mol. Biol.* 31,349-370,1968). Control of hybridization conditions, and the relationships between hybridization conditions and degree of homology are understood by those skilled in the art. See, e.g., Sambrook et al., *Molecular Cloning. A Laboratory Manual*, 2ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, 1989. Further examples of stringent conditions can be found in Goeddel et al., U.S. Patent No. 5,789,550.

**[0029]** In a non-limiting example, "stringent conditions" can be provided in a variety of ways such as overnight incubation at 42°C in a solution comprising: 20% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA. Alternatively, the stringent conditions are characterized by a hybridization buffer comprising 30% forma-



made in 5 x SSPE (0.18 M NaCl, 0.01 M NaPO<sub>4</sub>, pH 7.7, 0.0001 M EDTA) buffer at a temperature of 42°C, and subsequent washing at 42°C with 0.2 x SSPE. Preferably, stringent conditions involve the use of a hybridization buffer comprising 50% formamide in 5 x SSPE at a temperature of 42°C and washing at the same temperature with 0.2 x SSPE.

**[0030]** As used herein, by "preserves progenitor cells" is meant an ability of a FRIL family member molecule (or mutant thereof or fusion protein comprising a FRIL family member molecule or mutant thereof) to retain (*i.e.*, preserve) progenitor cells in an undifferentiated state, which can be determined using the assays described below (*e.g.*, the SCID mouse reconstituting cell assay and the methylcellulose or other semi-solid medium based hematopoietic progenitor cell assay). In accordance with the invention, "progenitor cell" refers to any normal somatic cell that has the capacity to generate fully differentiated, functional progeny by differentiation and proliferation. Progenitor cells include progenitors from any tissue or organ system, including, but not limited to, blood, mesenchymal, embryonic, nerve, muscle, skin, gut, bone, kidney, liver, pancreas, thymus, brain and the like. Progenitor cells are distinguished from "differentiated cells," the latter being defined as those cells that may or may not have the capacity to proliferate, *i.e.*, self-replicate, but that are unable to undergo further differentiation to a different cell type under normal physiological conditions. Moreover, progenitor cells are further distinguished from abnormal cells such as neoplastic cells, as defined herein. For example, leukemia cells proliferate (self-replicate), but generally do not further differentiate, despite appearing to be immature or undifferentiated.

**[0031]** Progenitor cells include all the cells in a lineage of differentiation and proliferation prior to the most differentiated or the fully mature cell. Thus, for example, progenitors include the skin progenitor in the mature individual. The skin progenitor is capable of differentiation to only one type of cell, but is itself not fully mature or fully differentiated.

**[0032]** By "hematopoiesis" is meant the development of mature, functional blood cells. The progenitor cells that give rise to mature, functional blood cells are called hematopoietic progenitor cells. The most primitive, undifferentiated hematopoietic progenitor cell is called a hematopoietic stem cell. Hematopoietic stem cells typically reside in the bone marrow primarily in a quiescent state, and may form identical daughter cells through a process called "self-renewal."

**[0033]** Production of some mature, functional blood cells results from proliferation and differentiation of "unipotential progenitors," *i.e.*, those progenitors that have the capacity to make only one type of blood cell. For red blood cell (erythrocyte) production, a unipotential progenitor called a "CFU-E" (colony forming unit-erythroid) has the capacity to generate two to 32 mature progeny cells. Various other hematopoietic progenitors have been characterized. For example, hematopoietic progenitor cells include those cells that are capable of successive cycles of differentiation and proliferation to yield up to eight different mature hematopoietic cell lineages.

**[0034]** Uncommitted progenitor cells, such as hematopoietic stem cells, can be described as being "totipotent," *i.e.*, both necessary and sufficient for generating all types of mature cells. Progenitor cells that retain a capacity to generate all cell lineages, but that can not self-renew, are termed "pluripotent." Cells that can produce some but not all blood lineages and cannot self-renew are termed "multipotent."

**[0035]** Progenitor cells can be defined by mRNA levels of genes that either specifically regulate progenitors or serve as markers of lineage commitment. For example, genes induced in primitive human hematopoietic progenitor cells include those encoding the shared beta subunits of the IL3, IL5, and/or granulocyte-macrophage colony-stimulating factor (GM-CSF) receptors, termed the beta common chain (McClanahan et al., *Blood* 81:3903-2915, 1993); CD34 genes; and/or the receptors for Kit (Turner et al., *Blood* 88:3383-3390, 1996), FLT1, FLT4 (Galland et al., *Oncogene* 8:3233-1240, 1993), FLK1 (Broxmeyer et al., *Int. J. Hematol.* 62:303-215, 1995), and FLT3 (Lyman and Jacobsen, *Blood* 91:3101-1134., 1998). Those genes for intermediate progenitors include the c-Fms, G-CSF receptor, and/or CD34 genes; and the IL-7 receptor gene, a gene induced for B lymphopoiesis.

**[0036]** Murine primitive progenitor populations include receptors for interleukin-1 alpha (IL-1 $\alpha$ ), IL-3, IL-6, granulocyte colony-stimulating factor (G-CSF), and/or FLK1-1 (the murine homologue of human KDR which binds VEGF) (Broxmeyer, *supra*), but lack receptors for macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), and leukemia inhibitory factor (LIF). Cells within the intermediate progenitor cell population include receptors for GM-CSF, G-CSF, IL-6, and/or IL-1 $\alpha$ .

**[0037]** In accordance with the invention, the terms "bind," "binds," or "bound" are used interchangeably to mean that a FRIL family member molecule of the invention binds to a normally glycosylated FLT3 receptor with an affinity higher than the affinity with which the FLT3-Ligand binds the FLT3 receptor. Preferably, a FRIL family member molecule binds to a normally glycosylated FLT3 receptor with an affinity that is at least as high as the affinity with which an antibody binds its specific ligand. Even more preferably, a FRIL family member molecule of the invention binds to a normally glycosylated FLT3 receptor with an affinity that is higher than the affinity with which an antibody binds its specific ligand. Still more preferably, a FRIL family member molecule of the invention binds to a normally glycosylated FLT3 receptor with a dissociation constant ( $K_D$ ) of at least  $10^{-7}$  M, more preferably  $10^{-8}$  M, even more preferably  $10^{-9}$  M, still more preferably, at least  $10^{-10}$  M, and most preferably, a FRIL family member molecule of the invention binds to a normally glycosylated FLT3 receptor with a dissociation constant ( $K_D$ ) of at least  $10^{-11}$  M. Standard methods for determining binding and binding affinity are known.

**[0038]** In accordance with the invention, by "normally glycosylated FLT3 receptor" is meant an FLT3 receptor that has a glycosylation pattern of an FLT3 cell glycosylated by a normal cell. By "normal cell," as used herein in accordance with the present invention, is meant a cell that is not neoplastic. As used herein, by "neoplastic cell" is meant a cell that shows aberrant proliferation, particularly increased proliferation, that is not regulated by such factors as cell-cell contact inhibition and soluble regulators (e.g., cytokines or hormones), and that abnormally glycosylates the FLT3 receptor such that the glycosylation pattern on the FLT3 receptor on the neoplastic cells is abnormal and such that the FLT3 receptor on the neoplastic cell is not bound by a FRIL family member molecule.

**[0039]** In accordance with the invention, by "essentially pure" means a molecule, such as a nucleic acid or protein (e.g., a FRIL family member molecule), or composition of a molecule that is more free from other organic molecules (e.g., carbohydrates, nucleic acids, proteins, and lipids) that naturally occur with an impure molecule, and is substantially free as well of materials used during the purification process. For example, a protein or nucleic acid molecule is considered to be essentially pure if it is at least approximately 60%, preferably at least approximately 75%, more preferably approximately at least 85%, most preferably approximately at least 90%, and optimally approximately at least 95% pure, i.e., free from other organic molecules with which it naturally occurs and free from materials used during the purification process. Methods for purifying proteins are known in the art and include, without limitation, HPLC, SDS-PAGE, immunoprecipitation, recombinant protein production, affinity chromatography using specific antibodies, ion-exchange, size-exclusion, and hydrophobic interaction chromatography, or a combination of any of these methods. These and other suitable methods are described, e.g., in Marston, "The purification of eukaryotic proteins expressed in *E. coli*," in *DNA Cloning*, Glover D.M., ed., Volume III, IRL Press Ltd., Oxford, 1987; Marston and Hartley, "Solubilization of protein aggregates," pp. 266-267 in *Guide to Protein Purification*, Deutscher MP, ed., Academic Press, San Diego, 1990; Laemmli, U.K., *Nature* 227:680-685, 1970. A FRIL family member can also be purified by binding to a mannose, which may be coupled on a solid support (e.g., a sepharose bead).

**[0040]** Methods for purifying nucleic acids are known in the art and include, without limitation, Guanidine-HCl extraction, polymerase chain reaction, CsCl gradient fractionation, phenol: chloroform extraction, ethanol precipitation, and standard recombinant DNA methodologies. Standard methods for purifying both proteins and nucleic acid molecules are provided in, e.g., Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1994; Sambrook et al., *supra*.

**[0041]** A FRIL family member molecule may be purified from a natural source by methods well known in the art. For example, the purification of DI-FRIL from *Dolichos lab lab* is described below in Example 1. The purification of Pv-FRIL from *Phaseolus vulgaris* is described below in Example 5. The purification of YarnFRIL from *Sphenostylis stenocarpa* is described below in Example 22. Such methods also include, for example, those described by Moore in PCT application PCT/US97/22486 and by Gowda et al., *supra*. A suitable natural source from which to purify a FRIL family member molecule includes plants, especially legume plants. Legumes, such as the garden pea or the common bean, are plants ("leguminous plants") from a family (*Leguminosae*) of dicotyledonous herbs, shrubs, and trees bearing (nitrogen-fixing bacteria) nodules on their roots. These plants are commonly associated with their seeds (e.g., the garden pea or the common bean).

**[0042]** More specifically, a FRIL family member molecule can be purified from members of the tribe *Phaseoleae*. For example, a FRIL family member molecule can be purified from *Dolichos lab lab* (e.g., hyacinth beans, which is also known by other common names throughout the world). Alternatively, a FRIL family member molecule can be purified from varieties of the common bean (*Phaseolus vulgaris*) (e.g., red kidney beans and white kidney beans), from the yarn bean (*Sphenostylis stenocarpa*) or from *Vigna sinensis*, commonly known as the black-eyed pea.

**[0043]** As demonstrated in the examples below, purification of a FRIL family member molecule from a legume is rapid and inexpensive, and results in a large amount of essentially pure lectin. A native FRIL family member molecule can be easily purified from legumes, such as hyacinth beans (pesticide-free), by mannose-affinity chromatography or ovalbumin affinity chromatography, and is more than 100 times cheaper to produce than recombinant cytokines.

**[0044]** Most preferably, a FRIL family member molecule is a mannose/glucose-specific legume lectin. By "lectin" is meant a protein that binds sugar residues with high affinity.

**[0045]** As demonstrated in the examples below, FRIL family member molecules, and compositions of FRIL family member molecules, have many attributes as reagents to either alleviate the progenitor cell-depleting activity of a therapeutic (e.g., a chemotherapeutic) or to alleviate the symptoms of a condition where the patient's progenitor cells are depleted. For example, FRIL family members have unique properties and are the first soluble regulators reported to preserve hematopoietic stem cells and progenitors in a dormant state for extended periods, even in the presence of potent stimulators of proliferation and differentiation. Moreover, because mice tolerate very high levels of compositions of FRIL family members, this may permit more effective protection of stem cells and progenitors by preventing their recruitment during aggressive dose intensification regimens aimed at increasing frequency and dosage levels of chemotherapy. While the biological activity of DI-FRIL is similar to cytokines (ng/ml range), as demonstrated in the examples below, mice tolerated up to a 1,000-fold more DI-FRIL than cytokines.

**[0046]** In addition, by preserving hematopoietic stem cells and other progenitor cells in a dormant state, one or more

members of the FRIL family allows for the administration of a broad range of cell cycle active chemotherapy drugs with greater frequency and higher dose. Thus, administration of a composition of one or more FRIL family members may permit more aggressive dose-intensification chemotherapy regimens for a broad range of chemotherapy drugs. Administration of a composition of a FRIL family member also provides for a larger reservoir of progenitor cells which could rapidly respond to stimulatory signals after completing chemotherapy.

**[0047]** The FRIL family member which is used according to the invention is from a legume (e.g., a bean plant), i.e., from a red kidney bean (i.e., *Phaseolus vulgaris*). The FRIL family member of the invention isolated from a red kidney bean has the amino acid sequence of SEQ ID NO: 6, and, even more preferably, is encoded by a nucleic acid having the nucleic acid sequence of SEQ ID NO: 5.

**[0048]** In the following described but not claimed are the FRIL family member molecule which is a fusion protein comprising a first portion and a second portion, wherein the first portion is derived from a second member of the FRIL family. By "fusion protein" is meant a molecule comprising at least two proteins or polypeptide fragments thereof joined together, wherein the proteins or polypeptide fragments thereof are not joined together in the naturally-occurring organism from which the proteins or polypeptide fragments thereof were derived. The two proteins or polypeptide fragments thereof of a fusion protein may be joined by any means, including, without limitation, a chemical linker, a peptide bond, or a noncovalent bond, such as an ionic bond. By "protein" or "polypeptide" is meant a chain of two or more amino acid residues joined with a peptide bond regardless of length or post-translational modification such as acetylation, glycosylation, lipidation, or phosphorylation.

**[0049]** A FRIL family member molecule that is a fusion protein may comprise a first portion derived from a FRIL family member and a second portion derived from a protein or other molecule not related to the FRIL family (e.g., the heavy chain of an antibody).

**[0050]** An additional FRIL family member molecule that is a fusion protein is FRIL family member comprising the  $\alpha$  subunit from a first FRIL family member and a  $\beta$  subunit from a second FRIL family member. Such a fusion protein may be generated, for example, by joining a nucleic acid sequence encoding the  $\alpha$  subunit of the first FRIL family member in frame with a nucleic acid sequence encoding the  $\beta$  subunit of the second FRIL family member. The nucleic acid encoding such a fusion protein can be engineered to encode an enzyme-specific cleavage site between the portion encoding the  $\alpha$  subunit of the first FRIL family member and the portion encoding the  $\beta$  subunit of the second FRIL family member.

**[0051]** Where a FRIL family member is a fusion protein, identity of the fusion protein as a FRIL family member is determined by the sequence identity between the FRIL family member-derived portion of the fusion protein and a second FRIL family member, where the FRIL family member-derived portion of the fusion protein and the second FRIL family member share at least about 45% amino acid sequence identity, even more preferably at least about 50% identity, even more preferably at least about 55% identity, still more preferably at least about 60% identity, still more preferably at least about 65% identity yet more preferably at least about 75% sequence identity, still more preferably at least about 85% identity, and most preferably at least about 95% identity, with the amino acid sequence of a second member of the FRIL family.

**[0052]** A FRIL family member in accordance with the first aspect of the invention can also be a recombinant protein made by expressing a recombinant nucleic acid that encodes FRIL in a suitable host. Thus, in a second aspect, the invention features an essentially pure nucleic acid molecule encoding a member of the FRIL family of progenitor cell preservation factors for the use as claimed. Exemplary purifications of the nucleic acid molecules of the invention from *Dolichos lab lab* and *Phaseolus vulgaris* are described below. As is well known, if an amino acid sequence (primary structure) is known, a family of nucleic acids can then be constructed, each having a sequence that differs from the others by at least one nucleotide, but where each different nucleic acid still encodes the same protein. For example, if a protein has been sequenced but its corresponding gene has not been identified, the gene can be acquired through amplification of genomic DNA using a set of degenerate primers that specify all possible sequences encoding the protein. Thus, a nucleic acid in accordance to this aspect of the invention need not have a naturally occurring sequence, but need only encode a FRIL family member according to the first aspect of the invention.

**[0053]** By a "recombinant nucleic acid" is meant a nucleic acid which encodes a FRIL family member molecule, or a portion encoding at least 15 contiguous amino acids thereof, or a mutant thereof, or a fusion protein comprising the molecule, portion thereof or mutant thereof or is capable of expressing an antisense molecule specifically complementary thereto, or a sense molecule that shares nucleic acid sequence identity thereto wherein the recombinant nucleic acid may be in the form of linear DNA or RNA, covalently closed circular DNA or RNA, or as part of a chromosome, provided however that it cannot be the native chromosomal locus for a FRIL family member molecule. Preferred recombinant nucleic acids of the invention are vectors, which may include an origin of replication and are thus capable of replication in one or more cell type. Certain preferred recombinant nucleic acids are expression vectors, and further comprise at least a promoter and passive terminator, thereby allowing transcription of the recombinant nucleic acid in a bacterial, fungal, plant, insect or mammalian cell.

By "nucleic acid" or "nucleic acid molecule" as used herein, means any deoxyribonucleic acid (DNA) or ribonucleic

acid (RNA), including, without limitation, complementary DNA (cDNA), genomic DNA, RNA, hnRNA, messenger RNA (mRNA), DNA/RNA hybrids, or synthetic nucleic acids (e.g., an oligonucleotide) comprising ribonucleic and/or deoxyribonucleic acids or synthetic variants thereof. The nucleic acid of the invention includes, without limitation, an oligonucleotide or a polynucleotide. The nucleic acid can be single stranded, or partially or completely double stranded (duplex). Duplex nucleic acids can be homoduplex or heteroduplex.

**[0054]** In accordance with the second aspect of the invention, a nucleic acid encoding a FRIL family member has at least about 50% nucleic acid sequence identity with a nucleic acid encoding another member of the FRIL family, preferably at least about 55% nucleic acid sequence identity, more preferably at least about 60% nucleic acid sequence identity, more preferably at least about 65% nucleic acid sequence identity, still more preferably at least about 75% nucleic acid sequence identity, still more preferably at least about 85% nucleic acid sequence identity, and most preferably at least about 95% nucleic acid sequence identity with a nucleic acid encoding another member of the FRIL family. Percentage nucleic acid sequence identity can be determined as described for the first aspect of the invention.

**[0055]** A recombinant nucleic acid can also be chemically synthesized by methods known in the art. For example, recombinant DNA can be synthesized chemically from the four nucleotides in whole or in part by methods known in the art. Such methods include those described in Caruthers, M.H., *Science* 230(4723):281-285, 1985. DNA can also be synthesized by preparing overlapping double-stranded oligonucleotides, filling in the gaps, and ligating the ends together. See, generally, Sambrook et al., *supra*, and Glover and Hames, eds., *DNA Cloning*, 2d ed., Vols. 1-4, IRL Press, Oxford, 1995.

**[0056]** A recombinant nucleic acid molecule encoding a mutant FRIL family member can be prepared from wild-type DNA by site-directed mutagenesis (see, for example, Zoller and Smith, *Nucleic. Acids. Res.* 10:6487-6500, 1982; Zoller, M.J., *Methods Enzymol.* 100:468-500, 1983; Zoller, M.J., *DNA* 3(6):479-488, 1984; and McPherson, M.J., ed., *Directed Mutagenesis: A Practical Approach*, IRL Press, Oxford, 1991. A recombinant nucleic acid can be amplified by methods known in the art. One suitable method is the polymerase chain reaction (PCR) method described in Saiki et al., *Science* 239:487, 1988, Mullis et al., U.S. Patent No. 4,683,195, and Sambrook et al., *supra*. It is convenient to amplify the clones in the lambda-gt10 or lambda-gt11 vectors using lambda-gt10- or lambda-gt11-specific oligomers as the amplimers (available from Clontech, Palo Alto, CA). Larger synthetic nucleic acid structures can also be manufactured having specific and recognizable utilities according to the invention. For example, vectors (e.g., recombinant expression vectors) are known which permit the incorporation of recombinant nucleic acids of interest for cloning and transformation of other cells. Thus, the invention further includes vectors (e.g., plasmids, phages, and cosmids) which incorporate a nucleotide sequence of the invention, especially vectors which include the recombinant nucleic acid molecule of the invention for expression of a FRIL family member.

**[0057]** A recombinant nucleic acid of the invention can be replicated and used to express a FRIL family member following insertion into a wide variety of host cells in a wide variety of cloning and expression vectors. The host can be prokaryotic or eukaryotic. The nucleic acid can be obtained from natural sources and, optionally, modified. The genes can also be synthesized in whole or in part.

**[0058]** Cloning vectors can comprise segments of chromosomal, non-chromosomal and synthetic DNA sequences. Some suitable prokaryotic cloning vectors include plasmids from *E. coli*, such as colE1, pCR1, pBR322, pMB9, pUC, pKSM, and RP4. Prokaryotic vectors also include derivatives of phage DNA such as M13fd, and other filamentous single-stranded DNA phages.

**[0059]** Vectors for expressing proteins in bacteria, especially *E. coli*, are also known. Such vectors include the pK233 (or any of the *tac* family of plasmids), T7, and lambda P<sub>L</sub>. Examples of vectors that express fusion proteins are PATH vectors described in Dieckmann and Tzagoloff (*J. Biol. Chem.* 260(3):1513-1520, 1985). These vectors contain DNA sequences that encode anthranilate synthetase (TrpE) followed by a polylinker at the carboxy terminus. Other expression vector systems are based on beta-galactosidase (pEX); maltose binding protein (pMAL); glutathione S-transferase (pGST) (see, e.g., Smith, D. B., *Gene* 67:31-40, 1988 and Abath, F.G., *Peptide Research* 3(4):167-168, 1990).

**[0060]** Vectors useful for cloning and expression in yeast are also available. A suitable example is the 2μ circle plasmid.

**[0061]** Suitable cloning/expression vectors for use in mammalian cells are also known. Such vectors include well-known derivatives of SV-40, adenovirus, cytomegalovirus (CMV) and retrovirus-derived DNA sequences. Any such vectors, when coupled with vectors derived from a combination of plasmids and phage DNA, i.e., shuttle vectors, allow for the isolation and identification of protein coding sequences in prokaryotes.

**[0062]** Further eukaryotic expression vectors are known in the art (e.g., Southern and Berg, *J. Mol. Appl. Genet.* 1: 327-341, 1982; Subramani et al., *Mol. Cell. Biol.* 1:854-864, 1981; Kaufmann and Sharp, *J. Mol. Biol.* 159:601-621, 1982; Kaufmann and Sharp, *Mol. Cell. Biol.* 159:601-664, 1982; Scahill et al., *Proc. Natl. Acad. Sci. USA* 80:4654-4659, 1983; Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA* 77:4216-4220, 1980).

**[0063]** The expression vectors useful in the present invention contain at least one expression control sequence that is operatively linked to the recombinant nucleic acid molecule or fragment thereof to be expressed. The control sequence is inserted in the vector in order to control and to regulate the expression of the recombinant nucleic acid of

the invention. Examples of useful expression control sequences are the *lac* system, the *trp* system, the *tac* system, the *trc* system, major operator and promoter regions of phage lambda, the control region of fd coat protein, the glycolytic promoters of yeast, e.g., the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, e.g., Pho5, the promoters of the yeast alpha-mating factors, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, e.g., the early and late promoters of SV40, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells and their viruses or combinations thereof.

**[0064]** Useful expression hosts for expressing the recombinant nucleic acids of the invention include well-known prokaryotic and eukaryotic cells. Some suitable prokaryotic hosts include, for example, *E. coli*, such as *E. coli* SG-936, *E. coli* HB101, *E. coli* W3110, *E. coli* X1776, *E. coli* X2282, *E. coli* DHI, and *E. coli* MRCI, *Pseudomonas*, *Bacillus*, such as *B. subtilis*, and *Streptomyces*. Suitable eukaryotic cells include yeasts and other fungi, insect, animal cells, such as COS cells and CHO cells, human cells and plant cells in tissue culture.

**[0065]** Given the recombinant nucleic acid sequences disclosed herein, the artisan can further design recombinant nucleic acids having particular functions in various types of applications. For example, the artisan can construct oligonucleotides or polynucleotides for use as primers in nucleic acid amplification procedures, such as the polymerase chain reaction (PCR), ligase chain reaction (LCR), Repair Chain Reaction (RCR), PCR oligonucleotide ligation assay (PCR-OLA), and the like. Oligonucleotides useful as probes in hybridization studies, such as *in situ* hybridization, can be constructed. Numerous methods for labeling such probes with radioisotopes, fluorescent tags, enzymes, binding moieties (e.g., biotin), and the like are known, so that the probes can be adapted for easy detectability.

**[0066]** Oligonucleotides can also be designed and manufactured for other purposes. For example, the invention enables the artisan to design antisense oligonucleotides, and triplex-forming oligonucleotides, and the like, for use in the study of structure/function relationships. Homologous recombination can be implemented by adaptation of the nucleic acid of the invention for use as targeting means.

**[0067]** Recombinant nucleic acids of the invention produced as described above can further be modified to alter biophysical or biological properties by means of techniques known in the art. For example, the recombinant nucleic acid can be modified to increase its stability against nucleases (e.g., "end-capping"), or to modify its lipophilicity, solubility, or binding affinity to complementary sequences. Methods for modifying nucleic acids to achieve specific purposes are disclosed in the art, for example, in Sambrook et al., *supra*. Moreover, the recombinant nucleic acid of the invention can include one or more portions of nucleotide sequence that are non-coding for a FRIL family member.

**[0068]** The invention provides a pharmaceutical formulation for the claimed use comprising an essentially pure composition of one or more members of the FRIL family of progenitor cell preservation factors and a pharmaceutically acceptable carrier. By "pharmaceutically acceptable carrier" is meant any inert carrier that is non-toxic to the animal to which it is administered and that retains the therapeutic properties of the compound with which it is administered (*i.e.*, the FRIL family member). Pharmaceutically acceptable carriers and their formulations are well-known and generally described in, for example, Remington's Pharmaceutical Sciences (18th Edition, ed. A. Gennaro, Mack Publishing Co., Easton, PA, 1990). One exemplary pharmaceutically acceptable carrier is physiological saline. Pharmaceutical formulations of the invention may employ any pharmaceutically acceptable carrier, depending upon the route of administration of the composition.

**[0069]** Compositions of FRIL family members may be used safely and efficaciously as therapeutics. The gastrointestinal tracts of animals come in constant contact with lectins, such as FRIL family members, in raw and/or cooked vegetables and fruits. Many lectins pass through the gastrointestinal tract biologically intact (Pusztai, A., *Eur. J. Clin. Nutr.* 47: 691-699, 1993). Some lectins interact with the gut and are transported into the peripheral blood circulation. For example, a recent study found peanut agglutinin (PNA) in the blood of humans at levels of 1-5 µg/ml an hour after ingesting 200 g of raw peanuts (Wang et al., *Lancet* 352: 1831-1832, 1998). Antibodies to dietary lectins are commonly found in people at levels of ~1 µg/ml (Tchemychev and Wilchek, *FEBS Lett.* 397: 139-142, 1996). These circulating antibodies do not block carbohydrate binding of the lectins.

**[0070]** The invention describes administration of a therapeutically effective amount of the pharmaceutical formulation to a patient suffering from a condition whereby the patient's hematopoietic progenitor cells are depleted alleviates and/or reduces the condition in the patient.

**[0071]** In accordance with the invention, by "therapeutically effective amount" is meant a dosage of a composition of a FRIL family member or pharmaceutical formulation comprising a composition of a FRIL family member that is effective to alleviate and/or reduce either a condition whereby the patient's hematopoietic progenitor cells are depleted or a hematopoietic progenitor cell-depleting activity of a therapeutic (e.g., a chemotherapeutic). Preferably, such administration is systemic (e.g., by intravenous injection). When administered systemically, a therapeutically effective amount is an amount of between about 500 ng of the FRIL family member/kg total body weight and about 5 mg/kg total body weight per day. Preferably, a therapeutically effective amount is between about 500 ng/kg and 500 µg/kg total body weight of the FRIL family member per day. Still more preferably, a therapeutically effective amount is between about 5 µg/kg and 50 µg/kg total body weight of the FRIL family member per day. Most preferably, a therapeutically effective amount is an amount that delivers about 50 µg/kg total body weight of the FRIL family member per day.

**[0072]** A composition of a FRIL family member of the invention and pharmaceutical formulation comprising a composition of a FRIL family member of the invention may be administered to patients having, or predisposed to developing, a condition whereby the patient's hematopoietic progenitor cells are depleted. Such a condition may be congenital. For example, the patient may have severe combined immunodeficiency or aplastic anemia.

**[0073]** The condition may also be induced by a drug. Thus, in certain embodiments of the third aspect of the invention, administration of a therapeutically effective amount of the pharmaceutical formulation to a patient prior to treatment of the patient with a therapeutic treatment having a hematopoietic progenitor cell-depleting activity alleviates the hematopoietic progenitor cell-depleting activity of the therapeutic in the patient. For example, cancer patients are often treated with radiotherapeutics or chemotherapeutics that have hematopoietic progenitor cell-depleting activity. By "hematopoietic progenitor cell-depleting activity" is meant an activity of a therapeutic treatment whereby the hematopoietic progenitor cells in the patient being treated with the therapeutic treatment are depleted, either by killing the progenitor cells or by inducing the progenitor cells to undergo irreversible differentiation. Non-limiting examples of therapeutic treatments having hematopoietic progenitor cell-depleting activity are the chemotherapeutic agents cytarabine (Ara-C), doxorubicin (Dox), daunorubicin, and 5-fluorouracil (5-FU).

**[0074]** In certain embodiments, administration of the pharmaceutical formulation of the invention to a patient prior to the treatment of the patient with a therapeutic treatment having a hematopoietic progenitor cell-depleting activity enables treatment of the patient with a higher dosage of the therapeutic treatment. The higher dosage of the therapeutic treatment may be accomplished by either an increased dose of the therapeutic treatment and/or an increased duration of treatment with the therapeutic treatment. For example, a child diagnosed with childhood Acute Myelogenous Leukemia (AML) is typically initially treated for the first seven days with daunorubicin at 45 mg/m<sup>2</sup> on Days 1-3 plus Ara-C at 100 mg/m<sup>2</sup> for 7 days plus GTG at 100 mg/m<sup>2</sup> for 7 days. The same child pretreated with a composition in accordance with this aspect of the invention may be able to tolerate a higher dosage (*i.e.*, higher dose and/or prolonged treatment period) of any or all of these chemotherapeutics. Such an increase in dosage tolerance of a therapeutic treatment (*e.g.*, a chemotherapeutic) having a hematopoietic progenitor cell-depleting activity in a cancer patient is desirable since a higher dosage may result in the destruction of more cancerous cells.

**[0075]** The pharmaceutical formulations and/or compositions of the invention may be administered by any appropriate means. For example, the pharmaceutical formulations and/or compositions of the invention may be administered to a mammal within a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form according to conventional pharmaceutical practice. Administration may begin before the mammal is symptomatic for a condition whereby the patient's hematopoietic progenitor cells are depleted. For example, administration of the pharmaceutical formulations of the third aspect of the invention to a cancer patient may begin before the patient receives radiotherapy and/or chemotherapy treatment.

**[0076]** Any appropriate route of administration of a pharmaceutical formulation and/or composition of the invention may be employed, including, without limitation, parenteral, intravenous, intra-arterial, subcutaneous, sublingual, transdermal, topical, intrapulmonary, intramuscular, intraperitoneal, by inhalation, intranasal, aerosol, intrarectal, intravaginal, or by oral administration. Pharmaceutical formulations and/or compositions of the invention may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols. The pharmaceutical formulations and/or compositions may be administered locally to the area affected by a condition whereby the patient's hematopoietic progenitor cells are depleted. For example, the pharmaceutical formulations and/or compositions of the invention may be administered directly into the patient's bone marrow. The pharmaceutical formulations and/or compositions of the invention may be administered systemically.

**[0077]** According to the invention, the patient is a human or a domesticated animal. By "domesticated animal" is meant an animal domesticated by humans, including, without limitation, a cat, a dog, an elephant, a horse, a sheep, a cow, a pig, and a goat. In some embodiments, the patient has cancer.

**[0078]** The treatment is a radiotherapeutic or a chemotherapeutic treatment, including, without limitation, cytarabine (Ara-C), doxorubicin (Dox), or 5-fluorouracil (5-FU), or a combination of a radiotherapeutic and a chemotherapeutic.

**[0079]** The invention relates to the claimed use for preserving progenitor cells *in vivo*, comprising administering to a patient a therapeutically effective amount of a composition of a FRIL family member for a therapeutically effective period of time, wherein the progenitor cells in the patient are rendered quiescent "FRIL family member" and "progenitor cell" are as described above for the first aspect of the invention. "Therapeutically effective amount" is as described for the third aspect of the invention.

**[0080]** By "therapeutically effective period of time" is meant treatment for a period of time effective to preserve progenitor cells. Where administered to a patient, preferably, such administration is systemic (*e.g.*, by intravenous injection). Effective amounts and effective periods of time can be determined using the models and assays described herein. For example, the examples below describe the preservation of progenitor cells that have SCID-reconstituting ability. In accordance with the invention, a therapeutically effective period of time is injecting a therapeutically effective amount of a composition and/or pharmaceutical formulation of a FRIL family member between about 5 days before the patient

receives treatment with a therapeutic treatment (e.g., a chemotherapeutic) having a progenitor cell-depleting activity to about 2 hours prior to treatment with the therapeutic treatment, wherein the therapeutically effective amount of a composition and/or pharmaceutical formulation of the FRIL family member is administered daily. In accordance with the invention, a preferred therapeutically effective period of time is injecting a patient (e.g., a cancer patient) with a therapeutically effective amount of a composition and/or pharmaceutical formulation of a FRIL family member between about 2 days before the patient receives treatment with a therapeutic treatment (e.g., a chemotherapeutic) having a progenitor cell-depleting activity to about 1 day prior to treatment with the therapeutic treatment, wherein the therapeutically effective amount of a composition and/or pharmaceutical formulation of a FRIL is administered daily. It will be understood that once the patient starts to receive treatment with a therapeutic treatment having a progenitor cell-depleting activity, the therapeutically effective amount of a composition and/or pharmaceutical formulation of a FRIL family member may be different from the therapeutically effective amount of the a composition and/or pharmaceutical formulation of a FRIL family member that the patient received prior to receiving treatment with the therapeutic treatment [0081] Preferably, the patient is a human or a domesticated animal. "Domesticated animal" is as described in the invention.

[0082] In certain embodiments of the invention, the patient is a cancer patient. In some embodiments, the effective amount of the composition of the FRIL family member is administered prior to the treatment of the patient with a therapeutic treatment having a hematopoietic progenitor cell-depleting activity. In some embodiments, the therapeutic treatment is a radiotherapeutic or a chemotherapeutic treatment, including, without limitation, cytarabine (Ara-C), doxorubicin (Dox), or 5-fluorouracil (5-FU), or a combination of a radiotherapeutic and a chemotherapeutic.

[0083] The following examples are intended to further illustrate certain preferred embodiments of the invention. Embodiments in the following examples which are not within the scope of the claims are only for illustrative purposes.

#### EXAMPLE 1

##### Purification and Cloning of a FRIL family member, DI-FRIL

###### Purification of DI-FRIL from *Dolichos lab lab*

[0084] Seeds from the hyacinth beans (*Dolichos lab lab*) were purchased from Stokes Seeds (Buffalo, NY) and grown in a greenhouse. Dry seeds were ground in a coffee mill and the powder was extracted in 5 volumes of 50 mM Tris/HCl containing 1 mM each of MgCl<sub>2</sub> and CaCl<sub>2</sub> for 4 hours at 4°C. Bean solids were pelleted by centrifugation at 10,000 x g for 20 min. The pH of the supernatant was acidified to pH 4.0 with acetic acid, followed by a second centrifugation to clarify the supernatant, and finally the pH was readjusted to 8.0 with sodium hydroxide. This crude extract was stored at -20°C.

[0085] Single-step purification of the FRIL family member was achieved by binding to a mannose-Sepharose matrix (Sigma). The gel (i.e., matrix) was tumbled with the thawed crude extract for 4-12 hours at 4°C, carefully washed several times with 50 mM Tris/HCl containing 1 mM each of MgCl<sub>2</sub> and CaCl<sub>2</sub>, and then eluted with 20 mM α-methyl α-D-mannoside. Because this FRIL family member was isolated from *Dolichos lab lab*, it is referred to herein as DI-FRIL.

##### RNA Isolation and cDNA Synthesis of DI-FRIL

[0086] Total RNA was prepared from mid-maturatio *Dolichos lab lab* seeds stored at -70°C following the procedure of Pawloski et al. *Mol. Plant Biol. Manual* 5: 1- 13, 1994. Poly (A<sup>+</sup>) RNA was obtained from this total RNA using the PolyATract mRNA Isolation System (Promega) according to the manufacturer's instructions. Avian myeloblastosis virus reverse transcriptase (Promega) was used to generate cDNA from 0.5 μg poly(A<sup>+</sup>) RNA, or from 3.0 μg of total RNA, using 1 μg of oligo(dT) in standard reaction conditions (Sambrook et al., *Molecular Cloning. A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, 1989).

##### Polymerase Chain Reaction and cDNA Cloning of DI-FRIL

[0087] Based on the amino acid sequence published by Gowda et al., *J. Biol. Chem.* 269:18789-18793, 1994, two degenerate oligonucleotide primers were designed using *Phaseolus* codon usage (Devereux et al., *Nucleic Acids Res.* 12:387-394, 1984):

MLA AA(AG)TT(TC)GA(TC)CC(AT)AA(TC)CA(AG)GA(AG)GA (SEQ ID NO: 13)

MLZ TT(AT)CC(AG)TT(TC)TGCCA(AG)TCCCA

(SEQ ID NO: 14)

**[0088]** A 500+ bp product was amplified from cDNA prepared as described above, by 30 cycles of polymerase chain reaction (PCR), each cycle comprising 40 seconds at 94°C, 40 seconds at 50°C, 60 seconds at 72°C, followed by an extension step at 72°C for 10 min. Reactions were performed in 50 µL containing 30 pmol of each primer, 0.2 mM deoxyribonucleotides, and 0.5 unit of AmpliTaq polymerase (Perkin Elmer, Norwalk, CT) in the corresponding buffer.

**[0089]** The 500 bp product obtained by PCR was cloned in the cloning vector, pCR2.1 (Invitrogen, Carlsbad, CA), and sequenced by sequenase dideoxy chain termination (United States Biochemicals) using the following primers:

GTACCGAGCTCGGAT

(SEQ ID NO: 15)

TCTAGATGCATGCTCGAG

(SEQ ID NO: 16)

**[0090]** This sequence was designated "DI-FRILa," as relating to the gene encoding the protein of interest, designated "DI-FRIL" as noted above.

**[0091]** Based on the sequence of the DI-FRILa amplified product, a specific primer was prepared:

MLX GTTGGACGTCAATTCGGATGTG

(SEQ ID NO: 17)

**[0092]** A degenerate primer corresponding to the first five amino acids of the sequence published by Gowda et al., *J. Biol. Chem.* 269:18789-18793, 1994 was also prepared:

MLI GC(TC)CA(AG)TC(TC)CT(TC)TC(TC)TT

(SEQ ID NO: 18)

**[0093]** The MLX and MLI primers were used in combination to amplify a 480 bp product from cDNA prepared as described above, through 30 PCR cycles using the same conditions described above. This secondary amplified fragment was cloned in the pCR2.1 vector and sequenced as described above, and was designated "DI-FRILb."

**[0094]** The 3' end of *DI-FRIL* was obtained through rapid amplification of cDNA ends by polymerase chain reaction (RACE-PCR) (see, e.g., Frohman "RACE: Rapid amplification of cDNA ends," pp. 28-38 in *PCR Protocols: A Guide to Methods and Applications*, Innis MA, Gelfand DH, Sninsky JJ, and White TJ, eds., Academic Press, San Diego, 1990) using the 5'/3' RACEKTT (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. In the cDNA synthesis for the 3' RACE, an oligo(dT) anchor primer ("AP") supplied with the kit was used, at a concentration of 32.5 µM, using the standard conditions described earlier in this Example.

AP GACCACGCGTATCGATGTCGAC

(SEQ ID NO: 19)

**[0095]** Nested PCR amplifications were performed using the AP anchor primer in combination with a specific primer having the following sequence:

MLB AAGTTAGACAGTGCAGGAAAC

(SEQ ID NO: 20).

**[0096]** The amplification conditions were again 30 cycles of 40 seconds at 94°C, 40 seconds at 55°C, 60 seconds at 72°C each, with an extension step at 72°C for 10 min. A 900+ bp product was obtained, which was subcloned in pCR2.1 and sequenced as described above, and was designated "DI-FRILc" (SEQ ID NO: 1).

**[0097]** To obtain the full length cDNA clone, the anchor primer AP was used in combination with a specific primer corresponding to the first 5 amino acids encoded at the 5'-terminus:

MLII GCACAGTCATTGTCATTAG

(SEQ ID NO: 21).



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The full length cDNA was obtained through 30 cycles of PCR, each cycle comprising 60 seconds at 94°C, 60 seconds at 58°C, 90 seconds at 72°C, with an extension step at 72°C for 10 min. The reaction was performed in 100 µL containing 30 pmol of each primer, 0.2 mM deoxyribonucleotide, 1.0 unit of Pfu polymerase (Stratagene, La Jolla, CA). The MLII and AP primers were designed to generate an *EcoRI* site at each end (3' and 5') of the polynucleotide sequence. The full length cDNA was ligated into the *EcoRI* site of the cloning vector pCR2.1, resulting in the final product "pCR2.1-DLA" illustrated schematically in Fig. 1.

## The Nucleotide Sequence of *DI-FRIL*

**[0098]** The DI-FRILc clone was sequenced completely using the dideoxy chain termination method. The nucleotide sequence of the full-length cDNA was determined to be:

```

1    GCACAGTCAT TGTCATTTAG TTTCACCAAG TTTGATCCTA ACCAAGAGGA
51   TCTTATCTTC CAAGGTCATG CCACTTCTAC AAACAATGTC TTACAAGTCA
101  CCAAGTTAGA CAGTGCAGGA AACCTGTGA GTTCTAGTGC GGAAGAGTG
151  TTATATTCTG CACCATGCG CTTTGGGAA GACTCTGCGG TATTGACAAG
201  CTTTGACACC ATTATCAACT TTGAAATCTC AACACCTTAC ACTTCTCGTA
251  TAGCTGATGG CTTGGCCTTC TTCATTGCAC CACCTGACTC TGTCATCAGT
301  TATCATGGTG GTTTTCTTGG ACTCTTTCCC AACGCAAACA CTCTCAACAA
351  CTCTTCCACC TCTGAAAACC AAACCACCAC TAAGGCTGCA TCAAGCAACG
401  TTGTTGCTGT TGAATTTGAC ACCTATCTTA ATCCCGATTA TGGTGATCCA
451  AACTACATAC ACATCGGAAT TGACGTCAAC TCTATTAGAT CCAAGGTAAC
501  TGCTAAGTGG GACTGGCAAA ATGGGAAAAT AGCCACTGCA CACATTAGCT
551  ATAACCTCTGT CTCTAAAAGA CTATCTGTTA CTAGTTATTA TGCTGGGAGT
601  AAACCTGCGA CTCTCTCCTA TGATATTGAG TTACATACAG TGCTTCCTGA
651  ATGGGTCAGA GTAGGGTTAT CTGCTTCAAC TGGACAAGAT AAAGAAAGAA
701  ATACCGTTCA CTCATGGTCT TTCACTTCAA GCTTGTGGAC CAATGTGGCG
751  AAGAAGGAGA ATGAAAACAA GTATATTACA AGAGGCGTTC TGTGATGATA
801  TATGTGTATC AATGATTTTC TATGTTATAA GCATGTAATG TGCGATGAGT
851  CAATAATCAC AAGTACAGTG TAGTACTTGT ATGTTGTTTG TGTAAGAGTC
901  AGTTTGCTTT TAATAATAAC AAGTGCAGTT AGTACTTGT (SEQ ID NO:1)

```

**[0099]** The *DI-FRIL* nucleotide sequence enabled inference of the following derived amino acid sequence for the DI-FRIL protein:

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AQSLSFSFTK FDPNQEDLIF QGHATSTNNV LQVTKLDSAG NPVSSSAGRV
LYSAPLRLWE DSAVLTSFDT IINFEISTPY TSRIADGLAF FIAPPDSVIS
YHGGFLGLFP NANTLNNST SENQTTTKAA SSNVVAVEFD TYLNPDYGDP
NYIHIGIDVN SIRSKVTA KW DWQNGKIATA HISYNSVSKR LSVTSYYAGS
KPATLSYDIE LHTVLPWVR VGLSASTGQD KERNTVHSWS FTSSLWTNVA
KKENENKYIT RGVL (SEQ ID NO:2)

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**[0100]** The naturally-occurring signal sequence from the FRIL family member isolated from *Dolichos lab lab* (i.e., DI-FRIL) has the following sequence:

MASSNLLTLA LFLVLLTHAN SA (SEQ ID NO: 4)

**[0101]** This sequence is located directly N-terminal to the first amino acid of SEQ ID NO: 2. The nucleic acid sequence of the naturally-occurring DI-FRIL protein is provided below.

```

1  ATGGCTTCCT CCAACTTACT CACCCTAGCC CTCTTCCTTG TGCTTCTCAC
51  CCACGCAAAC TCAGCCGCAC AGTCATTGTC ATTTAGTTTC ACCAAGTTTG
101 ATCCTAACCA AGAGGATCTT ATCTTCCAAG GTCATGCCAC TTCTACAAAC
151 AATGTCTTAC AAGTCACCAA GTTAGACAGT GCAGGAAACC CTGTGAGTTC
201 TAGTGCGGGA AGAGTGTTAT ATTCTGCACC ATTGCGCCTT TGGGAAGACT
251 CTGCGGTATT GACAAGCTTT GACACCATTA TCAACTTTGA AATCTCAACA
301 CCTTACACTT CTCGTATAGC TGATGGCTTG GCCTTCTTCA TTGCACCACC
351 TGA CTCTGTC ATCAGTTATC ATGGTGGTTT TCTTGGACTC TTTCCCAACG
401 CAAACACTCT CAACAACCTT TCCACCTCTG AAAACCAAAC CACCACTAAG
451 GCTGCATCAA GCAACGTTGT TGCTGTTGAA TTTGACACCT ATCTTAATCC
501 CGATTATGGT GATCCAAACT ACATACACAT CGGAATTGAC GTCAACTCTA
551 TTAGATCCAA GGTAAC TGCT AAGTGGGACT GGCAAAATGG GAAAATAGCC
601 ACTGCACACA TTAGCTATAA CTCTGTCTCT AAAAGACTAT CTGTTACTAG
651 TTATTATGCT GGGAGTAAAC CTGCGACTCT CTCCTATGAT ATTGAGTTAC
701 ATACAGTGCT TCCTGAATGG GTCAGAGTAG GGTTATCTGC TTCAACTGGA
751 CAAGATAAAG AAAGAAATAC CGTTCACTCA TGGTCTTTCA CTTCAAGCTT
801 GTGGACCAAT GTGGCGAAGA AGGAGAATGA AAACAAGTAT ATTACAAGAG
851 GCGTTCTGTG ATGATATATG TGTATCAATG ATTTTCTATG TTATAAGCAT
901 GTAATGTGCG ATGAGTCAAT AATCACAAGT ACAGTG TAGT ACTTGATATGT
951 TCTTTGTGTA AGAGTCAGTT TGCTTTTAAT AATAACAAGT GCAGTTAGTA
1001 CTTGT (SEQ ID NO: 3)

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**[0102]** A comparative illustration of the derived DI-FRIL amino acid sequence with the reported amino acid sequence of the mannoside lectin as determined by Gowda et al. (*J. Biol. Chem.* 269:18789-18793, 1994) is shown in Fig. 2. The single sequence derived for DI-FRIL protein comprises domains that correspond directly and with substantial homology to the  $\alpha$  subunit (SEQ ID NO: 12) and  $\beta$  subunit (SEQ ID NO: 11) of the protein described by Gowda et al., *supra*. When the  $\beta$  subunit of the Gowda et al. (*supra*) protein is assigned to the N-terminal domain and is followed linearly by the  $\alpha$  subunit, the arrangement of the polypeptides shows homology to other legume lectins. The derived DI-FRIL amino acid sequence, however, comprises an additional of eight amino acid residues (aa27-34) that does not occur in the amino acid sequence described Gowda et al., *supra*. Several other differences between the amino acid sequences of DI-FRIL and the amino acid sequence described by Gowda et al., *supra*, are also readily discernible from Fig. 2.

#### Site-Specific Mutagenesis

**[0103]** To establish functionality of homologs of the protein encoded by the DI-FRIL cDNA, a mutation was made in the DI-FRIL cDNA clone. The domains of the derived protein and the pea lectin that include the mutation site are shown below:

DI-FRIL . Y L N P D Y G . D P N Y I H I G I D V

(SEQ ID NO: 22)

Pea

F Y . N A A W D P S N R D R H I G I D V

(SEQ ID NO: 25)

[0104] It is known that the asparagine residue (the highlighted "N") in the pea lectin is involved in binding to its saccharide ligand. The corresponding asparagine in DI-FRIL (position 141 of the amino acid sequence of SEQ ID NO: 2) was mutated to aspartic acid ("D"). This mutation was designated "N141D" for convenience.

[0105] To introduce the mutation, recombinant-PCR was performed (see, e.g., Higuchi, R, *PCR Protocols: A Guide to Methods and Applications*, Innis M.A., Gelfand D.H., Sninsky J.J., and White T.J, eds., Academic Press, San Diego, 1990). Two PCR reactions were carried out separately on the full length cDNA using two primers that contain the same mutation and produce two products with an overlapping region:

MutI

CCATAATCGGGATCAAGATAGGTG

(SEQ ID NO: 26)

MutII

CACCTATCTTGATCCCGATTATGG

(SEQ ID NO: 27)

The primary PCR products were purified with the QIAquick PCR Purification kit (QIAGEN, Valencia, CA), according to the manufacturer's instructions. The overlapping primary products were then combined and amplified together in a single second reaction using flanking primers:

M1 Forw . AACTCAGCCGCACAGTCATTGTCA

(SEQ ID NO: 28)

APEcoRI

GAATTCGACCACGCGTATCGATGTCGAC

(SEQ ID NO: 29)

[0106] Both the primary and the secondary PCR reactions were performed in 100  $\mu$ L containing 50 pmol of each primer, 0.4 mM deoxyribonucleotide and 1.0 unit Pfu polymerase (Stratagene) in the corresponding buffer. The primary PCR reaction amplified the two separate fragments in 30 cycles, each cycle comprising 40 seconds at 94°C, 40 seconds at 50°C, 60 seconds at 72°C, with an extension step at 72°C for 10 min. The second PCR reaction amplified the recombinant fragment in 12 cycles using the same conditions described above.

[0107] The resulting full-length fragment contained the mutation. The recombinant mutated product was cloned in the EcoRI site of the cloning vector pCR2.1, as illustrated schematically in Fig. 3, and sequenced as described above. This plasmid is referred to as "pCR2.1-DLA(D)."

#### Construction of DI-FRIL-Expressing Plant Expression Vectors and *Nicotiana tabacum* Transformation

[0108] Recombinant PCR was used to modify the 5' ends of both the wild-type and the mutant DI-FRIL clones, to introduce a signal peptide for entry of the protein into the endoplasmic reticulum. Following the procedure of Higuchi, *supra*, the sequence encoding the signal peptide and the full-length cDNA clones were amplified in two separate primary PCR reactions. The signal peptide sequence was obtained from the amplification of the binary vector pTA4, harboring the complete sequence of the  $\alpha$ -amylase inhibitor gene (Hoffman et al., *Nucleic Acids Res.* 10:7819-7828, 1982; Moreno et al., *Proc. Natl. Acad. Sci. USA* 86:7885-7889, 1989).

[0109] The following primers were used for amplification of the signal peptide sequence:

Sigforw

GAATTCATGGCTTCCTCCAAC

(SEQ ID NO: 30)

Sigrev

TGA CTGTGCGGCTGAGTTT GCGTGGGTG

(SEQ ID NO: 31)

[0110] The primers M1Forw (SEQ ID NO: 28) and APEcoRI (SEQ ID NO: 29) used for amplification of the DI-FRIL cDNA described above, were again used to amplify the DI-FRIL cDNA.

**[0111]** The primers used for the secondary reactions were Sigforw and APEcoRI, which were designed to generate *EcoRI* sites at the 5' and the 3' ends. Both the primary and the secondary PCR reactions were performed as discussed above for the site-directed mutagenesis.

**[0112]** The wild-type recombinant product SpDLA was cloned in the *EcoRI* site of the pBluescript SK+ cloning vector (Stratagene) to give the vector pBS-SpDLA, as shown in Fig. 4. The mutant SpDLA(D) was cloned in the same site of the cloning vector pCR2.1 to give the vector pCR2.1-SpM1, as shown in Fig. 5. The nucleotide sequence of each PCR product was determined as described above to verify the correct attachment of the signal peptide. The nucleotide sequence of SpDLA is defined by SEQ ID NO:22, and the derived amino acid sequence is defined by SEQ ID NO:23.

**[0113]** The sequences of SEQ ID NO: 22 and SEQ ID NO: 23 are as follows:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3

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atggettcct ccaacttact caccctagcc ctcttccttg tgcttctcac ccacgcaaac 60
tcagccgcac agtcattgtc atttagtttc accaagtttg atcctaacca agaggatctt 120
atcttccaag gtcattgccac ttctacaaac aatgtcttac aagtcaccaa gttagacagt 180
gcaggaaacc ctgtgagttc tagtgcgga agagtgttat attctgcacc attgcgcctt 240
tggaagact ctgcggtatt gacaagcttt gacaccatta tcaactttga aatctcaaca 300
ccttacactt ctcgtagc tgatggcttg gccttcttca ttgcaccacc tgactctgtc 360
atcagttatc atgggtggtt tcttggaact tttcccaacg caaacactct caacaactct 420
tccacctctg aaaaccaaac caccactaag gctgcatcaa gcaacgttgt tgctgttgaa 480
tttgacacct atcttaatcc cgattatggt gatccaaact acatacacat cggaattgac 540
gtcaactcta ttagatccaa ggtaactgct aagtgggact ggcaaaatgg gaaaatagcc 600
actgcacaca ttagctataa ctctgtctct aaaagactat ctgttactag ttattatgct 660
gggagtaaac ctgcgactct ctctatgat attgagttac atacagtgtc tcctgaatgg 720
gtcagagtag ggttatctgc ttcaactgga caagataaag aaagaaatac cgttcactca 780
tggtctttca cttcaagctt gtggaccaat gtggcgaaga aggagaatga aaacaagtat 840
attacaagag gcgttctgtg atgatatatg tgtatcaatg attttctatg ttataagcat 900
gtaatgtgcg atgagtcaat aatcacaagt acagtgtagt acttgtatgt tgtttgtgta 960
agagtcagtt tgcttttaat aataacaagt gcagttagta cttgt 1005

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:



**[0116]** Recombinant DI-FRIL fusion proteins were cleaved by the transformed plant cells *in vivo*. Thus, the transformed plant cells produced mature DI-FRIL which, when purified, had a molecular mass of 60 kDa and comprised four subunits, two alpha subunits and two beta subunits (*i.e.*, an  $\alpha_2\beta_2$  heterodimer), where each subunit is about 15-18 kDa.

#### Expression of Recombinant DI-FRIL in *E. coli*

**[0117]** The DI-FRIL wild-type cDNA and mutant clones (without signal peptides), were ligated into the *EcoRI/SalI* and *EcoRI/XhoI* of the expression vector pGEX 4T-1 (Pharmacia Biotech, Uppsala, Sweden), to form the expression constructs pGEX-M1 and pGEX M1(D), respectively illustrated in Figs. 9 and 10. The host *E. coli* strain, BL21(D3), was purchased from Novagen (Madison, WI, and transformed with the above construct using the calcium chloride method (see Sambrook et al., *supra*; Gelvin and Schilperoort, *Plant Molecular Biology Manual*, Kluwer Academic Publishers, Dordrecht, The Netherlands, 1988; Altabella et al., *Plant Physiol* 93:805-810, 1990; and Pueyo et al., *Planta* 196:586-596, 1995). The induction of the tac promoter (Ptac) was achieved by adding IPTG (isopropyl-  $\beta$ -D-thiogalactopyranoside) (Sigma) at a 1.0 mM final concentration when the cells reached an optical density of 0.4 - 0.6 at 600 nm. The cultures were allowed to grow for 12 hours at 37°C after the addition of IPTG. Control non-induced cultures were maintained under similar conditions. The cells were lysed by treatment with 4 mg/mL lysozyme in phosphate-buffered saline containing 1% TRITON® X-100.

**[0118]** Total cellular protein was extracted from transformed *E. coli* cells and analyzed on SDS-PAGE on a 15% gel using a standard procedure (Sambrook et al., *supra*). The cells from 1 mL of *E. coli* culture were suspended in the same volume of loading buffer (50 mM Tris HCl pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, 0.1% bromophenol blue) and vortexed. Following transfer to a nitrocellulose membrane, protein was stained with Coomassie Brilliant Blue R250. A representative separation is shown in Fig. 11, with the lanes identified in Table 1, below.

**TABLE 1**  
Key to Fig. 11

Lane No.	Content
1	Molecular Mass Marker (Bio-Rad)
2	Total Protein Extract from Non-Induced BL21(D3) pGEX-M1
3	Total Protein Extract from Induced BL21(D3) pGEX-M1
4	Total Protein Extract from Non-Induced BL21(D3) pGEX-M1(D)
5	Total Protein Extract from Induced BL21(D3) pGEX-M1(D)

**[0119]** The separation of proteins in Fig. 11 shows that the induced cells (lanes 3, 5) both produced an abundant polypeptide having a molecular mass of about 60 kDa (indicated by arrow). The non-induced cells failed to produce any significant amount of this protein (Fig. 11, lanes 2, 4).

#### Purification of Recombinant DI-FRIL from Transformed *E. coli*

**[0120]** Induced *E. coli* cells (200 mL) as described above were harvested after 12 hour induction at 37°C by centrifugation at 5000 g for 10 min. The pellet was washed with 50 mM Tris-HCl pH 8.0, 2 mM EDTA, and resuspended in 1/10 vol of 1% TRITON surfactant in TBS (20 mM Tris pH 7.5, 500 mM NaCl). The cells were lysed by adding 4 mg/mL of lysozyme and incubating at room temperature for 30-60 min. After centrifugation at 5000 g, the supernatant containing the total soluble proteins was discarded and the resulting pellet, comprising the inclusion bodies and containing the accumulated the recombinant fusion protein, was extracted with 8 M guanidine-HCl (Martson and Hartley, "Solubilization of Protein Aggregates" in *Guide to Protein Purification* vol. 182, eds. Deutscher, M.P., pp 266-267, 1993).

**[0121]** The recombinant fusion protein solubilized by guanidine-HCl was purified on GST-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden) according the manufacturer's instructions and eluted in 1 mL of reduced glutathione

(Sigma). Samples of the purified fusion proteins were cleaved with thrombin (Novagen) using 5 cleavage units/mL purified fusion protein.

[0122] For immunoblot analysis (Western blot), the purified proteins were separated by SDS-PAGE in general accordance with the method described above. The gel was equilibrated in transfer buffer (25 mM Tris pH 8.3, 192 mM Glycine, 20% MeOH) and blotted onto nitrocellulose (Bio-Rad, Hercules, CA) for 1 hour at 100 V using a Bio-Rad electrotransfer apparatus. Non-specific binding was blocked by incubating the blots for at least 1 hour in 1X TBS (20 mM Tris pH 7.5, 500 mM NaCl) containing 3% gelatin. Blotting was followed by incubation with a primary antibody (a polyclonal rabbit serum raised against the N-terminal peptide of the  $\beta$ -subunit of *Phaseolus vulgaris* FRIL (*i.e.*, Pv-FRIL), 1:100 dilution, 3 hours; described below in Example 5), followed by incubation with a secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase at 1:1000 dilution for 1 hour). The blots were washed and the color developed with the color development reagent (Bio-Rad). A representative result is shown in Fig. 12, with the lanes identified in Table 2, below.

**TABLE 2**  
Key to Fig. 12

Lane No.	Content
1	Purified Fusion Protein M1
2	Purified Fusion Protein M1(D)
3	Control
4	Purified Fusion Protein M1 After Cleavage with Thrombin
5	Purified Fusion Protein M1(D) After Cleavage with Thrombin
6	Control

[0123] The separation shown in Fig. 12 demonstrates that the two forms of fusion protein have similar molecular masses of about 60 kDa, and that thrombin cleaved both types of fusion protein to produce a new polypeptide of molecular mass 30 kDa.

## EXAMPLE 2

### Recombinant DI-FRIL Specifically Stimulates Proliferation of 3T3 Cells Expressing the FLT3 Receptor

[0124] DI-FRIL interacts with the mammalian FLK2/FLT3 tyrosine kinase receptor. A specific and quantitative biological assay using NIH 3T3 fibroblasts transfected either with a climeric receptor having the extracellular portion of the murine FLT3 receptor combined with the intracellular portion of the human Fms receptor (Dosil et al., *Mol. Cell. Biol.* 13(10):6572-6585 1993) or with the full length human receptor (Small et al., *Proc. Natl. Acad. Sci. USA* 91:459-463, 1994) can be used to evaluate lectin biological activity during purification. Serial two-fold dilutions of lectin samples across rows of a 96 well plate allowed for greater than a thousand-fold range to access FLT3 3T3 biological activity. Either the murine or human FLT3 ligand (FL) (Lyman et al., *Cell* 75:1157-1167, 1993; Hannum et al., *Nature* 368: 643-648, 1994) or the FRIL was found to rescue FLT3-transfected cells from death in this assay.

[0125] Specifically, 3T3 cells cultured in tissue culture plates (Becton Dickinson Labware, Lincoln Park, NJ) were removed from the plates by washing cells twice in Hank's buffered saline solution (HBSS; Gibco Laboratories, Grand Island, NY). Non-enzymatic cell dissociation buffer (Gibco) was added for 15 minutes at room temperature. The resulting cells were washed in medium. FLT3 3T3 cells were cultured at a final concentration of 3,000 cells per well in a volume of 100  $\mu$ L of serum-defined medium containing 10 mg/mL rhIL-1 $\alpha$ , 10% AIMV (Gibco, Grand Island, NY) and 90% Dulbecco's modification of Eagle's medium (DMEM; Gibco) in 96 well plates. Under these assay conditions, cells die after two to four days of culture in a humidified incubator at 37°C and 5% CO<sub>2</sub> unless exogenously added ligand rescues cells from death. Each 96 well plate contained wells of cells containing calf serum, which stimulates all 3T3 cells, as a positive control and wells of cells containing medium only as a negative control ("blank"). Full-length Fms-transfected 3T3 cells (biological response shown in Tessler et al., *J. Biol. Chem.*, 269:12456-12461, 1994) served as receptor-transfected control target cells, and parent 3T3 cells served as untransfected control cells. Proliferation and

cell survival was quantitated by addition of XTT (2, 3-bis[Methoxy-4-nitro-5sulfophenyl]-2H-tetrazolium-5 carboxanilide inner salt) (Diagnostic Chemicals Ltd, Charlottetown, Prince Edward Island, Canada), which is a tetraformazan salt cleaved by actively respiring cells (Roehm et al., *J. Immunol. Methods* 142: 257-265, 1991). Proliferation and cell survival was quantitated spectrophotometrically using a Vmax kinetic plate reader (Molecular Devices Corp., Mountain View, CA), and recorded as either relative activity (units/mL) or as specific activity (units/mg). One unit of biological activity was defined as the reciprocal dilution at which half-maximal stimulation of cells is detected.

**[0126]** The crude protein extract from the *E. coli* cultures described in Example 1, above, was tested to determine whether expressed recombinant DI-FRIL possessed any capacity to stimulate FLT3 3T3 cells using this assay. The data from this experiment are summarized in Figs. 13A and 13B. Specifically, Fig. 13A is a graph showing that the crude extract of the *E. coli* culture containing expressed DI-FRIL specifically stimulates hFLT3 cells; Figure 13B is a graph showing that the same extract does not stimulate untransfected 3T3 cells. In Figs. 13A and 13B, medium control is represented by a solid line. The ordinate (absorbance) indicates cell viability measured by XTT at three days; the abscissa shows the reciprocal dilution of the extract sample. The apparent inhibition of proliferation observed at higher concentrations (Fig. 13A) is not understood, but may relate to toxic components in the crude *E. coli* extract or the consequences of dose-related preservation of the 3T3 fibroblasts.

### EXAMPLE 3

#### Recombinant DI-FRIL Preserves Mononuclear Cells and Progenitors in Liquid Culture

**[0127]** The recombinant DI-FRIL protein preserved functional progenitors for at least four weeks in liquid culture. Figs. 14A and 14B, and Table 3 illustrate the results of experiments in which recombinant DI-FRIL was shown to act in a dose-responsive manner to preserve human cord blood progenitors.

**[0128]** To do this, umbilical cord blood from healthy donors was collected in 100 units/ml of heparin. Cord blood mononuclear cells (CB mnc) were isolated within 4 hours of collection by FICOLL-PAQUE® separation (Pharmacia Biotech, Piscataway, NJ) following manufacturer's instruction and washed in X-VIVO 10 medium (BioWhittaker, Walkersville, MD). CB mnc were then cultured in six well tissue culture plates (Corning Inc., Corning, NY) at a concentration of 200,000 cells/mL in a volume of 4 mL of X-VIVO 10 (*i.e.*, 800,000 cells total per well). DI-FRIL and/or recombinant *E. coli* Flt3-L (recFL; BioSource International, Camarillo, CA) were added at a concentration of 40 ng/ml at the outset (with no addition as a control). Cultures were incubated in humidified chambers without medium changes for up to 29 days.

**[0129]** After incubation, the cultured CB mnc cells were harvested by washing in X-VIVO 10 (*i.e.*, harvested cells were pelleted and resuspended in X-VIVO 10) to remove the DI-FRIL and/or recFL, and then determining viable cell number by trypan blue (Sigma) exclusion. These results are shown in Fig. 14A. The progenitor number and capacity of harvested cells were assessed by plating the washed cells in triplicate in fetal bovine serum-free, methylcellulose colony assay medium containing IL-2, granulocyte-macrophage CSF, and kit ligand (StemCell Technologies, Vancouver, BC, Canada). After two weeks, the resultant colonies from each of the triplicate wells were scored and the results are shown in Fig. 14B. Thus, Figs. 14A and 14B show that recombinant DI-FRIL preserved cord blood mononuclear cells and progenitors in a dose-responsive manner in liquid culture.

**[0130]** Table 3 shows the resulting colonies after 15, 21, or 29 days of incubation, demonstrating that DI-FRIL, but not recFL, preserved progenitors in suspension culture.

TABLE 3

Day	Medium	Myeloid*	Erythroid*	Mix*	Blast*
15	DI-FRIL	1,033 ± 12	67 ± 12	7 ± 12	0
	rccFL	40 ± 69	0	0	0
	DI-FRIL + recFL	933 ± 250	167 ± 95	0	0
	No Addition	0	0	0	0
21	DI-FRIL	387 ± 83	7 ± 12	0	167 ± 64
	recFL	0	0	0	0
	DI-FRIL + recFL	473 ± 133	53 ± 42	0	300 ± 34
	No Addition	0	0	0	0

\*Data reported as ± SD of the three values from the triplicate methylcellulose colony assay.



TABLE 3 (continued)

Day	Medium	Myeloid*	Erythroid*	Mix*	Blast*
29	DI-FRIL	0	0	0	80 ± 72
	recFL	0	0	0	0
	DI-FRIL + recFL	0	0	0	40 ± 20
	No Addition	0	0	0	0

\*Data reported as ± SD of the three values from the triplicate methylcellulose colony assay.

**[0131]** The reported experiment is a representative of four experiments.

**[0132]** In Figs. 14A and 14B and in Table 3, "blast" refers to colonies consisting of primitive, morphologically undifferentiated cells; "mix" refers to colonies consisting of myeloid and erythroid cells; "erythroid" refers to colonies consisting of erythroid cells; and "myeloid" refers to colonies consisting of myeloid cells. In Figs. 14A and 14B, cell number (Fig. 14A) or colony number (Fig. 14B) is shown on the ordinate; the abscissa shows the reciprocal dilution of the sample.

**[0133]** As shown in Table 3, colonies derived from mature myeloid and erythroid progenitors formed from cells cultured for 15 days in either FRIL or FRIL plus recFL; 24-fold fewer mature colonies formed from cells cultured in recFL alone; and no colonies appeared if neither was present. After 21 days in culture, Table 3 shows that myeloid and erythroid colonies formed only from cells exposed to DI-FRIL. The frequency of myeloid colonies in DI-FRIL-only cultures (based on the initial number of CB mnc) decreased by 2.7 fold from 1 in 774 after 2 weeks in culture to 1 in 2,067 after 3 weeks; erythroid colonies decreased in frequency by 9.6 fold from 1 in 11,940 to 1 in 114,287 (see Table 3).

**[0134]** The colonies from this assay were photographed at various time points. As shown in Fig. 15A, in addition to myeloid and erythroid colonies, day 21 cultures contained small colonies consisting of undifferentiated cells. Fig. 15B shows that only blast-like colonies were observed when the cells were cultured in DI-FRIL for 29 days (see also Table 3).

**[0135]** The progenitor capacity of the blast-like colonies was examined further for cells initially cultured for 3 weeks in either DI-FRIL, recFL, no addition, or DI-FRIL + recFL, and then without these regulators for an additional 6 weeks in a methylcellulose colony assay. No colonies were detected from the cells cultured for 21 days in either recFL alone or medium control. Viable cells were harvested from the cells initially cultured in DI-FRIL alone and replated in an colony assay (in methylcellulose colony assay medium) for an additional 4 weeks. A schematic diagram of this experiment is shown in Fig. 16. As schematically diagrammed in Fig. 16, the frequency of blast-like colonies cultured in DI-FRIL alone decreased by 2.1 fold from day 21 to day 29, from 1 in 4,790 to 1 in 10,000, and 7.5 fold in DI-FRIL + recFL cultures from 1 in 2,667 to 1 in 20,000 of the initial CB mnc cells cultures. Following the protocol schematically diagrammed in Fig. 16, small, diffuse, blast-like colonies were detected at a frequency of 1 in 67 (900 colonies/600,000 CB mnc) exclusively in dishes of cells initially cultured in DI-FRIL alone, and at a frequency of 1 in 132 (990 colonies/131,000 CB mnc) for cells cultured in DI-FRIL + recFL (see exemplary colonies in Fig.17).

#### EXAMPLE 4

##### Recombinant DI-FRIL Acts Directly on Progenitor Cells

**[0136]** To assess whether recombinant DI-FRIL acts directly or indirectly through accessory cells to preserve progenitor cells, cord blood mononuclear cells were first enriched for progenitors expressing the CD34 antigen by immunomagnetic bead isolation (Dynal Corp., Lake Success, NY). Five hundred CD34<sup>+</sup> cells were placed into wells containing 100 µL of serum-free medium (BIT9500, StemCell Technologies, Vancouver, BC, Canada) either in the presence of recFL (PeproTech, Princeton, NJ) or a cytokine cocktail of recombinant human interleukin 3 (rhIL3) + recombinant human interleukin 6 (rhIL6) + recombinant human interleukin 11 (rhIL11) + rhTpo Thrombopoietin + FL (FLT3-Ligand) (BioSource International, Camarillo, CA) in 96-well plates and cultured for four weeks without medium changes. The numbers of functional progenitors from these cultures were assessed by plating cells in complete serum-free methylcellulose colony assay medium (StemCell Technologies). After two weeks, the resultant colonies were scored and the results are shown in Fig. 18 (solid bars = recombinant DI-FRIL; open bars = cytokine cocktail). Clearly, progenitors were preserved only in the recombinant DI-FRIL-containing cultures (Fig. 18). Thus, purified recombinant DI-FRIL acts directly on primitive hematopoietic progenitors.

**EXAMPLE 5**Identification and Cloning of Pv-FRIL, a second FRIL family memberFRIL activity in PHA-LCM media

**[0137]** A biological screening assay to search for novel stimulators of the Flt3 receptor was developed using NIH 3T3 cells transfected with expression vectors containing cDNA of murine and human Flt3 and the related Fms receptor. The mFlt3/Fms 3T3 cell line is a 3T3 cell line transfected with nucleic acid encoding a fusion protein consisting of the murine extracellular domain of the Flt3 receptor fused to the transmembrane and intracellular domains of the human Fms (provided by Dr. Ihor Lemischka, Princeton University, Princeton, NJ). The Stk 3T3 cell line is a 3T3 cell line transfected with the full-length human Flt3 receptor (provided by Dr. Donald Small, Johns Hopkins University, Baltimore, MD). The human FMS 3T3 cell line is a 3T3 cell line transfected with the full-length human Fms receptor (provided by Dr. Charles Sherr, Saint Jude Children's Research Hospital, Memphis, TN). Parent 3T3 cells were purchased from American Type Culture Collection ("ATCC"; Manassas, VA). Receptor-transfected cells contained Neo resistance genes and were maintained in medium containing 750 g/ml of G418 (Life Technologies, Rockville, MD).

**[0138]** To create factor-dependence for receptor-transfected 3T3 cells, growth conditions were compromised to permit only ligands to rescue cells from death. To do this, 3T3 cell lines were assayed in 96 well plates (Becton Dickinson Labware, Lincoln Park, NJ) containing 3,000 cells in 100  $\mu$ L of serum-free medium consisting of 10% AIMV (Life Technologies) and 90% DMEM. In each experiment, samples were serially diluted two-fold across rows starting at a 1:10 dilution. Viable cells were quantitated after 3-5 days by XTT (2,3-bis[Methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt) (Sigma, St. Louis, MO) (Roehm et al., *supra*). Relative activity (units/ml) and specific activity (units/mg) are defined as the reciprocal dilution at which half-maximal stimulation of cells was detected.

**[0139]** The FMS 3T3 cells (transfected with cDNA encoding the human Fms tyrosine kinase receptor) and its ligand, human M-CSF, served as a model system. Recombinant human M-CSF stock of 1  $\mu$ g/mL was serially diluted (where the first dilution, 1:20, was 50 ng/mL) was used to stimulate Fms3T3 cells. As shown in Fig. 19A, Fms 3T3 cells responded to M-CSF in a dose-responsive manner. Neither the mFlt3/Fms 3T3 cells nor the parent untransfected 3T3 cells responded to M-CSF (Fig. 19A).

**[0140]** Various sources of conditioned medium were screened for the presence of Flt3 3T3 stimulatory activity. The most potent source was conditioned medium harvested from human peripheral blood cells activated to secrete high levels and a broad range of cytokines by the mitogenic legume lectin, phytohemagglutinin (PHA), which is derived from impure red kidney bean extracts. This source, commonly called PHA leukocyte-conditioned medium (PHA-LCM), has been used as a standard positive control in hematopoietic colony assays for over two decades (Sharon and Lis, *Science* 246: 227, 1989). To make PHA-LCM, leukopherized blood from normal volunteers was purchased from North American Biologicals Inc., Miami, FL. Mononuclear cells were isolated by FICOLL-PAQUE®, washed in AIMV, and cultured at a concentration of  $2 \times 10^6$  cells/ml in AIMV containing a 1% volume of crude red kidney bean extract containing PHA from Life Technologies (catalog number 10576-015) in either T150 flasks (Becton Dickinson Labware, Lincoln Park, NJ) or roller bottles (Becton Dickinson Labware) for one week. Cells and debris were removed by centrifugation and conditioned medium was stored at -20°C.

**[0141]** PHA-LCM induced proliferation of mFlt3/Fms 3T3 cells and Stk 3T3 (expressing the human Flt3 receptor) in an indistinguishable manner at approximately 200 units/mL (Fig. 19B). Untransfected 3T3 cells did not respond to PHA-LCM (Figure 19B) and Fms 3T3 cells responded weakly (data not shown).

Purification of Pv-FRIL from PHA-stimulated Leukocyte Conditioned Media

**[0142]** Each batch of PHA-LCM was generated in serum-free medium with cells from individual normal donors. To start purification, the PHA-LCM with Flt3 3T3 activity was pooled in 30 liter lots with approximately  $10^7$  Flt3 3T3 units. Twenty-five liters of PHA-LCM was diafiltered into 50 mM Tris-HCl, pH 7.6, 50 mM NaCl and then concentrated to 2-2.5 liters by tangential flow ultrafiltration on a 10 kDa molecular weight cutoff membrane (Pellicon, Millipore, Bedford, MA). A Blue-sepharose FF (Pharmacia Biotech) column (10 cm x 15 cm) was equilibrated with 50 mM Tris-HCl, pH 7.4, 50 mM NaCl. To remove the human serum albumin, concentrated PHA-LCM was applied to the column at 25 ml/min by pump and the flow through was collected. The flow-through fraction from Blue-sepharose was subjected to anion exchange chromatography by being applied to a Q-sepharose FF (Pharmacia Biotech) column (5 cm x 5 cm) equilibrated with 10 mM Tris-HCl, pH 7.6. The column was washed with equilibration buffer and then eluted at 12 ml/min with a continuous gradient of 0-0.7 M NaCl in 10 mM Tris. Fractions were collected (6 ml/fraction) and an aliquot of each fraction was tested for Flt3 3T3 activity (*i.e.*, an ability to stimulate mFlt3/fms 3T3 cells and/or Stk 3T3 cells) in 96 well plates.

**[0143]** After validation that a fraction had Flt3 3T3 activity, a phenyl-sepharose HP (Pharmacia Biotech) column (1.6

cm x 10 cm) was equilibrated with 20 mM phosphate, pH 7, 1.5 M  $\text{NH}_4\text{SO}_4$ . The pooled sample from Q-sepharose was adjusted to 1.5 M  $\text{NH}_4\text{SO}_4$  and applied to the phenyl-sepharose column. The gradient of 15-0.1 M  $\text{NH}_4\text{SO}_4$  in 20 mM phosphate, pH 7. Fractions (1 ml) were collected and tested for Flt3 3T3 activity. Fractions with Flt3 3T3 activity

were pooled and dialyzed against 50 mM Tris-HCl, pH 7.2, 100 mM NaCl and concentrated by vacuum centrifugation.

**[0144]** A Superdex 75 (Pharmacia Biotech) column (1.6 cm x 60 cm) was equilibrated with 50 mM Tris-HCl, pH 7.4, 100 mM NaCl. The pooled sample from phenyl-sepharose was applied to the Superdex 75 column and eluted at 0.6 ml/min. Fractions (1.8 ml) were collected and tested for Flt3 3T3 activity. The active fractions were dialyzed against Tris-HCl, pH 7.2 and concentrated by vacuum centrifugation.

**[0145]** A C4 reverse-phase column (4.6 mm x 100 mm, Vydac, Hesperia, CA) was equilibrated with 0.1% trifluoroacetic acid (TFA) in HPLC grade  $\text{H}_2\text{O}$ . Pooled and concentrated sample from Superdex 75 chromatography was applied to the C4 column and the column was eluted with a gradient of 10-55% acetonitrile, 0.1% TFA over 70 min at 0.5 ml/min. Fractions of 0.5 mL were collected and evaporated by vacuum centrifugation.

**[0146]** Analysis by ELISA for cytokines (IL1- $\alpha$ , IL1- $\beta$ , IL2, IL3, IL4, IL6, GM-CSF, G-CSF and SCF) during purification of Pv-FRIL were performed using kits purchased from R&D Systems (Minneapolis, MN).

#### Pv-FRIL Specific Rabbit Anti-Serum

**[0147]** Throughout purification of Pv-FRIL a New Zealand White rabbit (HRP, Denver, PA) was immunized with crude PHA-LCM, boosted with increasingly purified samples containing Flt3 3T3 activity, and finally immunized with a peptide corresponding to Pv-FRIL AQSLSF[N, C, S]FTKFDLD (SEQ ID NOS: 32-34), referred to as the AQS-peptide. Samples were glutaraldehyde conjugated to keyhole limpet hemocyanin (KLH, Sigma). The rabbit was immunized with KLH-AQS peptide-containing samples using either Complete Freund's Adjuvant (Sigma) or Hunter's Titermax (Vaxcel, Inc., Norcross, GA). Antiserum demonstrated a 1:5,000 titer to the AQS peptide in an ELISA (data not shown). Since the antiserum contained reactivities to other proteins, further enrichment for AQS peptide-specific antibodies was achieved by either depletion of cross-reactive antibodies or by affinity purification using a AQS peptide covalently linked to an agarose support (AminoLink coupling gel, Pierce).

**[0148]** An anti-AQS affinity column was prepared either by purifying IgG from high titer rabbit antiserum by protein A affinity chromatography (ImmunoPure kit, Pierce) or antibody isolated from the AQS peptide column and then covalently linking antibody to an activated agarose support (AminoLink coupling gel, Pierce).

#### Activity of Purified Pv-FRIL

**[0149]** To relate the observations of Pv-FRIL's activity on receptor-transfected 3T3 cells to Flt3 receptor-expressing hematopoietic progenitors, a suspension culture of human cord blood cells enriched for Flt3<sup>+</sup> progenitors by CD34 immunomagnetic bead selection was adapted to a 96 well plate format. To do this, umbilical cord blood from healthy donors was collected in 100 units/ml of heparin (Fujisawa Healthcare, Deerfield, IL). Mononuclear cells were isolated by FICOLL-PAQUE® (Pharmacia Biotech, Piscataway, NJ), washed in HBSS, and resuspended in serum-defined medium, either AIMV or XVIVO-10 (BioWhittaker, Walkersville, MD). Mononuclear cells were enriched for Flt3<sup>+</sup> progenitors by CD34 immunomagnetic bead selection (DynaL Corporation, Lake Success, NY). CD34<sup>+</sup> cells were cultured in 6 well plates (Becton Dickinson Labware) in at a concentration of  $10^5$  cells in 1 mL of DMEM containing 10 ng/mL recombinant human IL3 (BioSource International, Camarillo, CA) and 10% fetal calf serum. The number of refractive cells present in culture wells was scored microscopically.

**[0150]** Culture medium always contained IL-3 since early-acting cytokines require additional co-factor(s) for survival and proliferation. Fig. 20A shows that cord blood cells responded to column fractions in two regions of the material eluted from an anion exchange column. The first region of activity corresponded with Flt3 3T3 stimulatory activity (Figs. 20B and 20C); the second associated with an activity detected with Fms 3T3 cells (Fig. 20D); no response was detected in untransfected 3T3 cells (data not shown). The active material, corresponding to Flt3 3T3 activity (peak one in Fig. 20A), was further characterized and purified on different chromatographic matrices including a cation exchange resin, heparin sepharose, hydroxyapatite, ConA-sepharose, phenyl sepharose, and gel filtration (Superdex 75) (data not shown).

**[0151]** The further purified Pv-FRIL was used in the Flt3 3T3 assay described above. Plateau stimulation of Flt3 3T3 cells decreased with sequential purification steps (see Fig. 21A), suggesting removal of essential co-factor(s). Addition of suboptimal levels of crude PHA-LCM to Pv-FRIL obtained in the later stages of purification, restored activity of this partially purified Pv-FRIL to maximal plateau levels (Fig. 21B).

**[0152]** Analysis by ELISA for cytokines that act on hematopoietic progenitors (interleukin 1- $\alpha$  (IL1- $\alpha$ ), interleukin 1- $\beta$  (IL1- $\beta$ ), interleukin 2 (IL2), interleukin 3 (IL3), interleukin 4 (IL4), interleukin 6 (IL6), granulocyte-macrophage colony stimulating factor (GM-CSF), G-CSF (granulocyte colony stimulating factor), and stem cell factor (SCF)) in fractions containing Pv-FRIL purified to near homogeneity revealed that IL1- $\alpha$  had remained with Pv-FRIL through every step

of purification (data not shown).

[0153] Flt3 3T3 activity was depleted but not eliminated either by adding neutralizing antibodies to IL1 or by removing IL1 by antibody affinity chromatography (data not shown). However, at the levels (<1 ng/mL) found in fractions containing purified Pv-FRIL, exogenous recombinant hIL1- $\alpha$  by itself had no stimulatory activity (data not shown). This observation suggested the possibility that IL1- $\alpha$  may act as a necessary co-factor to obtain maximal stimulation by Pv-FRIL. In subsequent experiments when testing purified Pv-FRIL, the co-factor requirement was met by the addition of either IL1- $\alpha$  or PHA-LCM added at a concentration that did not stimulate Flt3 3T3 cells by itself.

[0154] Using this modified Flt3 3T3 assay with the addition of sub-stimulatory amounts of either IL1- or PHA-LCM, the active protein was purified to near homogeneity in three independent experiments. Table 4 summarizes the results of one such experiment in which a 1% recovery was accompanied by an 80,000-fold purification and resulted in a fraction with a specific activity of 244,500 units/mg.

TABLE 4

Purification Step	Total Protein (mg)	Total Activity (units)	Specific Activity (u/mg)	Fold Purification	Recovery (%)
PHA-LCM	231,774	7,500,000	3	1	100
Blue Sepharose FF	1,294	2,600,000		670	35
Q-Sepharose FF	347	1,400,000	4,035	1,345	19
Phenyl-Sepharose HP	14	1,200,000	85,714	28,571	16
Superdex 75	0.12	29,340	244,500	81,500	1
Pv-FRIL was purified by its ability to stimulate Flt3-expressing 3T3 cells using four different chromatographic media. This resulted in a 80,000-fold purification with a 1% yield.					

#### Purified Pv-FRIL

[0155] SDS-PAGE showed the purified material to contain a limited number of polypeptides and the molecular size of the active material was determined by eluting the protein from SDS-PAGE gel slices run under non-reducing conditions and assaying the activity of the eluted material. Flt3 3T3 activity was always found in a gel slice that contained 14-22 kDa polypeptides and sometimes in a gel slice containing 32-43 kDa polypeptides (data not shown).

[0156] The polypeptide(s) in the active fraction corresponding to the 14-22 kDa range were subjected to aminoterminal sequencing by Edman degradation. To do this, an 18 kDa species from C4 reverse-phase chromatography was resolved by SDS-PAGE, electroblotted onto polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore) and stained with Ponceau S (Bio-Rad Laboratories, Inc., Hercules, CA). The 18 kDa band was cut from the PVDF membrane and N-terminal sequence was determined by automated Edman degradation on an ABI model 477A protein sequencer (PE Applied Biosystems, Foster, CA). The derived peptide sequences were compared against the SwissProt protein sequence database.

[0157] In each of three experiments, the sequence AQSLSFXFTKDLD, (SEQ ID NO: 35) was obtained from a polypeptide of 18 kDa (where X is an unknown amino acid). For the material at the dye front (14 kDa and below) the aminoterminal sequence of TDSRWAVEFDXFP (SEQ ID NO: 36) was found twice. The amino terminus of a smooth muscle protein (SM22- $\alpha$ ) was found twice and the amino terminus of myoglobin identified once. Since the sequence starting with AQS was the only sequence identified in each experiment, this polypeptide was concluded to be responsible for Flt3 3T3 activity.

[0158] Further purification of Pv-FRIL was obtained by immunoaffinity chromatography using a rabbit antiserum described above that was generated against a synthetic peptide of 13 amino acids corresponding to the N-terminus obtained for the 18 kDa polypeptide (referred to as anti-AQS). Crude PHA-LCM was applied to a rabbit anti-AQS affinity column, and after washing, bound protein was eluted under acidic conditions. Four pools of fractions were assayed for activity in the two different assay systems. The Flt3 3T3 cells responded weakly (<100 u/mL) to the pooled fractions (data not shown). Figs. 22A-22D shows results of an experiment where the pooled fractions were assayed on cord blood cells in the presence of IL3. After two weeks of suspension culture, the number of viable cells and status of CD34 expression was evaluated. A representative of three AQS affinity chromatography experiments is shown in Figs. 22A-22D. Cell cultures supplemented with the two early column fraction pools contained approximately four-fold more cells (426,000 cells and 466,250 cells, respectively) than at the 100,000 cells seeded and no appreciable CD34 staining

(Figs. 22A and 22B). The increase in cell number and loss of CD34 expression is attributed to the expected consequences of IL3-induced proliferation and differentiation. In contrast, cell cultures treated with the late-eluting fraction pool (Fig. 22D) contained less than 10,000 cells, or a tenth of input cells, and a uniform population of cells expressing CD34. The late-eluting AQS affinity pool did not show the potent effects of IL3 (high cell numbers and exhausted medium); instead the persistence of viable cells expression CD34 after two weeks in suspension culture suggested that the active component might preserve CD34<sup>+</sup>Flt3<sup>+</sup> progenitors.

#### Pv-FRIL Is Derived From *Phaseolus vulgaris*

**[0159]** Because PHA is derived from red kidney bean extract and because a FRIL family member, DI-FRIL, was isolated from another legume, namely *Dolichos lab lab*, mannose-binding lectins were isolated from red kidney bean (*Phaseolus vulgaris*) extract using standard methods, such as the procedure of Rudiger, H., *Isolation of Plant Lectins*, H.-J. Gabius and S. Gabius, eds., pp. 31-46, Berlin, 1993). The kidney bean mannose-binding lectin consisted of polypeptides with molecular weights of 18 kDa and 15 kDa, and the aminoterminals of these two polypeptides started with AQSLSFXFKFDPN (SEQ ID NO: 37) and TDSRVVAVEDF (SEQ ID NO: 38), respectively (where X is an unknown amino acid).

**[0160]** Pv-FRIL isolated from *Phaseolus vulgaris* was tested for activity in the Flt3 3T3 cell assay. As shown in Fig. 23, Flt3 3T3 cells responded in a dose dependent manner to Pv-FRIL, while parent untransfected 3T3 cells did not

#### Purification of Pv-FRIL from *Phaseolus vulgaris*

**[0161]** Dry seeds from the red and white kidney beans (*Phaseolus vulgaris*) were purchased from W. Atlee Burpee & Company, Warminster, PA. Lectins were eluted using a standard protocol. Briefly, beans were pulverized in a home coffee grinder and added to buffer of 50 mM Tris/HCl, pH. 8.0, 1 mM each of MgCl<sub>2</sub> and CaCl<sub>2</sub> for 4 hours at 4°C with constant mixing. Bean solids were pelleted by centrifugation at 10,000 x g for 20 min. The pH of the supernatant was modified to pH 4.0 with acetic acid and constant mixing to remove contaminating storage proteins, followed by centrifugation to clarify the supernatant, and finally the pH was readjusted to 8.0 with sodium hydroxide before storing at -20°C.

**[0162]** Specific binding of Pv-FRIL to mannose enabled a single-step purification of the lectin from the supernatant of the bean extract. 50 µL extracts of either red or white kidney beans were incubated in a conical tube with 1 mL of mannose covalently bound to agarose beads (Sigma) at 4°C with constant mixing for 4 hours to overnight. Beads were washed gently by centrifugation (300 g, 5 min) in lectin binding buffer. Mannose binding protein was eluted from the mannose beads after washing by incubation either with 200 mM α-methyl mannoside (Sigma) or 100 mM glycine, pH 8.

#### DNA Isolation and PCR amplification of Pv-FRIL-Encoding Nucleic Acid

**[0163]** Total genomic DNA was isolated from young *Phaseolus vulgaris* shoots according to the procedure of Dellaporta ("Plant DNA miniprep and microprep: Versions 2.1-2.3," Freeling, M. and V. Walbot (Ed.). *The Maize Handbook XXVI+759p*. Springer-Verlag New York, Inc.: New York, New York, USA; Berlin, Germany.), and stored at -20°C. Based on the determined N-terminal amino acid sequences of Pv-FRIL, four degenerate oligonucleotides (PVBeta1, PVBeta2, PValfa1, PValfa2) were designed using *Phaseolus vulgaris* codon usage. The sequences of the primers is as follows:

**PVBeta1:** TTY ACY AAR TTY GAY YTN GA (SEQ ID NO: 39)

**PVBeta2:** ATY TTY CAR GGW GAY GC (SEQ ID NO: 40)

**PValfa1:** TTR ACR TCR ATW CCR ATR TG (SEQ ID NO: 41)

**PValfa2:** TAR TTW GGR TCR ATR TTR GCR TT (SEQ ID NO: 42)

**[0164]** Two sequential polymerase chain reactions (PCR) were performed. In the first reaction, 10 ng of bean genomic DNA was amplified by 30 cycles of PCR, each cycle comprising 40 seconds at 94 °C, 40 seconds at 50 °C, 60 seconds at 72 °C, and an extension step at 72 °C for 10 min. The reactions were performed in 50 µl containing 30 pmol of each primer, PVBeta 1 and PValfa1, 0.2 mM deoxyribonucleotides and 0.5 unit of Ampli-Taq polymerase (Perkin Elmer) in

the corresponding buffer. One microliter of the PCR product was amplified by 30 PCR cycles using the same conditions as described above. The reaction was performed in 50 µL containing 30 pmol of the two primers, PVbeta 2 and PV alfa2, using 0.2 mM deoxyribonucleotides and 0.5 unit of Ampli-Taq polymerase (Perkin Elmer) in the corresponding buffer. The 460 bp fragment obtained was cloned in a T/A plasmid, pCR2.1 (Invitrogen) and sequenced by sequenase dideoxy chain termination (United States Biochemicals).

#### RNA Isolation and cDNA synthesis of Pv-FRIL-Encoding Nucleic Acid

**[0165]** Total RNA was prepared from mid-maturation *Phaseolus vulgaris* seeds stored at -70°C following the procedure reported by Pawloski *et al.* (*Mol. Plant Biol. Manual* 5: 1 -13, 1994). The 5'/3' RACEKIT (Boehringer Mannheim) was used to generate cDNA from 5.0 µg total RNA used according to the manufacturer's instructions. In the cDNA synthesis for the 3' RACE, the oligo(dT) anchor primer was at the concentration of 32.5 µM, in the standard conditions. For the 5' RACE, a specific primer (SPV1) was used at the concentration of 32.5 µM. The cDNA purification and the subsequent tailing reaction was performed according to the manufacturer's instructions.

#### Polymerase Chain Reaction and cDNA cloning of Pv-FRIL-Encoding Nucleic Acid

**[0166]** The 3' end of Pv-FRIL was obtained through rapid amplification of cDNA ends by polymerase chain reaction (RACE-PCR) using the 5'/3' RACEKIT (Boehringer Mannheim) used according to the manufacturer's instructions. Nested PCR amplifications were performed using the PCR-Anchor primer with the specific primers (PV3 and PV4) in two successive amplification reactions. The sequences of these primers is as follows:

**PV3:** CAA TGT CTT ACA ACT CAC TAA G (SEQ ID NO: 43)

**PV4:** AGT CTG GGA AGA GTG TTA TTC (SEQ ID NO: 44)

**[0167]** The amplification conditions were 30 cycles of 40 seconds at 94 °C, 40 seconds at 55 °C, 60 seconds at 72 °C each and an extension step at 72 °C for 10 min. The reactions were performed in 50 µL containing 30 pmol of each primer, PV3 and PCR-Anchor primer in the first, and PV4 and PCR-Anchor primer in the second, 0.2 mM deoxyribonucleotides and 0.5 units of Ampli-Taq polymerase (Perkin Elmer) in the corresponding buffer. The 831 bp product obtained was sub-cloned in pCR2.1 and sequenced as above reported.

**[0168]** For the 5'RACE, again nested PCR reactions were performed using in combination with the Anchor Primer the specific primers SPV2 and SPV3. The sequences of these primers is as follows:

**SPV2:** ACC AAA GCT TTG GTT TTC AGA (SEQ ID NO: 45)

**SPV3:** TCT GAA AAC GTT TGA GTA GAG (SEQ ID NO: 46)

The amplification conditions for both reactions were 30 cycles of 40 seconds at 94 °C, 40 seconds at 50 °C, 60 seconds at 72 °C each and an extension step at 72 °C for 10 min. The reactions were performed in 50 µL containing 30 pmol of each primer, SPV2 and PCR-Anchor primer in the first, and SPV3 and PCR-Anchor primer in the second, 0.2 mM deoxyribonucleotides and 0.5 unit of Ampli-Taq polymerase (Perkin Elmer) in the corresponding buffer.

**[0169]** To obtain the full-length cDNA clone, recombinant PCR was performed (Higuchi R, *supra*). Two PCR reactions were carried out separately, one on the 5' fragment and the other on the 3' RACE product using primers with an overlapping region. The overlapping primary products were subsequently re-amplified using the flanking primers resulting in a full-length fragment.

**[0170]** The primary PCR products were purified with the QIAquick PCR Purification kit (QIAGEN) used according to the manufacturer's instructions, and amplified together in a single second reaction. For the second PCR reaction, the primers PVEcoRI and the APEcoRI were used. The sequences of these primers is as follows:

**PVEcoRI** TAC ATG AAT TCG CTC AGT CAT TAT CTT TTA AC (SEQ ID NO: 47)

**AP<sub>EcoRI</sub>:** GAA TTC GAC CAC GCG TAT CGA TGT CGAC (SEQ ID NO: 48)

**[0171]** Both primary and secondary PCR reactions were performed in 100 µL containing 50 pmol of each primer, 0.4 mM deoxyribonucleotide and 1.0 unit Pfu polymerase (Stratagene) in the corresponding buffer. The primary PCR reaction amplified the two separate fragments by 30 cycles, each cycle comprising 40 seconds at 94°C, 40 seconds at 50°C, 60 seconds at 72°C and an extension step at 72°C for 10 min. The second PCR reaction amplified the recombinant fragment in 12 cycles using the same conditions reported above. The full-length product was cloned in the EcoRI site of the cloning vector pCR2.1 (Fig. 24A) and sequenced as noted above. This plasmid is referred to as pCR2.1-Pv-FRIL.

**[0172]** The nucleic acid sequence of the Pv-FRIL cDNA is as follows:

```

1  GCTCAGTCAT TATCTTTTAA CTTTACCAAG TTTGATCTTG ACCAAAAAGA
15  51  TCTTATCTTC CAAGGTGATG CCACTTCTAC AAACAATGTC TTACAACTCA
    101 CTAAGTTAGA CAGTGGAGGA AACCTGTGG GTGCTAGTGT GGGAAGAGTG
    151 TTATTCTCTG CACCATTTC TCTTTGGGAA AACTCTATGG CAGTGTCAAG
    201 CTTTGAAACT AATCTCACCA TTCAAATCTC AACACCTCAC CCTTATTATG
    251 CAGCTGATGG CTTTGCCTTC TTCCTTGCAC CACATGACAC TGTCATCCCT

25  301 CCAAATTCTT GGGGCAAATT CCTTGGACTC TACTCAAACG TTTTCAGAAA
    351 CTCCCCCACC TCTGAAAACC AAAGCTTTGG TGATGTCAAT ACTGACTCAA
    401 GAGTTGTTGC TGTCGAATTT GACACCTTCC CTAATGCCAA TATTGATCCA
    451 AATTACAGAC ACATTGGAAT CGATGTGAAC TCTATTAAGT CCAAGGAAAC
30  501 TGCTAGGTGG GAGTGGCAAA ATGGGAAAAC GGCCACTGCA CGCATCAGCT
    551 ATAACCTCTG CTCTAAAAAA TCAACTGTTA CTACGTTTTA TCCTGGGATG
    601 GAAGTTGTGG CTCTCTCCCA TGATGTTGAC TTACATGCAG AGCTTCCTGA
35  651 ATGGGTTAGA GTAGGGTTAT CTGCTTCAAC TGGAGAGGAG AAACAAAAAA
    701 ATACCATTAT CTCATGGTCT TTTCACTTCAA GCTTGAAGAA CAACGAGGTG
    751 AAGGAGCCGA AAGAAGACAT GTATATTGCA AACGTTGTGC GATCATATAC
40  801 ATGGATCAAT GACGTTCTAT CTTATATAAG CAATAAATAA ATGTATGATG
    851 CACTCAATAA TAATCACAAG TACGTACGGT GTAGTACTTG TATGTTGTTT
    901 ATGAAAAAAA AAAA (SEQ ID NO: 5)

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**[0173]** The amino acid sequence of Pv-FRIL is as follows:

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AQSLSFNFTKFDLDQKDLIFQGDATSTNNVLQLTKLDSGGNPVGASVGRVLFSAFHLWENS
50 MAVSSFETNLTIQISTPHPYAADGFAFFLAPHDTVIPPNSWGKFLGLYSNVFRNSPTSENO
SFGDVNTDSRVVAVEFDTFPNANIDPNYRHIGIDVNSIKSKETARWEWQNGKTATARISYNS
ASKKSTVTTTFYPGMEVVALSHDVLHAELPEWVRVGLSASTGEEKQKNTIISWSFTSSLKNN
55 EVKEPKEDMYIANVVRSYTWINDVLSYISNK*MYDALNNNHKYVRCSTCMLFMKKK
(SEQ ID NO: 6)

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[0174] The amino acid sequence of Pv-FRIL was compared to the amino acid sequences of DI-FRIL and of the PHA-E lectin. This comparison is shown on Fig. 24B.

#### Pv-FRIL-Encoding Plant Expression Vectors and *Nicotiana tabacum* Transformation

[0175] Recombinant PCR was used to introduce a signal peptide for entry of Pv-FRIL into the endoplasmic reticulum at the 5' end of the Pv-FRIL cDNA clone. Following the procedure of Higuchi (*supra*) the signal peptide and the full length cDNA clone were amplified in two separate primary PCR reactions. The signal peptide was obtained from the amplification of the binary vector pTA4, harboring the complete sequence of the bean  $\alpha$ -amylase inhibitor gene (Hoffman et al., L.M., Y. Ma and R.F. Barker, *Nucleic Acid Res.* 10: 7819-7828, 1982; Moreno and Chrispeels, *Proc. Natl. Acad. Sci. USA* 86: 7885-7889, 1989).

[0176] The primers used for the two primary reactions are the following:

Amplification of the Signal Peptide

**Sigforw BglII:** AGA TCT ATG GCT TCC TCC AAC (SEQ ID NO: 49)

**Sigrew:** AAA GAT AAT GAC TGA GCG GCT GAG TTT GCG TG (SEQ ID NO: 50)

Amplification of the mannose lectin cDNA:

**SpMlforw:** CAC GCA AAC TCA GCC GCT CAG TCA TTA TCT TT (SEQ ID NO: 51)

**APXhoI:** CTC GAG GAC CAC GCG TAT CGA TGT CGA (SEQ ID NO: 52)

The primers used for the secondary reactions, Sigforw and AP, were designed to generate BglII sites at the 5' and XhoI at the 3' ends. Both, primary and secondary PCR reactions were performed as discussed above. The recombinant product SpPv-FRIL was incubated for 10 min. at 72°C with 0.5 units of Ampli-Taqpolymerase (Perkin Elmer) and cloned in the cloning vector pCR2.1 (Fig. 25). The nucleotide sequence of the PCR product was determined as described above to verify the correct attachment of the signal peptide.

[0177] A binary vector was constructed for constitutive expression of Pv-FRIL in tobacco plants. The recombinant SpPv-FRIL was cloned in BglII/XhoI sites of a plant expression vector resulting in the formation of pM-SpPv-FRIL (Fig. 26).

[0178] The binary vector was transferred in *Agrobacterium tumefaciens* strain C58 according to the freeze-thaw procedure reported of An *et al.*, *supra*. *Agrobacterium*-mediated transformation of *Nicotiana tabacum* leaf disks was carried out as described by Horsh *et al.* (*Science* 227: 1229 - 1231, 1985) using C58 harboring the expression vector pM-SpPv-FRIL. Kanamycin resistant plants were scored for their ability to form roots in two consecutive steps of propagation in Murashige-Skoog medium containing 3% sucrose and kanamycin sulfate (Sigma) at 100 mg/L.

[0179] Tobacco plants transformed with this construct were grown in a growth room under controlled conditions. The leaves (20 grams) of young plants were harvested and frozen in liquid nitrogen and powdered in a mortar with a pestle. The powder was stirred in a buffer mixture consisting of 1 x phosphate buffered saline containing 1mM CaCl<sub>2</sub> with a cocktail of protease inhibitors (PMSF, Pepstatin and Leupeptin). This slurry was centrifuged at 2000 rpm and the supernatant was centrifuged at 40,000 rpm in a Beckman ultracentrifuge. The clear supernatant was tumbled with 1 ml of ovalbumin-Sepharose for 3 hours. The beads were washed with the same buffer and tumbled overnight with 200 mM trehalose in 1/10 phosphate buffered saline containing the cocktail of protease inhibitors. Coomassie blue stained gel showed this preparation to be pure Pv-FRIL. An immunoblot showed the presence of both alpha and beta subunits, thus, the single polypeptide chain encoding both subunits was cleaved by the transformed cells *in vivo*. Binding to the ovalbumin-sepharose and release by trehalose shows that the product of the transgene is an active lectin.

#### EXAMPLE 6

##### DI-FRIL Supports Prolonged *ex vivo* Maintenance of Quiescent Human CD34+,CD38-/SCID-Repopulating Cells

[0180] To further characterize the progenitor cell preservation activity of DI-FRIL, a functional *in vivo* assay for prim-



itive human hematopoietic cells was used to determine the cells' ability to home to and repopulate the marrow of sublethally irradiated C.B-17 and NOD/LtSz mice homozygous for the severe combined immunodeficiency *Prkdc<sup>scid</sup>* mutation (Lapidot et al., *Science* 255:1137,1992; Larochelle et al., *Nat. Med.* 2:1329-1337, 1996). To do this, the following methods were used:

#### Preparation of Human Cells

**[0181]** Human umbilical cord blood (CB) samples were obtained from full term deliveries. The blood samples were diluted 1:1 in phosphate-buffered-saline (PBS) without  $Mg^{+2}/Ca^{+2}$ , supplemented with 10% fetal bovine serum (FBS). Low density mononuclear cells were collected after standard separation on Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden), and washed in RPMI with 10% FBS. Some samples were frozen in 10% DMSO, while the others were used fresh. Enrichment of CD34<sup>+</sup> cells was performed with mini MACS separation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of the enriched CD34<sup>+</sup> cells was 60-80% using one column. CD34<sup>+</sup>CD38<sup>-low</sup> cells were purified by FACS sorting (FACStar<sup>+</sup>, Becton Dickinson, San Jose, CA) after staining CD34<sup>+</sup> enriched cells with mAb anti human CD34-FITC (Becton Dickinson) and anti human CD38 PE (Coulter, Miami FL USA) (Purity >99%).

#### Mice

**[0182]** Eight week old NOD/LtSz-*Prkdc<sup>scid</sup>/Prkdc<sup>scid</sup>* (NOD-SCID) mice and NOD/SCID  $\beta 2$  microglobulin knockout mice, hereafter termed NOD/SCID B2M<sup>null</sup> (Christianson et al., *J. Immunol.* 158:3578-3586,1997), bred and maintained under defined flora conditions in sterile micro isolator cages, were irradiated with a sublethal dose of 375 cGy at 67cGy/min. from a cobalt (<sup>60</sup>Co) source prior to transplantation.

**[0183]** Human cells were injected into the tail vein of irradiated mice in 0.5 mL of RPMI with 10% FBS. In some experiments (as indicated) non engrafting irradiated (1500 cGy) CD34<sup>-</sup> cells served as carrier cells, and were cotransplanted with cultured cells at a final concentration of  $0.5 \times 10^6$  cells/mouse. Mice were sacrificed 1 month post transplantation, and bone marrow (BM) cells were flushed from the 8 bones of each mouse (femurs, tibiae, humeri, and pelvis).

#### Ex vivo cultures

**[0184]** Human CD34<sup>+</sup> enriched cells were cultured in 24 well plates ( $2-4 \times 10^5$  cells in 0.5 mL), containing RPMI supplemented with 10% FBS+1% BSA. *Ex vivo* cultures contained the following cytokine combination: Stem cell factor (SCF) - 100 ng/mL and Flt3 ligand (Flt3-L) - 100 ng/mL (R&D Systems Inc. Minneapolis, MN, USA), rhIL-6 - 50 ng/mL and sIL6R - 1280 ng/mL, (InterPharm Laboratories, Ares-Serono Group, Ness Ziona, Israel) or DI-FRIL - 10 ng/mL (ImClone Systems Inc., NY, USA). In some experiments where indicated, the growth factor cocktail included 300 ng/mL SCF, 300 ng/mL Flt3-L, 50 ng/mL G-CSF, 10 ng/mL IL-3 (R&D Systems), and 10 ng/mL IL-6. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. After 3, 6, 10 and 13 days, the cells were collected, counted, analyzed by FACS, seeded in to semisolid cultures and transplanted into NOD-SCID mice. For CD56<sup>+</sup> NK cell development, BM cells from engrafted mice were cultured with 100 ng/mL of SCF and IL-15 (R&D) for 10-14 days.

#### Preparation of DI-FRIL

**[0185]** DI-FRIL was isolated from the seeds of hyacinth beans (*Dolichos lab lab*) using the protocol described above in Example 1.

#### CFU assay.

**[0186]** Semisolid cultures were performed in order to detect the levels of human progenitors in *ex vivo* cultures, and in the marrow of transplanted mice. The cells were plated ( $4 \times 10^3$  cells/mL) in 0.9% methylcellulose (Sigma, St. Louis, MO, USA), 30% FBS,  $5 \times 10^{-5}$ M 2ME, 50 ng/mL SCF, 5 ng/mL IL-3, 5 ng/mL GM-CSF (R&D), and 2 u/mL Erythropoietin (Ortho Bio Tech, Don Mills, ON, Canada). Human cells from the BM of engrafted mice were plated ( $2 \times 10^5$  cells/mL) in 15% FBS + 15% human plasma, selective for human colonies only. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and scored 14 days later with an inverted microscope for myeloid, erythroid, and mixed colonies by morphologic criteria.

Cell cycle analysis.

**[0187]** Cells were analyzed for their DNA content by staining with propidium iodide (Sigma). The cells were cultured in *ex vivo* cultures as described, for 3, 6, 10 and 13 days as indicated. At each time point, the cells were collected, resuspended to a final concentration of  $0.1-1 \times 10^6$  cells/mL, and incubated with 0.1% Triton X 100 (Sigma) for 20 minutes on ice. 50 mg/mL propidium iodide (PI) were added before analysis. Flow cytometric analyses were performed using FACSsort (Becton Dickinson, San Jose, CA).

Flow cytometry analyses.

**[0188]** Human and mouse Fc receptors on BM cells from transplanted mice were blocked by using human plasma (1:50) and anti mouse Fc receptor blockers (anti mouse CD16/CD32 mAb, Pharmingen, San Diego, CA, USA). Isotype control mAb were used in order to exclude false positive cells (Coulter, Miami FL USA). The purity of enriched sub-populations after magnetic bead separation, were analyzed by two color staining, using anti-human CD34 FTTC (Becton Dickinson, San Jose, CA, USA) and anti-human CD38 PE (Coulter). The levels of human cells and lymphoid lineages in the marrow of engrafted mice were detected by double staining with anti-human CD45 FITC (*Immuno Quality Products, Groningen, The Netherlands*) together with anti-human CD19 PE (Coulter) for detection of pre B cells, or with anti-human CD56 PE (Coulter) for detection of NK cells. Cells were washed with PBS supplemented with 1% FBS and 0.02% azide, suspended to a volume of  $0.1-1 \times 10^6$  cells/mL, stained with direct-labeled mAb and incubated for 25 minutes on ice. After staining, cells were washed once in the same buffer and analyzed on a FACSsort (Becton Dickinson). Analysis was performed using CELLquest software (Becton Dickinson).

Human cell engraftment analysis.

**[0189]** The levels of human cell engraftment were determined by both flow cytometry for analysis of human myeloid CD45<sup>+</sup> and lymphoid CD45<sup>+</sup>CD19<sup>+</sup> pre B cells and quantification of human DNA as previously described (Lapidot et al., *Science* 255:1137, 1992; Larochelle et al., *Nat. Med.* 2:1329-1337, 1996; Peled et al., *Science* 283:845-848, 1999). Briefly, high molecular weight DNA was obtained from the BM of transplanted mice by phenol/chloroform extraction. DNA (5 g) was digested with EcoRI, subjected to electrophoresis on 0.6% agarose gel, blotted onto a nylon membrane, and hybridized with a human chromosome 17-specific  $\alpha$ -satellite probe (p17H8) labeled with  $^{32}\text{P}$  (Lapidot et al., *supra*). The intensity of the bands in the samples were compared to artificial human/mouse DNA mixtures (0%, 0.1%, 1%, and 10% human DNA) to quantify the human DNA (lanes to the right of lane 4 in Fig. 31). Multiple exposures of the autoradiographs were taken to ensure sensitivity down to 0.01% human DNA. A transplanted mouse was scored positive when both human myeloid and lymphoid cells and human DNA were detected in its BM.

**[0190]** From these experiments, the following results were obtained:

DI-FRIL maintains but does not expand CB CD34<sup>+</sup>progenitors in suspension culture

**[0191]** As shown above, DI-FRIL by itself preserves immature CB progenitor cells up to a month in suspension culture without medium changes (see, e.g., Figs 14A and 14B, and Table 3). To compare DI-FRIL's progenitor-preserving properties to a combination of cytokines (SCF, Flt3-L, IL-6, and sIL6-R) shown to maintain CB progenitor cells in suspension culture (Sui et al., *Proc. Natl. Acad. Sci. USA* 92:2859, 1995; Ebihara et al., *Blood* 90:4363-4368, 1997), enriched CB CD34<sup>+</sup> cells were cultured with either DI-FRIL or cytokines for 3, 6, 10, or 13 days in medium containing 10% FBS and 1% BSA in RPMI. Fresh media and DI-FRIL and/or cytokines were added on day 6. Cells harvested at each time point were counted and assayed for clonogenic progenitor cells by plating (*i.e.*, seeding) in semisolid media. **[0192]** As shown in Fig. 27A, the total number of cells in DI-FRIL cultures gradually declined over time from  $2 \times 10^5$  cells initially seeded to  $1.26 \times 10^5$  cells at day 13, in contrast to the expected 14.3-fold increase to  $2.8 \times 10^6$  cells in cytokine cultures at day 13. Similarly, as shown in Fig. 27B, the levels of progenitor cells in DI-FRIL cultures also remained relatively constant until day 10, after which they declined to 9 % of the starting population (from  $24.7 \times 10^3$  colonies on day 0 to  $2.3 \times 10^3$  colonies on day 13). Progenitor levels increased in cytokine cultures by 10-fold from the outset to day 13 of culture (Fig. 27B).

DI-FRIL maintains the expansion capacity of CD34<sup>+</sup>progenitors up to 2 weeks in *ex vivo* culture

**[0193]** To characterize the expansion capacity of DI-FRIL-preserved progenitor cells in suspension culture, CD34<sup>+</sup> cells cultured for 6 days with DI-FRIL were washed and exposed to cytokines (without DI-FRIL) for an additional 4 days. Total cell counts and clonogenic progenitor assays were performed and the results were compared to cells cultured for 10 days with DI-FRIL. Fresh media and DI-FRIL were added on day 6. Interestingly, the DI-FRIL-cultured

cells proliferated in response to cytokine stimulation, resulting in a 3.4-fold increase in cell numbers (Fig. 28A) and 13-fold increase in progenitor levels (Fig. 28B).

**[0194]** Further experiments tested DI-FRIL's ability to preserve cytokine-responsive CD34<sup>+</sup> progenitor cells cultured with either DI-FRIL for 10 days followed by 3 days of cytokine stimulation or only with DI-FRIL for the entire 13 days. Fresh media and DI-FRIL were added on day 6. A 2.9-fold increase in total cell numbers (Fig. 28C) and a 5.5-fold increase in progenitor levels (Fig. 28D) were observed for cells cultured with DI-FRIL for 10 days followed by an additional 3 days of cytokine stimulation compared to cultures with DI-FRIL alone for 13 days. These results provide evidence that DI-FRIL alone maintains the proliferative capacity of human progenitor cells up to 13 days in suspension culture and that subsequent stimulation of cells cultured for 10 days with DI-FRIL still respond to the proliferative signals of the cytokines.

#### DI-FRIL maintains SCID repopulating stem cells (SRC) in ex vivo cultures

**[0195]** At each time point during culture, the human CB CD34<sup>+</sup> cells were collected and the content of each well was assayed for progenitor levels (described above) and  $2 \times 10^5$  of the remaining cells were transplanted into one mouse. One month later, mice were sacrificed and their bone marrows were harvested and assayed for the presence of human myeloid and lymphoid cells. Before assaying, human DNA levels in the bone marrow of individual transplanted mice was quantitated by Southern blotting analysis. A representative Southern blot analysis showing the detection of a 0%, 0.1%, 1%, and 10% human DNA per murine DNA is provided in Fig. 29E.

**[0196]** Figs. 29A-29D show representative Southern blot analyses of the BM of mice transplanted with ex vivo cultured cells. Fig. 29A is a representative Southern blot showing human DNA in the marrow of mice transplanted with cells cultured with FRIL for 6 days (lane 1), FRIL for 10 days (lane 2), or with FRIL for 6 days followed by 4 days with cytokine stimulation (lane 3). In a typical experiment, the levels of engraftment by cells cultured with DI-FRIL for 10 days decreased by approximately 10-fold from cells cultured with DI-FRIL for 6 days (Fig. 29A, lanes 1 and 2). However, when cells cultured with DI-FRIL for 6 days were subsequently stimulated with cytokines for 4 days, there was an approximate 10-fold increase in the level of engraftment (Fig. 29A, lane 3) compared to DI-FRIL alone for 10 days (lane 2). Similar results were obtained when cells from other donors were transplanted as shown in Fig. 29B, lanes 3-4 (cells cultured with DI-FRIL for 6 days followed by 4 days of cytokine stimulation) compared to lanes 1-2 (cells cultured with DI-FRIL alone for 10 days) prior to transplantation. DI-FRIL cultures could be prolonged even to 13 days, as shown in Figs. 28C-28D, prior to transplantation into mice. A modest increase in the levels of engraftment was seen comparing the marrow of mice transplanted with the original cells, which were not cultured with DI-FRIL, prior to seeding (Fig. 29C, lane 1) with cells cultured with DI-FRIL for 13 days (Fig. 29C, lane 2). Fig. 28D shows the results of another experiment, where the difference between engraftment levels obtained by cells cultured with DI-FRIL for 10 days (lane 1) or with DI-FRIL for 6 days followed by 4 days of cytokine stimulation (lane 2) are minor. Nevertheless, a 10-fold increase was observed for cells cultured either with DI-FRIL for 13 days (Fig. 29D, lane 3) or with DI-FRIL for 10 days followed by 3 days of cytokine stimulation (Fig. 29D, lane 4) when compared to cells cultured for 10 days (Fig. 29D, lanes 1 and 2).

**[0197]** Levels of human engraftment in the marrow of mice transplanted with CD34<sup>+</sup> cells cultured with either DI-FRIL for 10 days (n=12) or DI-FRIL for 6 days (n=33) followed by cytokine stimulation for 4 days are summarized in Fig. 30. Cells cultured with DI-FRIL followed by cytokines, engrafted at levels 7.4-fold greater than cells cultured with DI-FRIL alone for 10 days (Fig. 30, p=0.05).

**[0198]** To test the effect of DI-FRIL on CB populations highly enriched for primitive human SRC, CD34<sup>+</sup> cells isolated by immunomagnetic beads were further sorted by flow cytometry based on the absence of CD38 expression. CD34<sup>+</sup>CD38<sup>-low</sup> cells (99% purity) cultured with either DI-FRIL alone or DI-FRIL followed by cytokines showed patterns of engraftment in NOD/SCID B2M<sup>null</sup> mice (Christianson et al., *supra*), similar to those observed for CD34<sup>+</sup> cells. These mice have been used successfully for secondary human stem cell transplantation (Peled et al., *supra*), have less innate immunity due to lack of NK activity and thus require fewer (10 fold) human cells for engraftment compared to NOD/SCID mice.

**[0199]** Fig. 31 is a representative Southern blot (1 out of 4 experiments) showing the relative level of engraftment of cells that were cultured with DI-FRIL for 10 days (lane 4) compared to 6 days with DI-FRIL followed by 4 days cytokine stimulation (lanes 1-3) prior to transplantation into mice. High molecular weight DNA was obtained from the BM of transplanted mice and subjected to Southern blotting analysis for the presence of human DNA.

**[0200]** Based on these results, an increased level of engraftment in cells exposed to cytokines after DI-FRIL was expected. Consequently, the same initial cell dose transplanted into one mouse in lane 4 was divided into 3 mice in lanes 1-3, indicating an expansion of DI-FRIL cultured SRC when they were subsequently exposed to cytokine stimulation. The level of engraftment in mice transplanted with cells that were treated with DI-FRIL followed by cytokines (Fig. 31, lanes 1-3) was about 10-fold higher compared to the mouse transplanted with cells cultured with DI-FRIL alone (Fig. 31, lane 4). Since the initial dose of cells for the DI-FRIL + cytokines sample was split into three mice, a 30-fold increase in the level of engraftment is observed for CD34<sup>+</sup>CD38<sup>-low</sup> cells stimulated by cytokines after DI-FRIL

compared to the culture with DI-FRIL alone. In addition, this result represents about a 3-fold greater level of expansion of SRC for CD34<sup>+</sup>CD38<sup>-/low</sup> cells than for total CD34<sup>+</sup> cells. Fig. 32 summarizes the fold increase observed in the engraftment levels of CD34<sup>+</sup>CD38<sup>-/low</sup> cells cultured with DI-FRIL alone compared to cells subsequently stimulated with cytokines in 6 experiments (3 experiments with NOD/SCID B2M<sup>null</sup> mice and 3 experiments with NOD/SCID mice, gave similar results). These data indicated a 30-fold expansion of SRC for CD34<sup>+</sup>CD38<sup>-/low</sup> cells and a 3-fold greater level than that of total CD34<sup>+</sup> cells in the corresponding experiments.

**[0201]** Further studies have demonstrated that DI-FRIL preserves long-term repopulating stem cells. Bone marrows harvested from NOD/SCID mice that were initially transplanted with CD34<sup>+</sup> cord blood cells cultured in DI-FRIL were transplanted into a second set of sublethally irradiated NOD/SCID mouse recipients. After one month, bone marrows from the second set of recipients were analyzed for the presence of human hematopoiesis, as determined using the assays described above. Multilineage engraftment was observed in the second set sublethally irradiated NOD/SCID mouse recipients. This observed persistence of repopulating cells in this serial transplantation study indicated the presence of long-term repopulating stem cells.

#### DI-FRIL preserves SRC potential of multilineage differentiation in the murine BM

**[0202]** Southern blot analysis detected and quantified the levels of human DNA in the marrow of transplanted mice without specifically indicating the differentiation status of human cells recovered from the murine BM. Since SCID repopulating stem cells (SRC) are defined by multilineage differentiation of myeloid and lymphoid cells in NOD/SCID mice, the *in vivo* differentiation processes of DI-FRIL-cultured CB CD34<sup>+</sup> cells in the murine BM were further studied. To do this, CD34<sup>+</sup> or sorted CD34<sup>+</sup>CD38<sup>-/low</sup> cells were cultured either with FRIL for 10 days or with FRIL for 6 days followed by cytokine stimulation for 4 days prior to transplantation. BM of transplanted mice was collected 1 month later and the cells were either seeded in semisolid media selective for human colonies (results shown in Fig. 33A) or were analyzed for lineage specific markers by flow cytometry (results shown in Figs. 33B-33F).

**[0203]** The progenitor capacity of human CB CD34<sup>+</sup> cells cultured for 10 days with DI-FRIL and harvested one month after transplantation into NOD/SCID mice was evaluated in semi solid-colony assays that selectively promoted growth of human progenitors. Fig. 33A shows that the levels of human progenitors in the bone marrow of transplanted mice increased 2.3-fold when SRC were stimulated with cytokines for 4 days after 6 days of DI-FRIL incubation compared to DI-FRIL alone for 10 days prior to transplantation. Moreover, both myeloid and erythroid colonies formed in the colony assays (data not shown) as well as human B cell differentiation that was determined by flow cytometry (Figs. 33C and 33D), indicating that incubation with DI-FRIL maintained the multilineage differentiation potential of SRC. A representative flow cytometry analysis demonstrated the presence of human CD45<sup>+</sup>CD19<sup>+</sup> pre-B cells in the marrow of mice transplanted with either CD34<sup>+</sup> cells (Fig. 33C) or CD34<sup>+</sup>CD38<sup>-/low</sup> cells (Fig. 33D) cultured with DI-FRIL for 10 days (1% or 10% CD19<sup>+</sup> cells, respectively).

**[0204]** To determine whether human lymphoid precursors in the marrow of transplanted mice have the potential to differentiate in addition to myeloid cells also into lymphoid NK cells, cells from transplanted murine bone marrow were also cultured with SCF and IL-15 for 10 days. Cells harvested from cultures were analyzed for human NK cells by flow cytometry using the human-specific monoclonal antibodies to CD45 and the NK cell antigen, CD56. Cells from mice transplanted with CD34<sup>+</sup> or CD34<sup>+</sup>CD38<sup>-/low</sup> cells are shown (1% or 7.7% of CD56<sup>+</sup> cells; Figs. 33E and 33F, respectively).

#### ex vivo preservation of early progenitors with DI-FRIL compared to Flt3 ligand

**[0205]** DI-FRIL was identified by its ability to stimulate proliferation of NIH 3T3 cells transfected with Flt3 and not by untransfected cells or cells transfected with the related Fms tyrosine kinase receptor (Moore et al., *Blood* 90 supp.1: 308,1997). Although DI-FRIL and Flt3-L both stimulate proliferation of Flt3 3T3 cells, they exert different activities on CB progenitor cells. Flt3-L induces quiescent primitive cells into cycle (Lyman and Jacobsen, *Blood* 91:1101-11345, 1998). In contrast, DI-FRIL by itself maintains progenitors in serum-defined medium from 15-29 days in culture without medium changes (Colucci et al., *Proc. Natl. Acad. Sci. USA* 96(2):646-650, 1999). The abilities of DI-FRIL or Flt3-L to maintain CB CD34<sup>+</sup> progenitors in suspension cultures consisting of 10% FBS and 1% BSA in RPMI for 10 days were compared in the presence and absence of cytokines. The cells were then were seeded for CFU assay.

**[0206]** As shown in Fig. 34A, the number of total cells harvested after 10 days of suspension culture in DI-FRIL increased minimally by 1.9-fold, whereas cells cultured in either Flt3-L alone or in combination with DI-FRIL increased by 4.5-fold and 5.4-fold, respectively ( $p < 0.05$ ). Cultures containing CB CD34<sup>+</sup> cells with cytokines, either alone or with DI-FRIL and Flt3-L, led to 10.9-12.5 fold increase in cell numbers; however cells cultured with DI-FRIL alone followed by cytokines, increased only by 4.6-fold (Fig. 34A,  $p < 0.05$ ).

**[0207]** Interestingly, cells cultured with DI-FRIL alone or followed by cytokines, selectively maintained a higher number and proportion of the primitive granulocyte-erythroid-macrophage-megakaryocyte colony forming-units

(CFU-GEMM) compared to other treatments (Fig. 34B,  $p < 0.05$ ). About 40% of the progenitor cells maintained by DI-FRIL were CFU-GEMM, a relative 2.6- to 3.7-fold increase with DI-FRIL cultures compared to other combinations (Fig. 34B,  $p < 0.05$ ). In contrast to Flt3-L, exposure of cells first cultured with DI-FRIL to cytokines, maintained the high percentage of CFU-GEMM, 18.2% versus 38.6%, respectively (Fig. 34B  $p < 0.05$ ). These results demonstrate that DI-FRIL and Flt3-L may act differently on primitive progenitor cells in culture.

DI-FRIL maintains higher levels of CD34<sup>+</sup> cells in G<sub>0</sub>/G<sub>1</sub> phase of cell cycle compared to cytokine treated cells

**[0208]** *In vivo*, immature CD34<sup>+</sup> cells are predominantly quiescent, non-cycling cells (Young et al., *Blood* 87:545, 1996; Movassagh et al., *Stem Cells* 15:214-222, 1997; Ladd et al., *Blood* 90:658-668, 1997). Since DI-FRIL maintains relatively constant levels of progenitor cells over two weeks in suspension culture that can subsequently respond to cytokine stimulation (see Fig. 27), the cell cycle status of CD34<sup>+</sup> cells incubated with DI-FRIL was investigated and compared to cultures with cytokine stimulation. DNA content of CB CD34<sup>+</sup> cells was analyzed by flow cytometry immediately after isolation of cells. A mean value of 96.6% of cells were observed in the G<sub>0</sub>/G<sub>1</sub> phase of cell cycle (Fig. 35A).

**[0209]** The cell cycle status of CB CD34<sup>+</sup> cells was analyzed in 5 independent experiments with cells cultured for 3-13 days with either DI-FRIL or a cytokine combination (SCF, Flt3-L, G-CSF, IL-3, IL-6) (Bhatia et al., *J. Exp. Med.* 186: 619-624, 1997; Conneally et al., *Proc. Natl. Acad. Sci. USA* 94:9836-9841, 1997). The percentage of cycling cells in SG<sub>2</sub>M phase in both cultures decreased by half from day 3 to day 13, from 17.7% to 9.1% for DI-FRIL and from 50.9% to 23.2% for cytokines (Table 1) (3.4% of cells initially seeded were in SG<sub>2</sub>M).

TABLE 5

Percentage and numbers of cycling CD34 <sup>+</sup> cells (in SG <sub>2</sub> M phase) cultured with DI-FRIL or cytokines for 3,6,10 or 13 days					
Days in culture	DI-FRIL (%)	Cytokines (%)	DI-FRIL (cells x10 <sup>3</sup> )	Cytokines (cells x10 <sup>3</sup> )	Fold increase induced by cytokines
day 3	17.7±1.9	50.8±1.4	35	212	6
day 6	20±2	26	43.6	389.5	9
day 10	10±1.9	29.7±1	26.4	553	21
day 13	9.1±4.1	23.14	11.4	664.6	60
CD34 <sup>+</sup> cells were cultured (2x10 <sup>5</sup> cells/0.5 ml) with DI-FRIL or SCF+Flt3 ligand+IL-3+GCSF+IL-6. Cell cycle analysis was performed with propidium iodide staining. Cell numbers were calculated by multiplying percent of cycling cells by total cell numbers. Data represents mean±SE values, from 5 experiments.					

**[0210]** More dramatically, the number of cells in SG<sub>2</sub>M phase during culture differed from the 6.8x 10<sup>3</sup> cells in SG<sub>2</sub>M phase initially seeded. The average number of cycling cells in DI-FRIL cultures remained relatively constant from 35x10<sup>3</sup> cells at day 3 and 43.6x10<sup>3</sup> cells at day 6 to a reduced level of 26.4x10<sup>3</sup> cells at day 10 and 11.4x10<sup>3</sup> cells at day 13. This pattern of cells in cycle explains the low levels of total cell numbers and progenitor cells (see Fig. 27). In contrast, the expected number of cycling cells in cytokine cultures increased dramatically. The average number of cells in SG<sub>2</sub>M phase in cytokine cultures increased by 31-fold to 212x10<sup>3</sup> cells at day 3, by 57-fold to 389.5x10<sup>3</sup> cells at day 6, by 81-fold to 553x10<sup>3</sup> cells at day 10, and by 97.7-fold to 664.6x10<sup>3</sup> cycling cells at day 13, compared to 6.8 x 10<sup>3</sup> cells in SG<sub>2</sub>M seeded in culture (Table 1). Taken together, these results support the notion that DI-FRIL preserves more immature progenitor cells in a quiescent state up to 2 weeks in culture compared to cytokine treated cultures.

**[0211]** To test whether DI-FRIL acts in a dominant manner to prevent cytokines from inducing the quiescent progenitor population into SG<sub>2</sub>M phase, the cell cycle status of cells cultured with either DI-FRIL or Flt3-L, in the presence and absence of cytokines was analyzed, after 3 days of suspension culture as indicated in Figs. 35A-35G and Table 6. Figs. 35A-35G show representative cell cycle histograms of CD34<sup>+</sup> cells cultured with no addition (Fig. 35A), DI-FRIL alone (Fig. 35B) or various cytokines or combinations thereof (Figs. 35C-35G) for 3 days, and the mean percentage of cells in each cell cycle phase is summarized in Table 6.

TABLE 6

Cell cycle status of CD34 <sup>+</sup> cells, <i>ex vivo</i> cultured for 3 days			
Culture conditions	Sub G <sub>0</sub> /G <sub>1</sub>	G <sub>0</sub> /G <sub>1</sub>	SG <sub>2</sub> M
DI-FRIL	9.4±2.8	72.9±2.8	17.7±1.9
Flt3-L	9.4±3.3	68.4±2.1*	22.2±5.4*
DI-FRIL+Flt3-L	8.6±3.9	67.1±1.8*	24.3±5.65*
Cytokines	2.6±1.7	46.5±4.5*	50.9±1.4*
Cytokines+DI-FRIL	2.5±0.6	47.5±2.2*	50.0±2.7*
Cytokines+Flt3-L	2.1±0.5	47.7±2.4**	50.2±2.9*

Data shown is mean percentage ± SE, from 4 independent experiments.  
p values = (vs. DI-FRIL): \* p<0.05.

**[0212]** As was also shown in Table 5, DI-FRIL maintains a significantly higher percentage of quiescent cells and lower percentage of cycling cells (Fig. 35B and Table 6) compared to stimulation with cytokines (Figs. 35C-35G and Table 6). Cultures with Flt3-L alone led to a modest, although significant, decrease percentage of cells in G<sub>0</sub>/G<sub>1</sub>, (p<0.05) and to a corresponding increase in SG<sub>2</sub>M phase, compared to cells cultured with DI-FRIL alone (Table 6). Cultures containing DI-FRIL and Flt3-L together slightly increased the percentage of cells in SG<sub>2</sub>M phase, from 17.7% (with DI-FRIL alone) to 24.3% (p<0.05, Table 6). As shown in Table 5, exposure of CB CD34<sup>+</sup> cells to cytokines for 3 days induced a substantially greater proportion of cells into SG<sub>2</sub>M phase compared to culture with DI-FRIL alone (p<0.05). The percentage of cells undergoing apoptosis, as determined by subG<sub>0</sub>/G<sub>1</sub> population, when cultured in DI-FRIL was slightly higher than under cytokine conditions. Neither DI-FRIL nor Flt3-L, under these culture conditions, effected cytokine induction into SG<sub>2</sub>M phase of the mostly quiescent CD34<sup>+</sup> cell population (Table 6).

#### EXAMPLE 7

##### DI-FRIL Preserves CB Progenitors in a Dormant State in the Presence of Potent Stimulators

**[0213]** DI-FRIL was purified from *Dolichos lab lab* as described above in Example 1. DI-FRIL was assayed over a 5-log dose range (10 ng/mL-1,000 ng/mL) on human CB MNC cultured in serum-defined medium containing agar-leukocyte conditioned medium, a potent source of a broad range of stimulators. The number of viable MNC and progenitors were evaluated after 5 days in culture. Results from 1 of 3 representative experiments are shown in Table 7 below. The number of viable MNC at the end of culture was reduced by 1.7 - to 5-fold in cultures containing 10-1,000 ng/ml of DI-FRIL. The frequencies of myeloid and erythroid progenitors in these cultures increased from 1.4- to 2.4-fold over the same dose range.

TABLE 7

DI-FRIL	MNC	Progenitor frequency	
(ng/ml)	(x 10 <sup>-4</sup> )	(Colonies/10 <sup>-5</sup> MNC)	
		Myeloid	Erythroid
1,000	20	90 +/- 85	150 +/- 42
100	55	44 +/- 15	85 +/- 12
10	70	77 +/- 12	103 +/- 6
1	120	16 +/- 2	38 +/- 7
0.1	115	21 +/- 7	50 +/- 7
0	120	38 +/- 7	63 +/- 32

**Culture of CB MNC in DI-FRIL results in fewer MNC and a greater frequency of progenitors.** 4 x 10<sup>6</sup> CB MNC were cultured in 2 mL of AIMV (Life Technologies) containing Agar-SCM (StemCell Technologies) and varying concentrations of DI-FRIL. Cultures were harvested after 5 days and the number of viable MNC and progenitors were assessed. The colony data shown were normalized as the frequency of progenitors per 10<sup>5</sup> MNC.

**[0214]** A reduction in cell number and a corresponding increase in the frequency of progenitors in cultures containing DI-FRIL in the presence of potent stimulators are consistent with our hypothesis that DI-FRIL can prevent cytokine-induced proliferation and differentiation of progenitors. Table 8 below shows the relative decrease of MNC in DI-FRIL-

containing cultures compared to controls and the relative increase in progenitor frequency of progenitors. The total number of progenitors was reduced as expected (progenitors are at varying stages of differentiation no direct correlation between progenitor number and MNC would be expected).

TABLE 8

DI-FRIL (ng/ml)	Fold DECREASE	Fold INCREASE	
	MNC	Progenitor frequency	
		Myeloid	Erythroid
1,000	5.0	2.4	2.4
100	2.0	1.2	1.4
10	1.7	2.1	1.6
1	1.0	0.4	0.6
0.1	1.0	0.6	0.8
0	1.0	1.0	1.0

**Reduction in MNC correlates with the relative increase in frequency of progenitors.** The relative reduction in MNC of DI-FRIL cultures is consistent with increased progenitor frequency in corresponding samples.

### EXAMPLE 8

DI-FRIL protects CB MNC from the toxicity of chemotherapy drugs

**[0215]** Experiments showing that DI-FRIL can prevent proliferation and differentiation of CB progenitors in cultures containing potent stimulators indicated that DI-FRIL protects progenitors from the toxicity of cell cycle-active chemotherapy drugs. The culture system described above was adapted to a 96 well plate format and the widely used chemotherapeutics, cytarabine (Ara-C) (Fig. 36A), doxorubicin (Dox) (Fig. 36B), and 5-fluorouracil (5-FU) (Fig. 36C) over a 5-log dose range were assayed on CB MNC cultured in the presence and absence of DI-FRIL (see Figs. 36A-36C). DI-FRIL was purified from *Dolichos lab lab* as described above in Example 1. Cultures containing DI-FRIL (either at 10 ng/ml or 100 ng/ml) resulted in a 2-3 log dose shift of CB MNC to Ara-C (Fig. 36A and Dox (Fig. 36B). As shown in Fig. 36C, the presence of DI-FRIL in 5-FU cultures increased viability over a large dose range. Differences between the dose shift of Ara-C and Dox by DI-FRIL compared to 5-FU may be explained by recent reports demonstrating that 5-FU acts via an RNA mechanism rather than as a DNA-specific drug (Bunz et al., *J. Clin. Invest.* 104:263-269,1999).

### EXAMPLE 9

Mice tolerate high levels of DI-FRIL

**[0216]** The *in vivo* toxicity of DI-FRIL was determined in mice. Initially, DI-FRIL was administered intravenously to mice over a 3-log dose range of 0.006-1 mg/kg (0.32-20 µg/mouse). DI-FRIL was well tolerated and these mice have subsequently received 2 monthly challenges of DI-FRIL without any observable short- or long-term adverse effects.

**[0217]** Protocols to test chemoprotective properties of cytokines in mice typically involve daily pre-treatment (bolus or continuous delivery) regimens of 4-10 days before starting chemotherapy. Using this framework as a starting point, DI-FRIL was injected at 5 mg/kg (100 µg/mouse) intravenously daily for 4 days. No gross adverse effects have been observed in over 150 mice treated with this dose regimen. DI-FRIL was purified from *Dolichos lab lab* as described above in Example 1.

**[0218]** The upper limits of DI-FRIL toxicity were next explored by injecting a single bolus intraperitoneal injection of DI-FRIL (to accommodate 1 mL volume) at 500 mg/kg and monitored the survival of mice for 48 hours. Of the four BALB/c mice receiving this treatment (2 males and 2 females, aged 5 months), only 1 mouse (a male) survived 48 hours. The surviving mouse's weight decreased by approximately 15% in the first 2 days and returned to normal by day 4. The surviving mouse's blood counts were in the normal range 3 days after injection of FRIL. The results demonstrate that even a very large dose of DI-FRIL is not completely toxic.

**[0219]** Additional dose range finding studies are performed to determine toxicity in mice receiving high doses of DI-FRIL administered by various routes (intravenous, intraperitoneal, subcutaneous, and oral).

**EXAMPLE 10***in vivo* modulation of progenitors by DI-FRIL in mice

**[0220]** Using the initial planned dose regimen of DI-FRIL (5 mg/kg x 4 days) for testing chemoprotection, hematopoietic parameters were examined in mice 3, 5, and 7 days after completing DI-FRIL treatment. DI-FRIL was purified from *Dolichos lab lab* as described above in Example 1. Weight-matched BALB/c mice (females, aged 8 weeks) were injected intravenously with either 0.2 ml of DI-FRIL (500 mg/ml) or 0.2 ml of HBSS daily for 4 days. Two mice from each group were evaluated at 3 days, 5 days, and 7 days after completing DI-FRIL treatment. Blood was collected in heparinized tubes by eye bleeds prior to sacrificing mice by CO<sub>2</sub>. Two femurs and a spleen were harvested from each mouse and the samples were processed within 1 hour. Progenitors were accessed using standard hematopoietic colony assays (StemCell Technologies). The results from this study are shown below in Table 9.

TABLE 9

Hematopoietic parameters 3, 5, and 7 days after DI-FRIL treatment (5 mg/kg x 4days)						
Cellularities						
Days	RBC		WBC	BM	Spleen	
3	0.58		0.34	0.59	1.01	
5	1.26		0.98	0.75	0.72	
7	0.95		0.82	0.91	1.12	
Bone marrow progenitors						
Days	CFU-C		CFU-E		BFU-E + Mix	
	Freq. Total		Freq	Total	Freq	Total
3	1.85	1.10	1.63	1.01	1.61	1.01
5	1.17	0.87	0.86	0.63	1.03	0.80
7	1.81	1.69	0.61	0.57	2.59	2.47
Spleen progenitors						
Days	CFU-C		CFU-E		BFU-E + Mix	
	Freq.	Total	Freq	Total	Freq	Total
3	-	-	2.83	3.35	-	-
5	2.41	1.68	0.95	0.63	2.12	1.42
7	-	-	1.74	1.78	-	-
DI-FRIL data are reported as relative to control values.						

**[0221]** As shown in Table 9, the peripheral blood counts (red blood cell (RBC) and white blood cell (WBC)) of DI-FRIL-treated mice were found to be reduced by 1.7- and 2.9-fold, respectively, at 3 days, and returned to normal by day 5. Bone marrow (BM) cellularity was also reduced by 2.5-fold at day 3, and returned to normal after 7 days. The spleen cellularity was lower at day 5 but normal at day 3 and day 7.

**[0222]** As shown in Table 9, the frequency of progenitors was slightly increased in bone marrow, by 1.6- to 1.85-fold at day 3, but the total number of progenitors in the bone marrow remained unchanged. In the spleen, a compensatory organ during hematopoiesis stress, a 2.83-fold higher frequency and 3-35-fold higher number of erythroid progenitors were observed in the spleen at day 3 (see Table 9). The frequencies and total number of progenitors in the bone marrow appeared normal at day 5 but fewer mature erythroid progenitors (CFU-E) and more primitive progenitors (BFU-E/mix) were observed at day 7. A similar reduction in CFU-E was observed in spleens at day 5; otherwise, the frequencies and total numbers of progenitors increased from days 3-7.

**EXAMPLE 11**DI-FRIL protects mice from 5-FU induced death in the critical first week

**[0223]** A dose regimen was established to determine whether FRIL protects mice from death resulting from hemat-



opoietic toxicity of Ara-C and Dox. This murine 5-FU chemoprotection model is based on studies showing that a single dose of 5-FU (150 mg/kg) resulted in >90% reduction of bone marrow cellularity but had limited cytotoxic effect on stem cells (Lerner and Harrison, *Exp. Hematol.* 18:114-118, 1990). This finding was consistent with the understanding that stem cells reside in the bone marrow in predominantly a quiescent state. Bone marrow cellularity in these mice was restored after 2 weeks by the recruitment of the dormant progenitors and responsive stem cells that escaped toxicity of 5-FU. Administration of a second dose of 5-FU (also at 150 mg/kg) 3-7 days after the initial dose killed those stem cells and progenitors recruited into S-phase in response to the first treatment of 5-FU (Lerner and Harrison, *supra*).

[0224] de Haan et al. (*Blood* 87:4581-4588, 1996) applied this model to test whether prophylactic treatment of mice with hematopoietic regulators, pegylated SCF + IL11, could expand the stem cell and primitive progenitor compartments and better protect mice from death by 5-FU induced hematopoietic toxicity. These experiments demonstrated that although SCF + IL11 pretreatment could accelerate hematopoietic recovery after it was underway by 4 days-5 days when compared to controls (from approximately 11 days to 7 days for 40% survival, the SCF + IL11 cytokine pretreatment strategy did not rescue mice from death when the second dose of 5-FU was administered in the critical first week when stem cells are ablated (de Haan et al. *supra*).

[0225] In this study described below, the pretreatment dose regimen for DI-FRIL described above (5 mg/kg x 4 days) was selected based on the requirement of treating animals with cytokines for several days prior to starting chemotherapy and because DI-FRIL-treated mice easily tolerated doses (5 mg/kg) that were 10- to 100-fold greater than that used for cytokines. Since FRIL and cytokines act on progenitors in the same concentration range (ng/ml), this pretreatment dose regimen was used to test whether DI-FRIL can protect mice from 5-FU administered at 2 intervals in the first week: 5-FU (150 mg/kg) was injected at day 0 and then a second dose (also at 150 mg/kg) was injected either on day 3 (d0/3 dose interval) or day 5 (d0/5 dose interval). No survival on the day 3 interval was observed by de Haan et al. (*supra*) at day 3.

[0226] DI-FRIL was purified from *Dolichos lab lab* as described above in Example 1.

[0227] Weight-matched BALB/c mice (10 mice/group) were injected intravenously with either with 0.2 mL of DI-FRIL (500 mg/mL) or 0.2 mL of HBSS daily for 4 days. Two hours after the final treatment of DI-FRIL, mice were injected intraperitoneally with 5-FU (150 mg/kg). Groups of mice received a second dose of 5-FU (150 mg/kg) at either day 3 or day 5. No mice died from a single dose of 5-FU.

[0228] As shown in Table 10, DI-FRIL pretreatment improved survival of mice in two separate experiments.

TABLE 10

Exp.	Mice	5-FU Dose Interval			
		D0/3		D0/5	
		FRIL	HBSS	FRIL	HBSS
1	Males, 16 wk	3/10	0/10	N.T.	N.T.
2	Females, 8 wk	0/10	0/10	4/10	1/10
Improved survival of mice pretreated with FRIL (5mg/kg x 4 days) prior to undergoing 5-FU dose intervals of d0/3 and d0/5.					

[0229] In the first experiment, 3 of 10 mice survived a d0/3 dose interval of 5-FU compared to no mice in the HBSS pretreatment control. In the second experiment, 4 of 10 mice pretreated with DI-FRIL survived a d0/5 dose interval of 5-FU compared to 1 of 10 for HBSS pretreated mice.

## EXAMPLE 12

### Optimization of the dose regiment of a FRIL family member to protect mice from 5-FU induced death

[0230] FRIL family members are relatively abundant in legumes. For example, DI-FRIL accounts for approximately 0.02% of the mass of hyacinth beans.

[0231] DI-FRIL is purified by carbohydrate affinity chromatography as described in Example 1, and is evaluated for purity by SDS-PAGE (5 discrete bands are visualized on an overloaded gel; see Fig. 37); is analyzed for mass and composition by amino acid analysis; and is assayed in the cord blood progenitor assay described above.

[0232] The murine 5-FU chemoprotection model (Lerner and Harrison, *supra*; de Haan, *supra*) is used to empirically derive the optimal dose regimen of DI-FRIL to protect mice from death. DI-FRIL is administered intravenously to mice over a 3-log dose range (5 - 5,000 mg/kg) under various regimens that include DI-FRIL treatment prior to chemotherapy (daily from 3 days to 2 hour before initiation of chemotherapy) and during chemotherapy.

[0233] The mice used in these studies are BALB/c female mice, 8-10 weeks at outset of experiments (Jackson

Laboratory, Bar Harbor, ME), weight matched each for experiment, where there are 10 mice per group. Organs from mice receiving a dose of 5,000 mg/kg of FRIL (with no 5-FU treatment) are collected for toxicity studies

**[0234]** The five doses of DI-FRIL are 0, 5, 50, 500, and 5,000 µg/kg. The four dose regimens of DI-FRIL will be -2hour; -1day and -2hour; -2day, -1day, and -2hour; - 3day, -2day, -1day, and -2hour prior to 5-FU treatment. The two maintenance regimens are either daily x 7 day (-2hour to day 7) or every other day (days 0, 2, 4, 6). Thus, one group of mice will receive a dose of DI-FRIL daily for 7 days; while the second group will receive a dose of DI-FRIL every other day for 7 days.

**[0235]** The 5-FU dose intervals of 150 mg/kg are at dose intervals of d0/3, d0/5, and d0/7.

### **EXAMPLE 13**

A FRIL family member has chemoprotective properties with widely used cell cycle-active chemotherapeutics

**[0236]** After establishing the optimal dose regimen of a FRIL family member, the FRIL family member's ability to protect mice from death by cytarabine (Ara-C) and doxorubicin.

**[0237]** Initial dose regimens of cytarabine (Ara-C) and doxorubicin are as follows: Doxorubicin-14 mg/kg as single bolus i.p. injection (Grzegorzewski et al., *J.Exp.Med.* 180:1047-1057, 1994); Ara-C - 300 mg/kg at time as an i.p. injection at 0 and 12 hours (Paukovits et al., *Blood* 77:1313-1319, 1991). Further studies are based on targeted clinical indication.

### **EXAMPLE 14**

Characterization of the hematopoietic status of mice during optimal dose regimen of a FRIL family member to protect mice from 5-FU induced death

**[0238]** Peripheral blood counts and the status of hematopoietic progenitors (frequency, total number, and cycling status) are characterized in mice during and after receiving the optimal dose regimen of a FRIL family member.

**[0239]** To do this, mice injected with a dose regimen of a FRIL family member with no 5-FU treatment are evaluated daily during and for one week after treatment with the FRIL family member. The mice are evaluated for the following hematopoietic parameters: WBC and RBC counts; bone marrow and spleen cellularities; and progenitor status, which includes hematopoietic colony assays (StemCell Technologies). The progenitors assayed are myeloid (CFU-C), erythroid (CFU-E), and primitive, multipotential (BFU-E/Mix). The frequencies and total numbers are determined, as well as the cycling status of these cells, as measured by <sup>3</sup>H-thymidine suicide assay (Moore et al., *Exp. Hematol.* 14: 222-229, 1986).

### **EXAMPLE 15**

Analysis of pharmacology and toxicology of FRIL in mice

**[0240]** The clearance of a FRIL family member from the circulation and its accumulation in the body is determined by preliminary pharmacokinetics.

**[0241]** For these studies, <sup>125</sup>I-FRIL is injected into mice (dosage of FRIL based on optimization results). Clearance of FRIL from the blood is evaluated at the following timepoints after injection: 5 min., 15 min., 30 min., 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, 36 hours, 48 hours. Two mice are evaluated at each timepoint. Following this study, the animals are sacrificed and the organs collected and evaluated.

**[0242]** Dose-range finding experiments determine the maximal tolerated dose of a FRIL family member by various routes of administration (intravenous, intraperitoneal, subcutaneous, and oral). To do this dose range finding study, four routes at four doses are used. The routes of administration are intravenous, intraperitoneal, subcutaneous, oral. The highest dose of a FRIL family member is 1 g/kg. The dosage of the FRIL family member is reduced by 2-fold until mice survive. Survival is measured at 48 hours after treatment. Other clinical observations are made, including behavioral, lethargy, vocalization, diarrhea. CBC analyses are made. The animals are evaluated for any necropsy (i.e., gross lesions). Following this study, the animals are sacrificed and the organs collected and evaluated.

**[0243]** Acute dose toxicity studies allow identify target organs that may develop lesions after exposure to a FRIL family member. For these acute dose toxicity studies, a dose is selected, and a FRIL family member is injected daily for 7 days. Acute toxicity is evaluated at 7 days, and recovery from acute toxicity is evaluated at 21 days. Blood chemistries, target organs, bone marrow and blood, and other health indicator are evaluated.

**[0244]** Hypersensitivity studies in guinea pigs are performed to test for any adverse immunologic reactions. To do this, fifteen guinea pigs (5 FRIL, 5 DNCB positive control, 5 saline negative control) are used. A FRIL family member is intradermally injected at 0.1 mL. Daily clinical observations at site for redness and edema are compared to the DNCB

positive control. The FRIL guinea pigs are challenged at 2 weeks with 0.05 mL of the FRIL family member, and daily clinical observations are made.

**[0245]** A determination of development of mouse anti-FRIL family member antibodies in mice receiving treatment with a FRIL family member is made to determine the extent and nature of the body's response to a FRIL family member. To do this, a FRIL family member is attached to Dynal's tosylactivated magnetic beads. The FRIL family member-coated beads are incubated with plasma (pooled or individual) from a mouse who has received treatment with the FRIL family member. Using rabbit and rat antiserum to FRIL used as positive control, the presence of FRIL family member-specific antibodies is evaluated by SDS-PAGE and Western blot analysis (horseradish peroxidase or chemiluminescence). Lastly, a determination is made as to whether sugar blocks antibody binding ( $\alpha$ -D-mannopyranoside and negative control).

## **EXAMPLE 16**

### Purification of Progenitor Cells using DI-FRIL-Coated Magnetic Beads

**[0246]** Using magnetic beads coated with a non-limiting FRIL family member, DI-FRIL, a population of progenitor cells was isolated and characterized. To do this, the following methods were used.

#### Preparation of FRIL-beads for cell isolation

**[0247]** DI-FRIL was purified from *Dolichos lab lab* seeds as described in Example 1. DI-FRIL can be immobilized on magnetic beads (M-280 Dynabeads Tosylactivated, Lake Success, NY) via amino- and sulfhydryl-groups of the lectin according to the manufacturer's directions. DI-FRIL can also be immobilized on magnetic beads by a biotin-streptavidin interaction.

**[0248]** In this example, DI-FRIL was immobilized on magnetic beads by a biotin-streptavidin interaction. Biotinylation of DI-FRIL via primary amine-groups (EZ-Link Sulfo-NHS-LC-LC-Biotin, Pierce Chemical Company, Rockford, IL) was carried out according to the manufacturer's directions: Biotinylated DI-FRIL was incubated with streptavidin-labeled magnetic beads (Dynal or Miltenyi Biotec, Auburn, CA) according to the manufacturer's directions.

#### Preparation of cells

**[0249]** Human cord blood (CB), peripheral blood, and bone marrow, collected in sterile receptacles containing anti-coagulant (e.g., heparin, EDTA), was processed to isolate mononuclear cells (mnc) within six hours of collection by density centrifugation on Ficoll-Paque PLUS (Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's directions. Mononuclear cells harvested at the interface of plasma and Ficoll-Paque were washed and resuspended in serum-defined medium (e.g., XVIVO-10, Biowhittaker, Walkerville, MD or AIM-V, Life Technologies, Rockville, MD).

#### DI-FRIL-bead cell isolation

**[0250]** DI-FRIL-coated beads specifically bound a minor mnc population found in CB, peripheral blood, and bone marrow. A ten-fold excess of DI-FRIL-beads was incubated with the cells. For CB, where DI-FRIL-beads captured approximately 1% of mnc, the ratio of beads to cells was 1:10, or 10-fold greater number of beads for every target cell. The ratio for other FLT3-expressing cell populations, was hematopoietic and non-hematopoietic, was experimentally determined by serial exposure of cells to fresh DI-FRIL-beads.

**[0251]** DI-FRIL-beads were washed twice in serum-defined medium prior to use. An aliquot of DI-FRIL-beads was added to 10 mL of serum-defined medium in a 15 mL conical centrifuge tube (Falcon, Becton-Dickinson, Lincoln, NJ), mixed, and placed in a magnet (Dynal or Miltenyi Biotec, depending on source of magnetic beads) for ten minutes. Medium was aspirated with a 10 mL pipette without disturbing beads bound to walls of centrifuge tube by the magnetic charge from the magnet. After washing, 0.5 mL of serum-defined medium was added to the tubes to wash the beads from the walls to the bottom of the conical tube. Medium was added to beads in a small volume (<2 mL) and the centrifuge tube was tumbled on a rocker in a cold room (i.e., at approximately 4°C) for one hour. After incubation, serum-defined medium was added to a final volume of 10 mL and the tube was placed in the magnet for ten minutes. Medium was removed by aspiration without disturbing cells bound to DI-FRIL-beads on the walls of the centrifuge tube via the magnetic charge. Cells were washed a second time by removing the conical tube from the magnet, adding 10 mL of serum-defined medium, mixing cells, and placing the conical tube back onto the magnet. Following aspiration of the medium, the final volume was adjusted to 2 mL.

Detachment of DI-FRILbeads from cells

**[0252]** For some applications, detachment of DI-FRIL-beads from cells is preferred. About half of DI-FRIL-beads detached from CB mnc after overnight incubation on a rocker in the cold room. Although binding studies have demonstrated that excess mannose and  $\alpha$ -methyl  $\alpha$ -D-mannoside prevent DI-FRIL from binding to Flt3, neither sugar released tightly bound DI-FRIL-beads from CB mnc. To remove DI-FRIL-beads from this subpopulation of cells, the cells were incubated in 100 mM trehalose (Sigma, St. Louis, MO) for one hour on a rocker in the cold room.

**[0253]** It should be noted that since Miltenyi beads are very small (approximately 50 nm) as compared to Dynal beads (approximately 10  $\mu$ m), when Miltenyi beads were used to purify DI-FRIL-binding progenitor cells, the beads were allowed to remain attached to the purified progenitor cells.

Receptor tyrosine kinase gene expression

**[0254]** Receptor tyrosine kinase gene expression was characterized by RT-PCR.

**[0255]** Using these methods, the following results were obtained:

Functional properties of DI-FRIL bead-selected CB mnc

**[0256]** The progenitor capacity of DI-FRIL-selected CB mnc was tested in a methylcellulose colony assay under conditions to promote proliferation and differentiation along either the hematopoietic or endothelial lineages (as described above. Table 11 shows the number of hematopoietic colonies (myeloid, erythroid, and mix) and endothelial colonies (other) that formed after culture of unselected cells (CB mnc), DI-FRIL-selected cells (DI-FRIL<sup>+</sup>) and CB mnc that did not bind to DI-FRIL-beads (DI-FRIL<sup>-</sup>).

TABLE 11

Response of DI-FRIL-selected cells to hematopoietic and endothelial stimuli						
Stimulator		Myeloid	Erythroid	Mix	Other	Total
<b>+ CSFs</b>	CB mnc	1	3	2	0	6
	DI-FRIL <sup>+</sup>	14	10	4	0	28
	DI-FRIL <sup>-</sup>	1	1	2	0	4
<b>+ VEGF</b>	CB mnc	0	0	0	2	2
	DI-FRIL <sup>+</sup>	0	0	0	19	19
	DI-FRIL <sup>-</sup>	0	0	0	5	5
Cord blood mononuclear cells were isolated by Ficoll-Paque, bead selected, and plated in MethoCult® StemCell Technologies, Vancouver, BC, Canada).						

**[0257]** As shown in Table 11, DI-FRIL-selection increased the number of hematopoietic colonies (stimulated with colony stimulating factors (CSFs) by 14-fold for myeloid colonies, 3.3- to 10-fold for erythroid colonies (CB mnc and DI-FRIL<sup>-</sup> cells, respectively), and by 2-fold for mixed colonies. Similar levels of DI-FRIL-bead enrichment was observed for endothelial colonies (stimulated with vascular endothelial growth factor (VEGF)): 9.5-fold over CB mnc and 3.8-fold for DI-FRIL cells.

Cell surface phenotypic properties of DI-FRIL bead-selected CB mnc

**[0258]** The cell surface phenotypic properties of DI-FRIL bead-selected CB mnc was characterized by flow cytometry. Table 12 shows the phenotypes (by percentage of cells expressing the indicated cell surface phenotype marker) of the three CB cell populations: (1) cells not selected by DI-FRIL-beads (DI-FRIL<sup>-</sup>); (2) cells that detached from DI-FRIL-beads after overnight incubation in the coldroom on a rocker (DI-FRIL<sup>+</sup>); and (3) cells that retained DI-FRIL-beads after overnight incubation (DI-FRIL<sup>++</sup>). The two DI-FRIL-binding cell populations were analyzed separately to see whether tightness of binding (avidity) related to type of cells selected. Isotype control levels were set at 2%; all values of 2% represent no reactivity with test antibody.

TABLE 12

Flow cytometric analysis of DI-FRIL-selected CB mnc				
Antigen	Cell Type	DI-FRIL <sup>-</sup> (%)	DI-FRIL <sup>+</sup> (%)	DI-FRIL <sup>++</sup> (%)
CD3	Mature T	26	35	6
CD11b	Mac-1, CR3	19	35	67
CD11c	LeuCAMc	10	22	32
CD13	Pan myeloid, CFU-GM	5	<2	<2
CD19	Pan B	4	5	12
CD32	Low affinity IgG Fcγ-R	5	19	26
CD33	Myeloid progenitors	3	2	8
CD34	Pan progenitors	<2	<2	<2
CD38	Activated T	88	96	93
CD42a	Platelet gplX	5	2	7
CD69	Early activation ag (EA-1)	6	8	14
CDw90	Thy-1, progenitor subset	8	14	13
CD117	c-kit, progenitors	4	2	2

**[0259]** As shown in Table 12, DI-FRIL-beads did not capture CB mnc that express CD34, the hallmark marker of hematopoietic stem cells and progenitors. This observation was unexpected because DI-FRIL-selected cells enrich for progenitors (see Table 11). Although CB CD34<sup>+</sup> cells uniformly express FLT3, only 70% of Flt3<sup>+</sup> CB mnc also express CD34 (Rappold et al., *Blood* 90:111-125, 1997). Consequently, 30% of CB mnc expressed the phenotype of CD34 Flt3<sup>+</sup>. DI-FRIL-beads appeared to capture this latter population of cells.

**[0260]** Cells expressing dendritic cell (DC) markers, CD11b and CD11c, were enriched approximately 2-fold in the DI-FRIL<sup>+</sup> cell population and over 3-fold in the DI-FRIL<sup>++</sup> cell population (Table 12). This observation is consistent with reports that Flt3 is involved in dendritic cell proliferation and maturation in mice (Pulendran et al., *J. Immunol.* 159: 2222-2231, 1997) and humans (Miller et al., *Blood* 93:96-106., 1999). The rare hematopoietic dendritic cell population is useful in inducing tumor regression and for the treatment of AIDS.

**[0261]** Differences of the cell surface phenotypes were observed between DI-FRIL<sup>+</sup> and DI-FRIL<sup>++</sup> CB cells (see Table 11). The percentage of CD3 T cells decreased from 35% for DI-FRIL<sup>+</sup> cells to 6% for DI-FRIL<sup>++</sup> cells. Conversely, the percentage of CD11b<sup>+</sup> cells and CD11c<sup>+</sup> cells increased from 35% to 67% and from 22% to 32% for DI-FRIL<sup>+</sup> and DI-FRIL<sup>++</sup> cell populations, respectively.

**[0262]** Cells that retained DI-FRIL-beads after overnight incubation on a rocker in the cold room (DI-FRIL<sup>++</sup> cells) were observed as single cells or as clumps of beadbound cells. These clumps could not be disrupted either by mechanical means or by elution with competing sugars, mannose or mannose derivatives (data not shown). From studies to characterize the carbohydrate-binding properties of DI-FRIL, α,α-trehalose demonstrated a 3.6-fold greater potency than mannose and a 1.6- to 2.1-fold greater potency than α-methyl α-D-mannoside derivatives that were tested (Mo et al., *Glycobiology* 9:173-179, 1999). Incubation of clumped DI-FRIL-bead bound cells with 100 mM Trehalose effectively disrupted the clumped cells and removed most of the DI-FRIL-beads from cells.

**[0263]** Two populations of trehalose-disrupted DI-FRIL<sup>++</sup> cells were analyzed by flow cytometry: cells that no longer bound beads (DI-FRIL<sup>++</sup>) and cells that still retained beads following incubation with trehalose (DI-FRIL<sup>+++</sup>). The results of one experiment are shown in Table 13.

TABLE 13

Flow cytometric analysis of DI-FRIL-selected CB mnc after exposure to trehalose			
Antigen	Cell Type	DI-FRIL <sup>++</sup> (%)	DI-FRIL <sup>+++</sup> (%)
CD3	Mature T	6	3
CD11b	Mac-1, CR3	52	76
CD11c	LeuCAMc	16	43
CD13	Pan myeloid, CFU-GM	72	46
CD19	Pan B	5	9
CD32	Low affinity IgG Fcγ	43	19
CD33	Myeloid progenitors	56	15

TABLE 13 (continued)

Flow cytometric analysis of DI-FRIL-selected CB mnc after exposure to trehalose			
Antigen	Cell Type	DI-FRIL <sup>++</sup> (%)	DI-FRIL <sup>+++</sup> (%)
CD34	Pan progenitors	2	2
CD117	c-kit, progenitors	2	12
CD135	Flt3, progenitors	2	5

**[0264]** The difference in cell surface phenotypes between DI-FRIL<sup>++</sup> cells and DI-FRIL<sup>+++</sup> cells in Table 13 was greater than those observed for DI-FRIL<sup>+</sup> cells and DI-FRIL<sup>++</sup> cells in Table 12. In Table 13, the percentage of CD3, CD13, CD32, and CD33 cells decreased by 1.6- to 3.7-fold in DI-FRIL<sup>+++</sup> cells compared to DI-FRIL<sup>++</sup> cells. Conversely, the percentage of CD11b, CD11c, CD19, CD117, and CD135 cells increased by 1.5- to 6-fold in DI-FRIL<sup>+++</sup> cells compared to DI-FRIL<sup>++</sup> cells. Again no CD34 was observed in either cell population.

**[0265]** The increase in percentage of cells that express CD117 and CD135, two tyrosine kinase receptors central to hematopoietic stem cell and progenitor function (Lyman and Jacobson, *Blood* 91:1101-1134, 1998), suggested that avidity of DI-FRIL-bead binding of cells might correspond to the primitive status of the cells. The studies described herein which characterize the carbohydrate binding properties of DI-FRIL supported this notion. DI-FRIL has neither an extending carbohydrate combining-binding site nor a hydrophobic binding site adjacent to it (Mo et al., *Glycobiology* 9:173-179, 1999). DI-FRIL binds most tightly to a trimannosyl structure that is the basis for N-linked glycosylation in mammals. Consequently, DI-FRIL may bind cells that have undergone less processing of glycosylation, which is consistent with more primitive cells. This property of DI-FRIL-binding may provide a unique method to isolate primitive cells not currently possible by antibodies to CD34. Thus, DI-FRIL binds the normally glycosylated FLT3 receptor more tightly than the FLT3-Ligand binds to normally glycosylated FLT3. DI-FRIL binds normally glycosylated FLT3 receptor more tightly than the typical antibody binds its specific ligand.

#### Receptor tyrosine kinase gene expression in DI-FRIL-selected CB cells

**[0266]** The number of cell surface receptors and markers increases as pluripotent hematopoietic stem cells proliferate and differentiate. The number of functional receptors on the most primitive cells is probably less than ten. The levels of detection for flow cytometry are probably in the range of several hundred cell surface molecules. Consequently, analysis of primitive cell populations cannot be analyzed by flow cytometry.

**[0267]** The presence or absence of functional tyrosine kinase receptors on primitive cells was further characterized by RT-PCR. Expression of Flt3, Kit, Fms, Flk1, Flt1, and Flt4 mRNA was determined for CB mnc, CD34-selected cells, and DI-FRIL-selected cells. For CB mnc, the PCR products for the receptors were either faint or not detectable. The pattern of gene expression for cells selected by CD34-beads or DI-FRIL-beads was the same; all tyrosine kinase receptors showed stronger PCR signals. These data suggest that receptors associated with stem cells (Flt3 and Kit) and primitive endothelial cells (Flk1, Flt1, and Flt4) are also detected in DI-FRIL selected cells.

#### EXAMPLE 17

##### Use of beads coated with a FRIL family member to isolate CD34<sup>+</sup> primitive stem cells

**[0268]** A rare human stem cell population with the phenotype of CD34<sup>+</sup>CD38<sup>-</sup>Lin<sup>-</sup> has been identified by its ability to establish multilineage engraftment in NOD/SCID mice (Bhatia et al., *Nat. Med.* 4:1038-1045, 1998). These repopulating cells give rise to stem cells that express the hallmark CD34 marker. Isolating CD34<sup>+</sup>CD38<sup>-</sup>Lin<sup>-</sup> cells is labor-intensive methods of negative selection that includes the use of immunomagnetic beads and flow cytometry to deplete cells that express CD34, CD38, and lineage markers. Rapid, efficient, positive selection of CD34<sup>+</sup>CD38<sup>-</sup>Lin<sup>-</sup> cells would be preferable for clinical uses. However, the absence of the highly expressed CD34 marker and low number of functional receptors on this rare population of cells will prevent use of antibodies for cell isolation.

**[0269]** FRIL attached to magnetic beads are used in a unique method to isolate the rare CD34<sup>+</sup>CD38<sup>-</sup>Lin<sup>-</sup> cell population by binding primitive cells that express this phenotype. Isolation of CD34<sup>+</sup>CD38<sup>-</sup>Lin<sup>-</sup> is achieved by a single-step cell isolation. However, since FRIL-beads also recognize cells that express CD11b, CD11c, and CD38, optimal isolation of CD34<sup>+</sup>CD38<sup>-</sup>Lin<sup>-</sup> cells is improved by first negatively selecting unwanted cells by immunomagnetic beads that bind to CD11b, CD11c, and/or CD38.

**EXAMPLE 18**

Use of beads coated with a FRIL family member to isolate normal stem cells from patients with leukemia

[0270] A majority of leukemias express the phenotype of CD34<sup>+</sup>Flt3<sup>+</sup> (Carow et al., *Blood* 87:1089-1096, 1996). Consequently, methods that rely on CD34 expression cannot distinguish normal stem cells and progenitors from leukemic cells in the bone marrow and peripheral blood of patients.

[0271] FRIL does not interact with two cell leukemic lines tested with the CD34<sup>+</sup>Flt3<sup>+</sup> phenotype (KG1-A and ML-1). FRIL neither effects growth of these leukemic cell lines nor do FRIL-beads capture appreciable numbers of cells (data not shown). Since FRIL-beads select normal progenitors with the phenotype of CD34<sup>+</sup>Flt3<sup>+</sup>, FRIL-beads provide a unique method that distinguishes between normal and leukemic cells.

[0272] A FRIL family member attached to magnetic beads is used to isolate normal hematopoietic stem cells and progenitors from the bone marrow and peripheral blood of leukemia patients. This is accomplished using a method similar to leukopheresis, where blood is passed through a device that retains cells of interest. In this example, FRIL-beads binds CD34<sup>+</sup>Flt3<sup>+</sup> normal cells. Since FRIL also interacts with Flt3-expressing CD11b and CD11c cells, prior exposure and removal of the cells that immunomagnetic beads that bind to CD11b and/or CD11c (*i.e.*, negative selection) may permit enrichment of primitive cells.

**EXAMPLE 19**

Use of FRIL-beads to isolate dendritic progenitors and mature cells from normal individuals

[0273] Dendritic cells (DC) are immune cells that capture antigens and initiate T cell-mediated immune responses (Banchereau and Steinman, *Nature* 392:245-252, 1998). DC act as first lines of defense in the skin, gut, and lymphoid organs. Antigens on DC can activate naive and quiescent T cells and small numbers of DC pulsed with lose dosages of antigens stimulate strong T cell responses. Under certain circumstances, DC also induce T cell tolerance. Consequently, the unique properties of DC has generated significant interest to use these cells to treat cancer and AIDS.

[0274] DC are derived from CD34<sup>+</sup> progenitors in the bone marrow of humans. The cytokines GM-CSF, TNF- $\alpha$ , and Flt3 ligand (FL) influence DC development (Banchereau and Steinman, *supra*; Pulendran et al., *J. Immunol.* 159: 2222-2231, 1997). Injection of FLT3-Ligand in mice dramatically increases the number of DC (Pulendran et al., *supra*).

[0275] FRIL interacts with the Flt3 receptor on DC, and FRIL-beads capture cells with the dendritic phenotype of CD11b and CD11c. Selecting dendritic cells with FRIL-beads from human bone marrow, peripheral blood, or cord blood allows the efficient and effective isolation of DC for clinical use.

**EXAMPLE 20**

Use of FRIL-beads to isolate endothelial stem cells and progenitors

[0276] Endothelial stem cells and progenitors give rise to cells that form blood vessels in a process called angiogenesis. During strokes and heart attacks, new blood vessels are needed to repair damage. Activation of endothelial stem cells and progenitors to produce more mature cells is mediated by the cytokines that activate the FLkT/KDR, Flt1, and Flt4 tyrosine kinase receptors. Flt3 is expressed on very primitive endothelial progenitors. FRIL-beads are used to capture a population of cells from cord blood that express all of these receptors.

**EXAMPLE 21**

Use of FRIL family members and Non-FRIL family member lectins to alter signal transduction and other cellular pathways

[0277] Drugs designed to alter signal transduction pathways need to specifically distinguish target cells. FRIL is used as a targeting vehicle to deliver small molecules to Flt3-expressing cells such as stem cells, progenitors, and dendritic cells. FRIL has several advantages for drug delivery: 1) FRIL is specific for Flt3; 2) FRIL is stable in the cytoplasm; 3) FRIL is capable of undergoing conjugation with small molecules; 4) FRIL can be delivered in dose-responsive manner; and 5) FRIL provides specificity for overlapping pathways of signal transduction.

[0278] Other legume- or bulb-derived lectins can also deliver small molecule drugs to specific cell populations. For example, the lectins PHA and ConA both bind to CD3-T cell receptor complex and the FC-gamma receptor (CD32) (Leca et al. *Scand. J. Immunol.* 23:535-544, 1986); UDA binds the V $\beta$  domain of the T cell receptor (Galelli et al., *J. Immunol.* 151:1821-1831, 1993).

[0279] Standard methods of conjugation are used to attach small molecules, oligos, or enzymes to plant lectins.

## EXAMPLE 22

### Purification and Cloning of YamFRIL from *Sphenostylis stenocarpa*

[0280] Dry seeds of Yam bean (*Sphenostylis stenocarpa*) were ground in a coffee mill and the powder was extracted with 5 volumes of 10 mM Na Acetate buffer, pH 5.2, containing 1 mM CaCl<sub>2</sub> for 1 hour at 4°C. After centrifugation, the clear supernatant was neutralized with Tris-HCl pH 8.0.

[0281] YamFril purification was achieved through and absorption on a ovalbumin gel affinity column (Sigma) and was eluted with 200 mM trehalose.

[0282] The resulting protein was fractionated into 2 polypeptides that were submitted to N-terminal amino acid sequences.

Beta band: AQSVSFTFTKFDSQ (SEQ ID NO: 9)

Alpha band: AASNNVVAVEFDTXPN (SEQ ID NO: 10)

[0283] Reverse-transcriptase PCR was performed on total RNA obtained from International Institute of Tropical Agriculture (Ibadan, Oyo State, Nigeria), using degenerate primers based on the alpha and beta N-terminus sequences (i.e., SEQ ID NO: 10 and SEQ ID NO: 9, respectively).

[0284] 3' RACE PCR was performed on total and polyA<sup>+</sup> RNA using gene specific primers with an Anchor Primer.

[0285] Partial cDNA clones were obtained and the following sequences deduced.

#### YamFril: partial mRNA sequence

ACGAAGTTCGACAGCGACCAAAAGGATCTTATGTTCCAAGGTCATACCATTCTAGCAGC  
AATGTCATACAACCTACCAAGTTAGACAGTAATGGAAACCCTGTGAGTACCAGTGTGGGA  
AGAGTGTATATACTCTGCACCATTGCGCCTTTGGGAAAGCTCTACAGTAGTGTCAACCTTT  
GAGACCACTTTTACCTTTCAAATCTCAACACCTTACACTAGTCCTCCTGGTGATGGGCTC  
GCCTTCTTCTTGCACCATATGACACTGTCAATCCCTCCAAATTCTGCTGGCAATCTTCTT  
GGACTCTTTCCTAACTTAAATGCTTTAAGAACTCCACCACCAGTAAAGAAACCACTATT  
GATGTCAATGCTGCATCTAACAACGTTGTTGCCGTTGAATTTGACACCTACCCTAACGAC  
AATATTGGTGATCCAAGATACAAACACATTGGAATCGATGTCAACTCTATCAGGTCCAAG  
GCAACTGTTGCGTGGGACTGGCAAAATGGGAAAACAGCCACTGCACACATCAGCTATAAC  
TCTGCCTCTAAAAGACTATCTGTTACTACTTTTTATCCTGGGGGTAAAGCTGTGAGTCTT  
TCCCATGACGTTGAGCTCACTCAAGTGCTTCCTCAATGGATTAGAGTAGGGTTCTCTGCT  
TCAACAGGATTAGAGAAA (SEQ ID NO: 7)

#### YamFril: deduced amino acid sequence

AQSVSFTFTKFDSQKDLMFQGH TISSNVIQLTKLDSNGNPVSTSVGRVLYSAPLRLWE  
SSTVVSTFETTFQISTPYTSPPGDGLAFFLAPYDTVIPPNSAGNLLGLFPNLNALRNS  
TTSKETIDVNAASNNVVAVEFD TY PNDNIGDPYKXIGIDVNSIRSKATVAWDWQNGKT



ATAHISYNSASKRLSVTTTFYPGGKAVSLSHDVELTQVLPQWIRVGFSASTGLEK (SEQ ID  
NO: 8)

5

## Claims

- 10 1. Use of a therapeutically effective amount of a composition containing a FRIL protein comprising the amine acid sequence set forth in SEQ ID NO: 6 for the preparation of a medicament for treatment of a condition **characterized by** hematopoietic progenitor cell-depleting activity of a radiotherapeutic or a chemotherapeutic treatment or a combination of a radiotherapeutic and a chemotherapeutic treatment, wherein the therapeutically effective amount of said composition alleviates or reduces the hematopoietic progenitor cell-depleting activity of said therapeutic treatment in the patient, and wherein the medicament is to be administered prior to the radio therapeutic or chemotherapeutic treatment; wherein the medicament is to be administered about 5 days to about 2 hours before the therapeutic treatment and is to be administered daily.
- 15 2. The use of claim 1, wherein said FRIL member is encoded by the nucleic acid sequence set forth in SEQ ID NO: 5.
- 20 3. The use of claim 1, wherein the hematopoietic progenitor cells in the patient being treated with the therapeutic treatment are depleted, either by killing the progenitor cells or by inducing the progenitor cells to undergo irreversible differentiation.
- 25 4. The use of claim 1, wherein the patient is human.
5. The use of claim 3, wherein the patient has cancer
- 30 6. The use of claim 1, wherein the chemotherapeutic is selected from the group consisting of cytarabine, doxorubicin, and 5-fluorouracil.

## Patentansprüche

- 35 1. Verwendung einer therapeutisch wirksamen Menge einer Zusammensetzung, die ein FRIL-Protein enthält, das die Aminosäuresequenz von SEQ ID NO: 6 umfasst, für die Herstellung eines Arzneimittels zur Behandlung eines Zustands, der durch eine hämatopoietische Vorläuferzell-depletierende Aktivität von einer radiotherapeutischen oder einer chemotherapeutischen Behandlung oder einer Kombination von einer radiotherapeutischen oder einer chemotherapeutischen Behandlung charakterisiert ist, wobei die therapeutische wirksame Menge der Zusammensetzung die hämatopoietische Vorläuferzell-depletierende Aktivität der therapeutischen Behandlung in dem Patienten lindert oder reduziert, und wobei das Arzneimittel vor der radiotherapeutischen oder chemotherapeutischen Behandlung verabreicht wird, wobei das Arzneimittel ungefähr 5 Tage bis ungefähr 2 Stunden vor der therapeutischen Behandlung verabreicht wird und täglich verabreicht wird.
- 40 2. Verwendung nach Anspruch 1, wobei das FRIL-Mitglied durch die Nukleinsäuresequenz von SEQ ID NO: 5 kodiert wird.
3. Verwendung nach Anspruch 1, wobei die hämatopoietischen Vorläuferzellen in dem Patienten, der mit der therapeutischen Behandlung behandelt wird, entweder durch Abtöten der Vorläuferzellen oder indem die Vorläuferzellen dazu induziert werden, eine irreversible Differenzierung durchzumachen, depletiert sind.
- 50 4. Verwendung nach Anspruch 1, wobei der Patient ein Mensch ist.
5. Verwendung nach Anspruch 3, wobei der Patient Krebs hat.
- 55 6. Verwendung nach Anspruch 1, wobei das Chemotherapeutikum ausgewählt ist aus der Gruppe bestehend aus Cytarabin, Doxorubicin und 5-Fluoruracil.

## Revendications

1. Utilisation d'une quantité thérapeutiquement efficace d'une composition contenant une protéine FRIL comprenant la séquence d'acides aminés indiquée dans SEQ ID NO: 6 pour la préparation d'un médicament pour le traitement d'un état **caractérisé par** une activité de déplétion de cellules progénitrices hématopoïétiques d'un traitement radiothérapeutique ou d'un traitement chimiothérapeutique ou d'une combinaison d'un traitement radiothérapeutique et d'un traitement chimiothérapeutique, tandis que la quantité thérapeutiquement efficace de ladite composition atténue ou réduit l'activité de déplétion de cellules progénitrices hématopoïétiques du dit traitement thérapeutique chez le patient, et tandis que le médicament est destiné à être administré avant le traitement radiothérapeutique ou chimiothérapeutique, tandis que le médicament est destiné à être administré environ 5 jours à environ 2 heures avant le traitement thérapeutique et est destiné à être administré quotidiennement.
2. Utilisation selon la revendication 1, dans laquelle ledit membre FRIL est codé par la séquence d'acide nucléique indiquée dans SEQ ID NO: 5.
3. Utilisation selon la revendication 1, dans laquelle les cellules progénitrices hématopoïétiques chez le patient qui est en train d'être traité avec le traitement thérapeutique sont déplétées, soit par destruction des cellules progénitrices soit par induction des cellules progénitrices à subir une différenciation irréversible.
4. Utilisation selon la revendication 1, dans laquelle le patient est un humain.
5. Utilisation selon la revendication 3, dans laquelle le patient a un cancer.
6. Utilisation selon la revendication 1, dans laquelle l'agent chimiothérapeutique est choisi dans le groupe consistant en cytarabine, doxorubicine, et 5-fluorouracile.

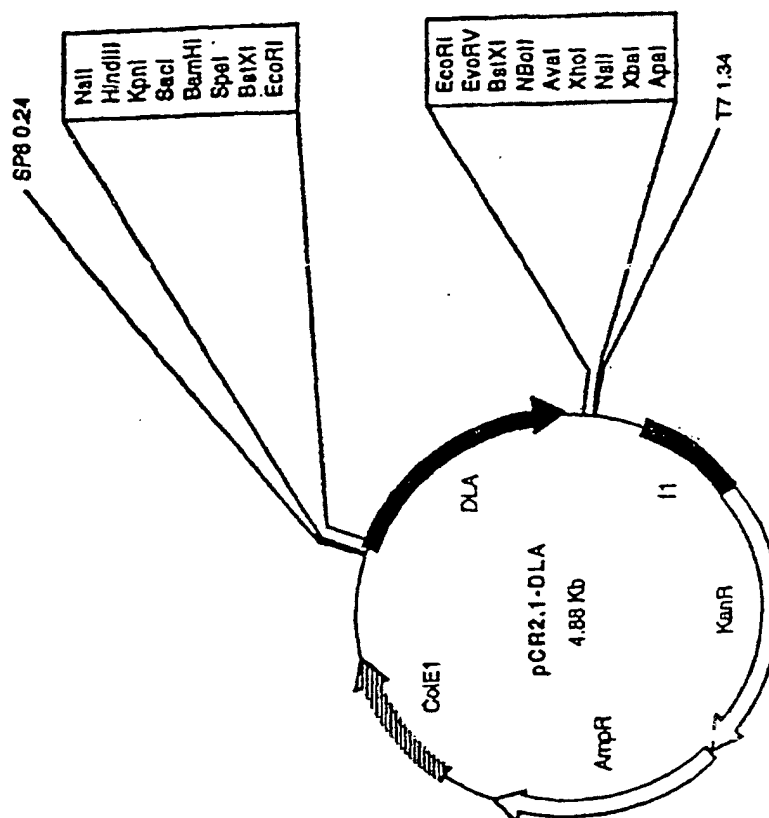


FIGURE 1

# β-Subunit

Gowda 1 AQLSFSFTKFDPNQEDLIFQGTATS.....KLD SAGNPVSSSAGRV 42  
 FRIL 1 AQLSFSFTKFDPNQEDLIFQGHATSTNNVLQVTKLDSAGNPVSSSAGRV 50  
 43 LYSAPLRLWEDSAVLTSFDPTIY..IFTNYSRIADGLA.FIAPPDSVIS 89  
 51 LYSAPLRLWEDSAVLTSFDTIINFEISTPYTSRIADGLAFFIAPPDSVIS 100  
 90 YHGGFLGLFPNAAESG..... 105 (SEQ ID NO:11) ←  
 101 YHGGFLGLFPNANTLNNSSTSEN 123

# α-Subunit

Gowda 1 .....IAESNVVAVEFDTDYLNPDYGDPNYIHIGIDVNSIRSKVTASWDW 45  
 FRIL 1 QTTTKAASSNVVAVEFDT.YLNPDYGDPNYIHIGIDVNSIRSKVTAKWDW 49  
 46 QNGKIATAHI:YNSVSKRLSVTTYYPGRGKPATSYDIELHTVLPWVRVG 95  
 50 QNGKIATAHI:YNSVSKRLSVTSYAGSKPATLSYDIELHTVLPWVRVG 99  
 96 LSASTGQNIERNVTVHSWSFTSSLWTNNAKVGVASISG..... 132 (SEQ ID NO:12) ←  
 100 LSASTGQDKERNVTVHSWSFTSSLWTNNAKKNENKYITRGVL\*YMCIND 149

FIGURE 2

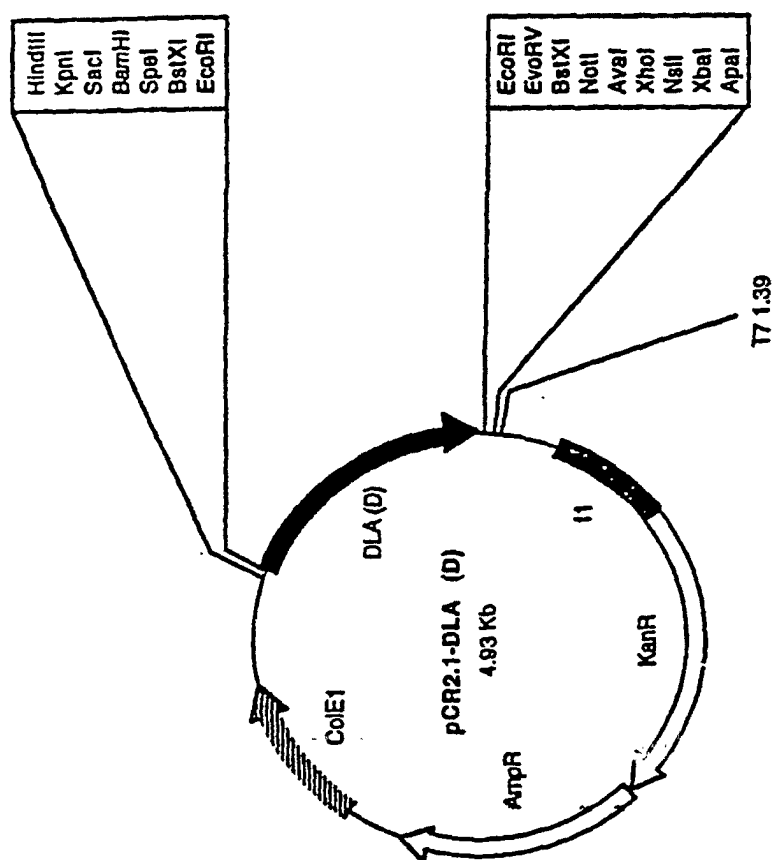
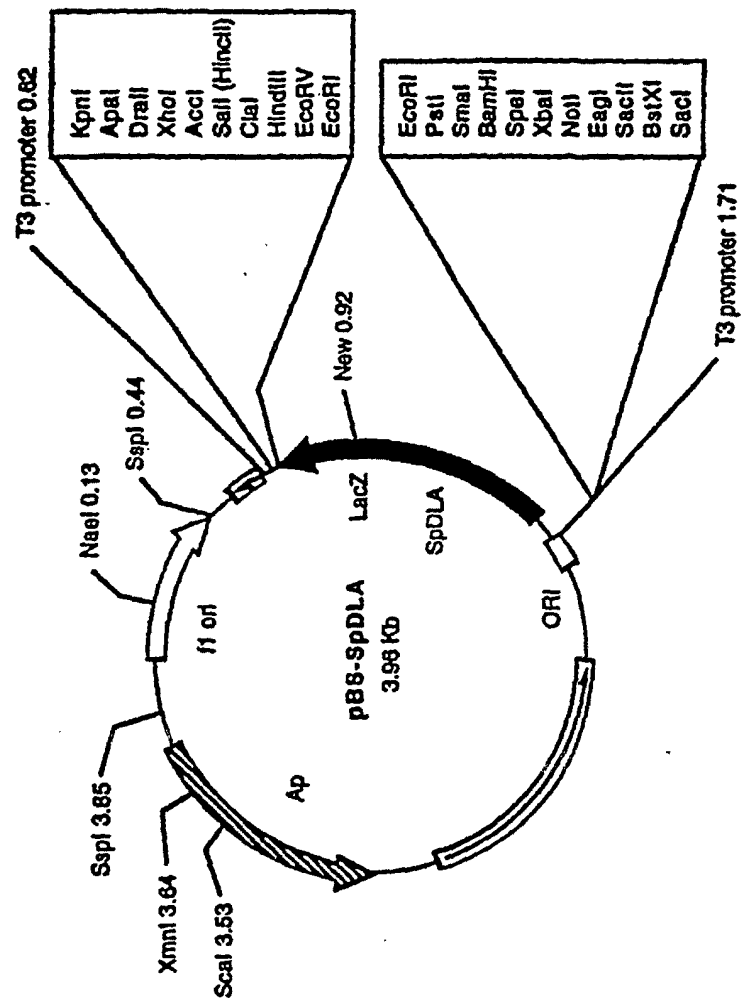


FIGURE 3



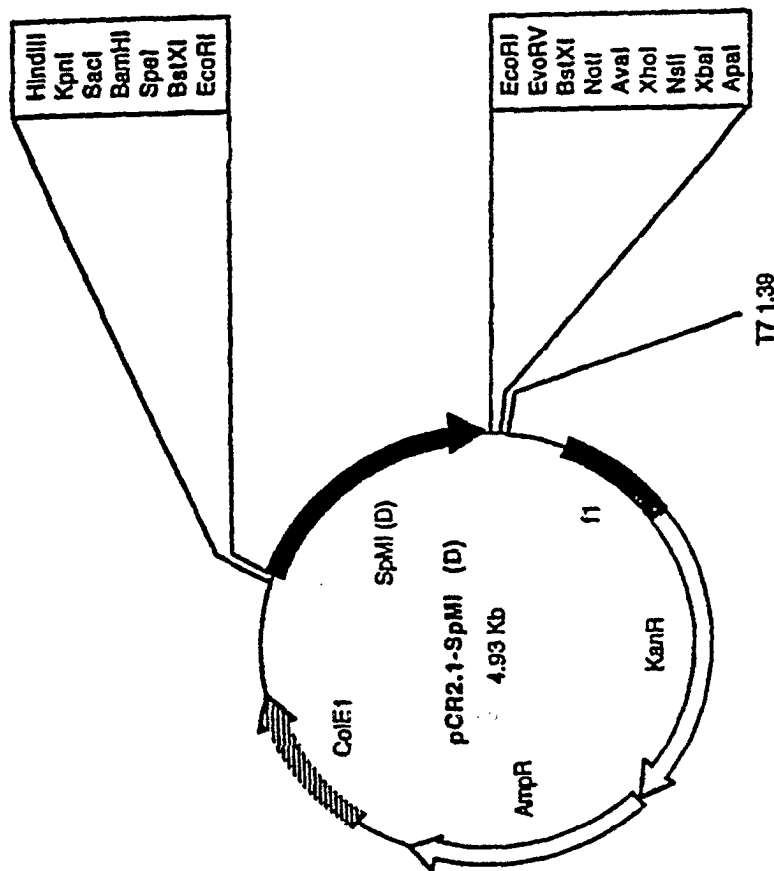


FIGURE 5

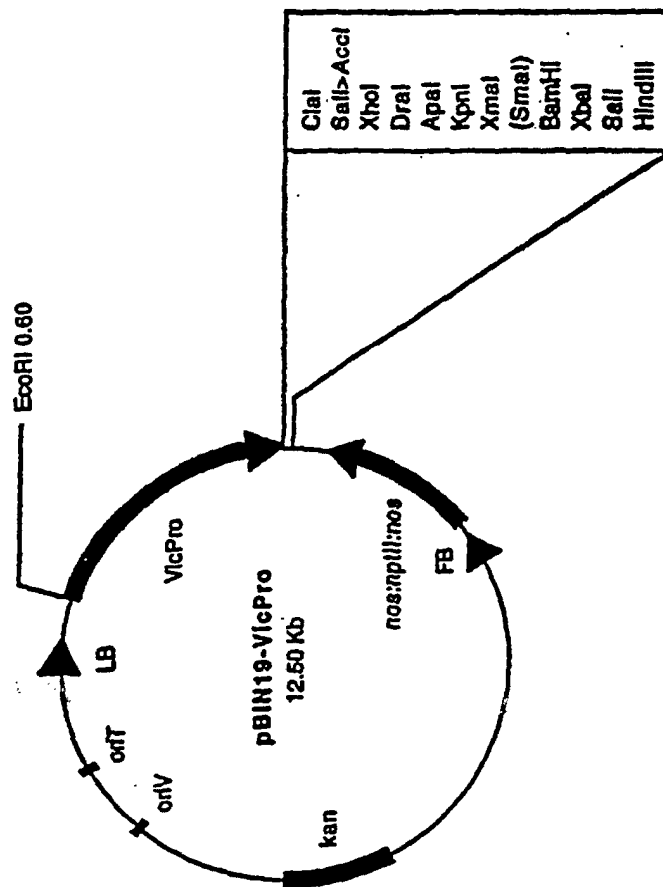


FIGURE 6



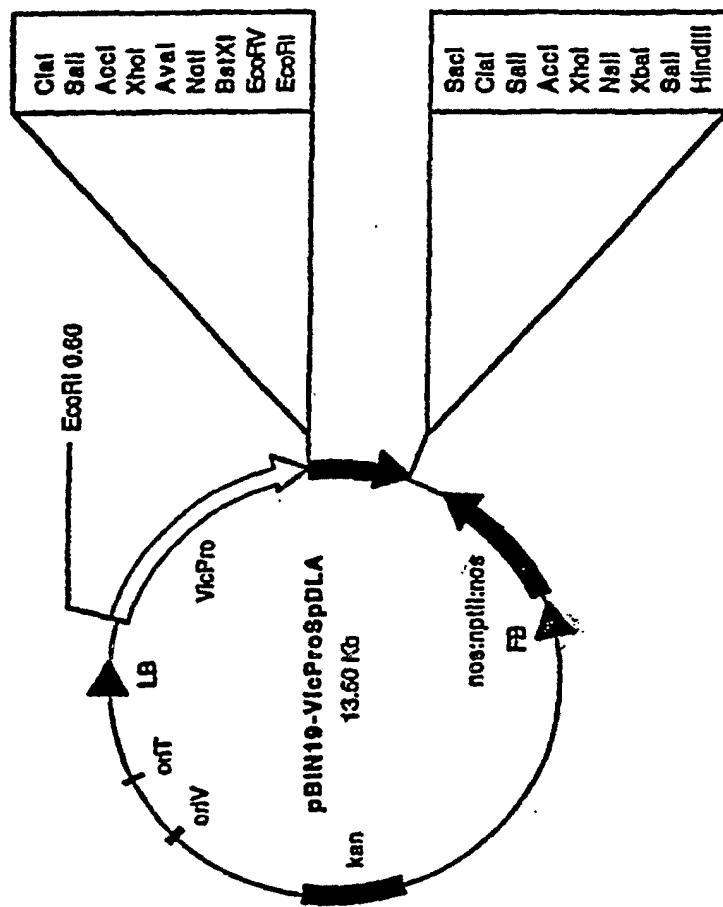


FIGURE 7

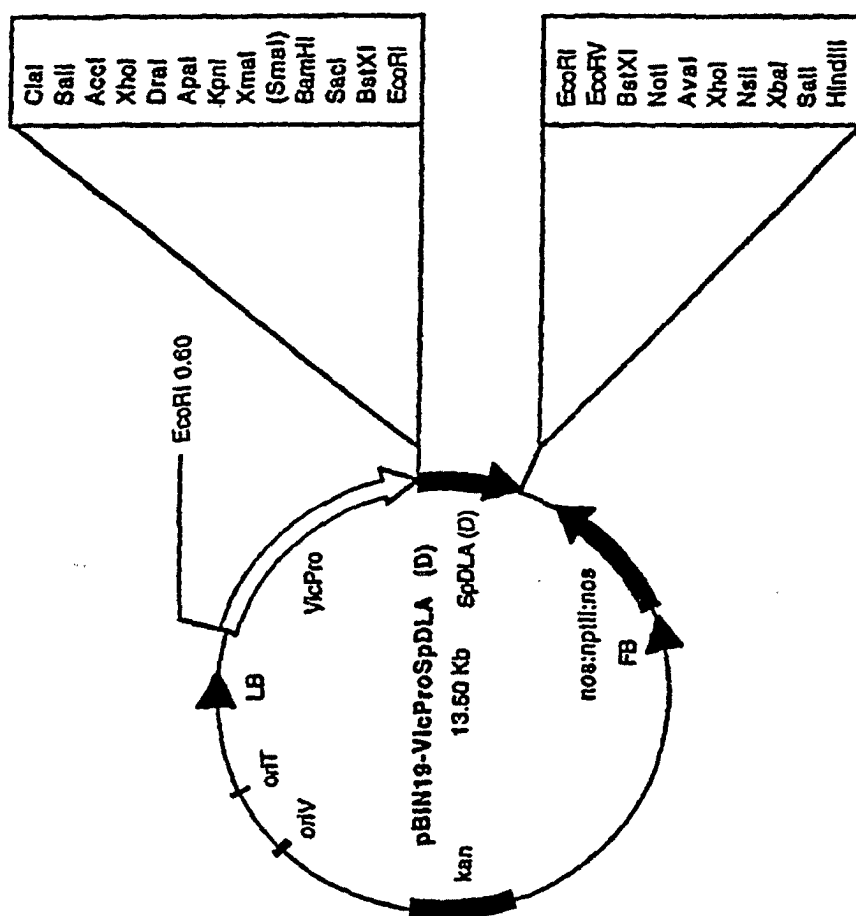
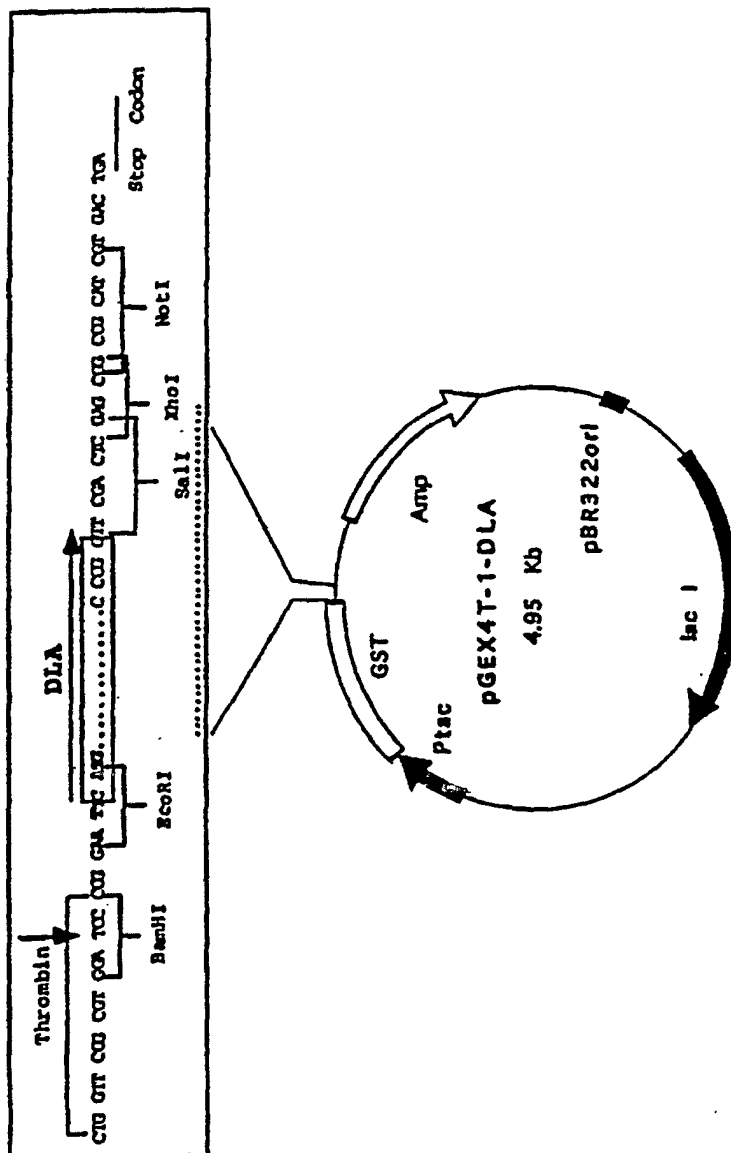


FIGURE 8



**FIGURE 9**

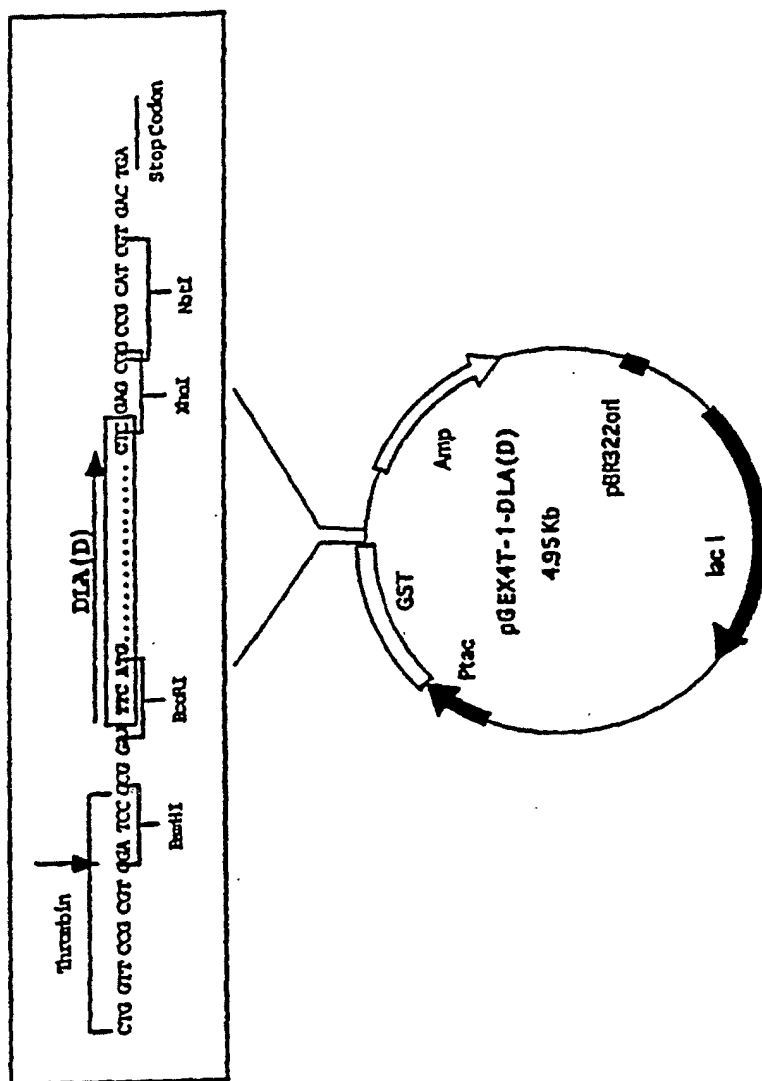


FIGURE 10

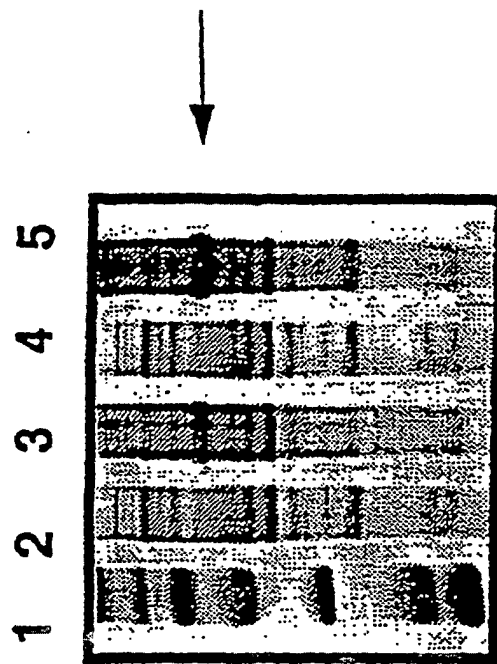


FIGURE 11

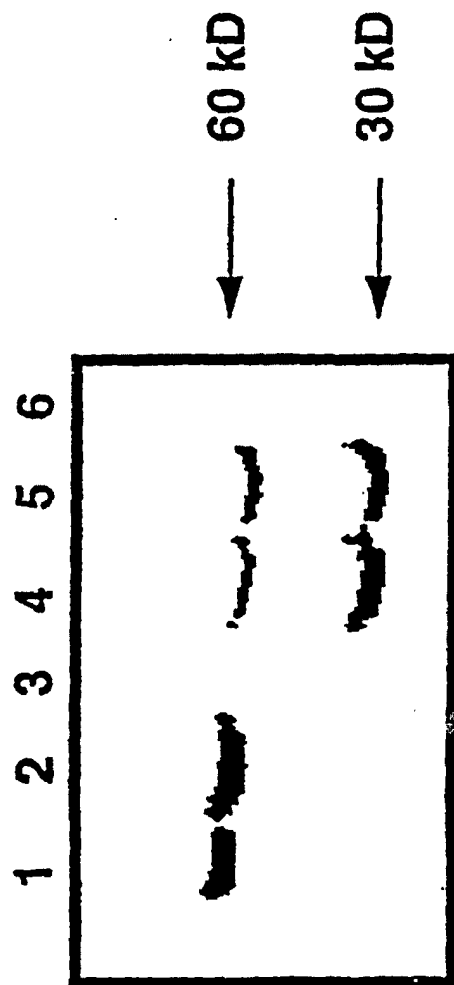
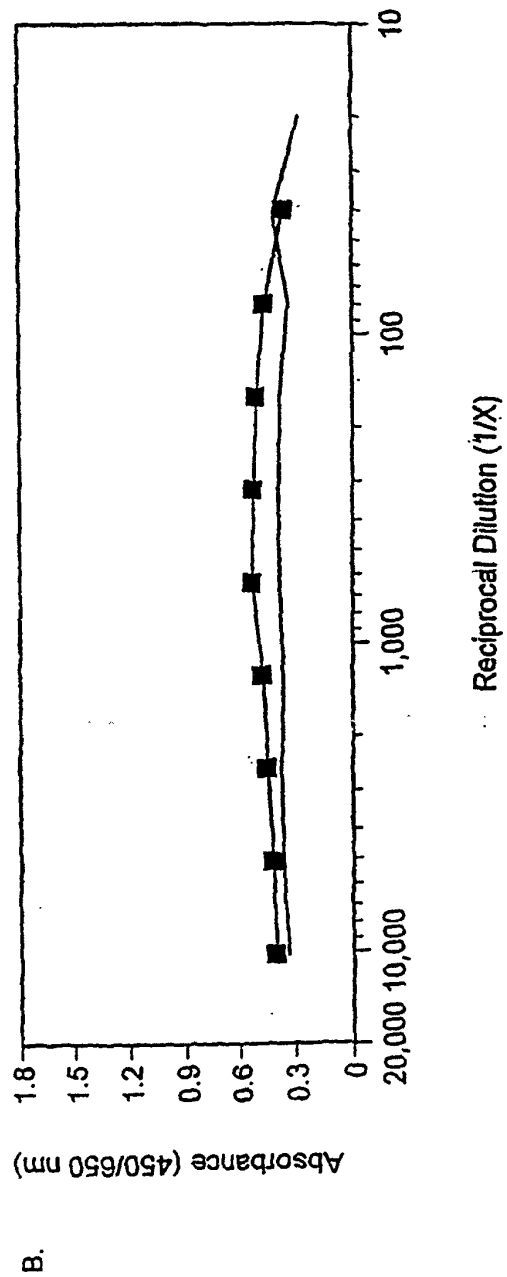
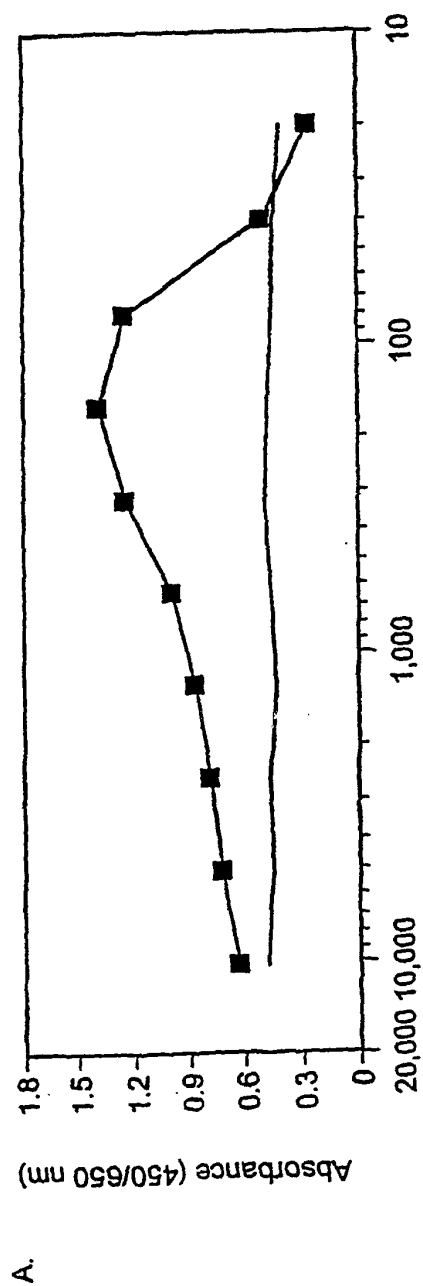
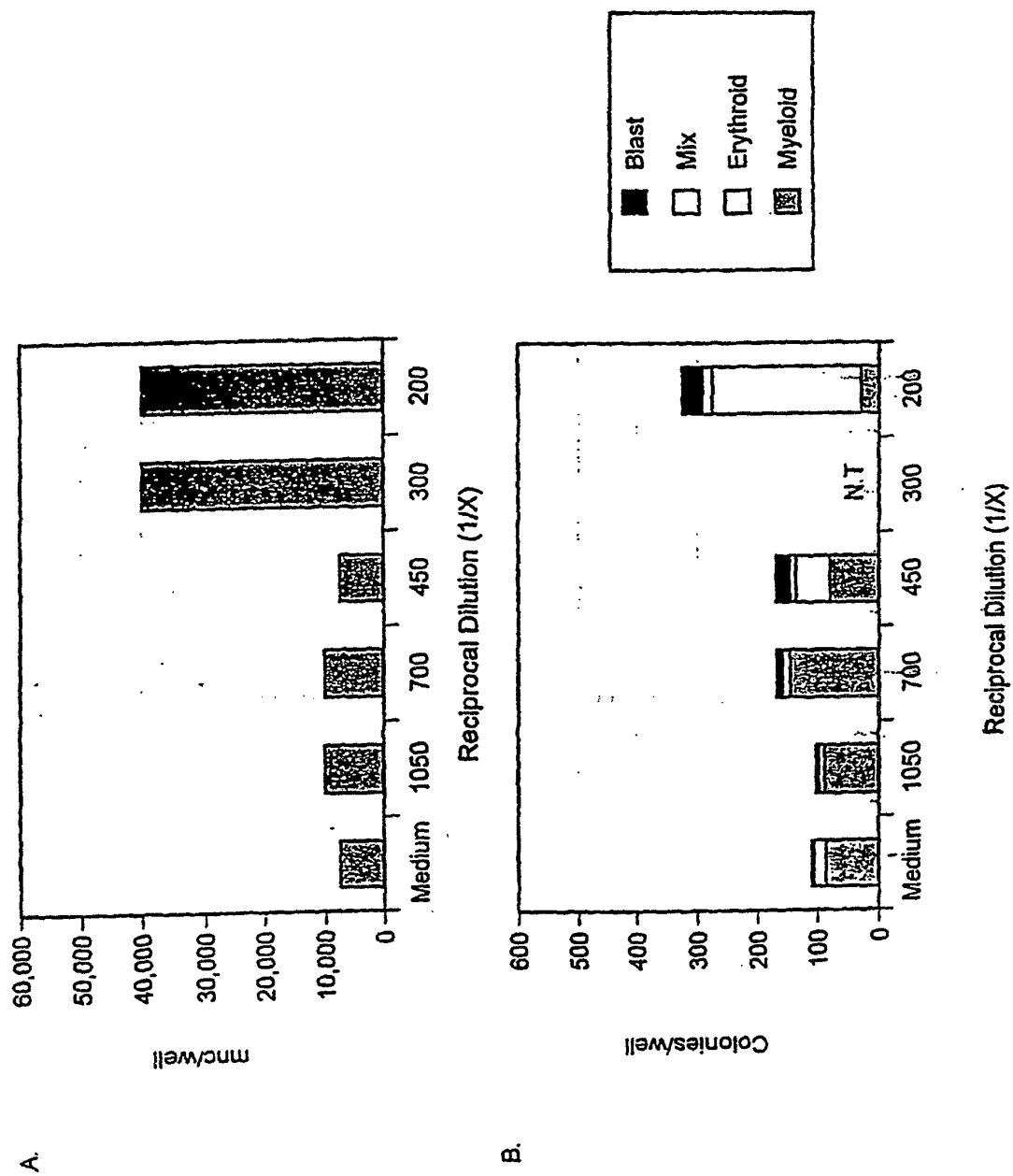


FIGURE 12

Figures 13A and 13B



Figures 14A and 14B





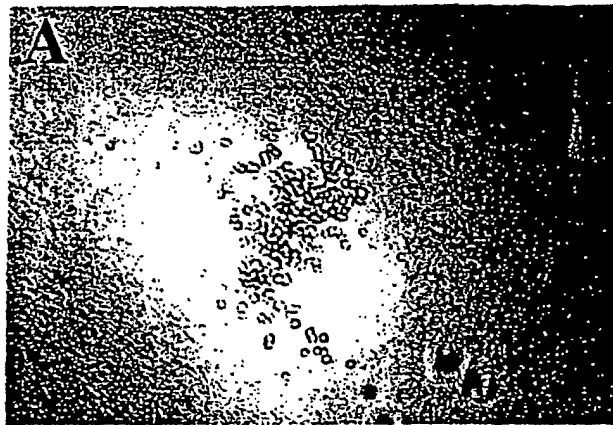


Figure 15A

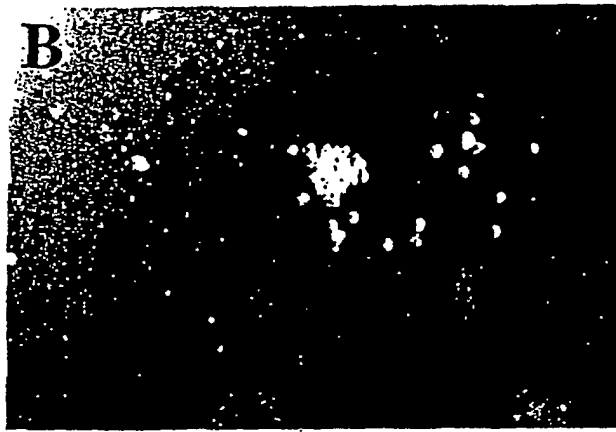


Figure 15B

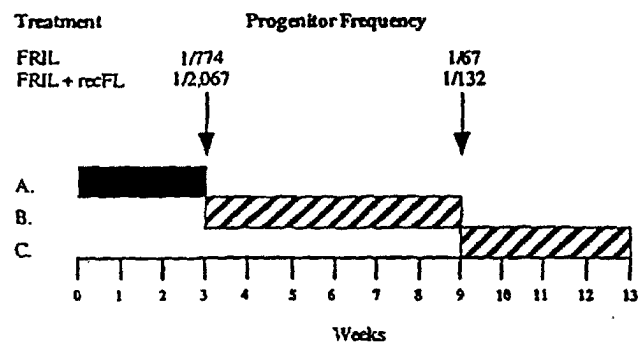


Figure 16

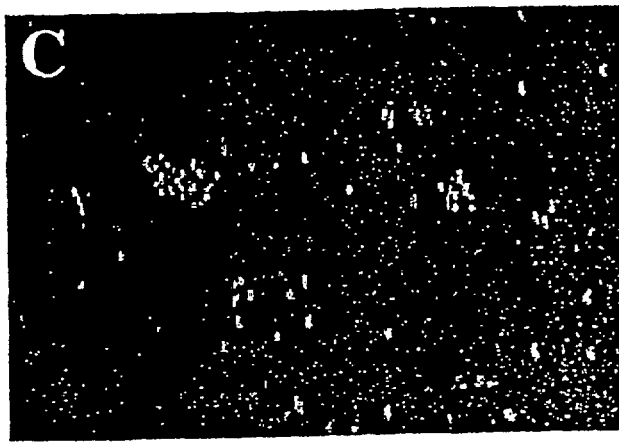
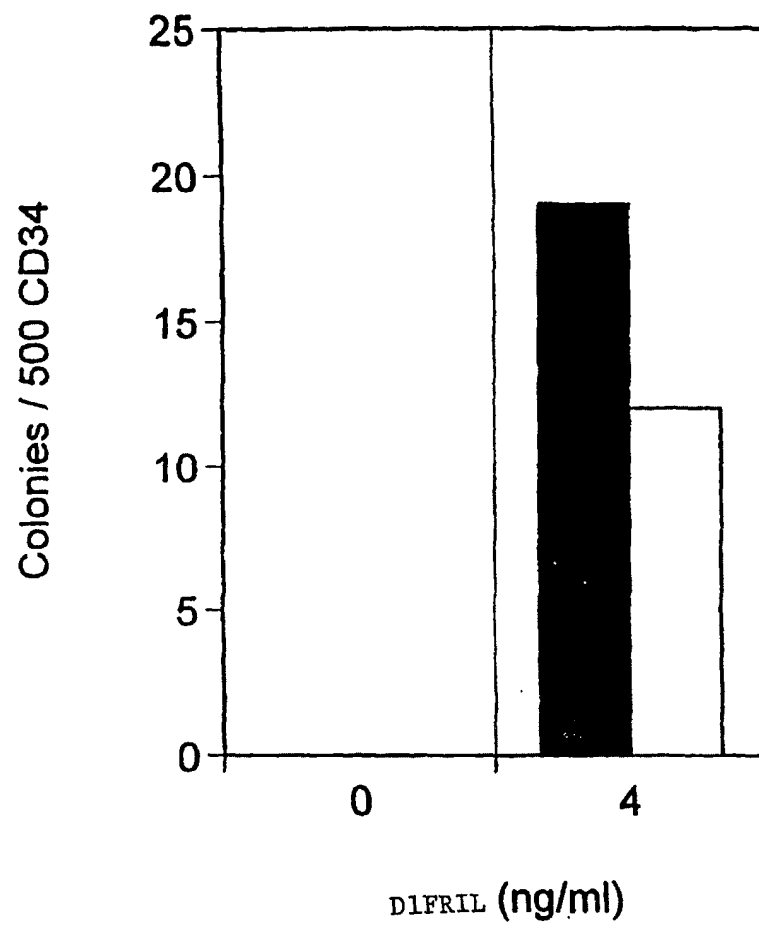


Figure 17

Figure 18



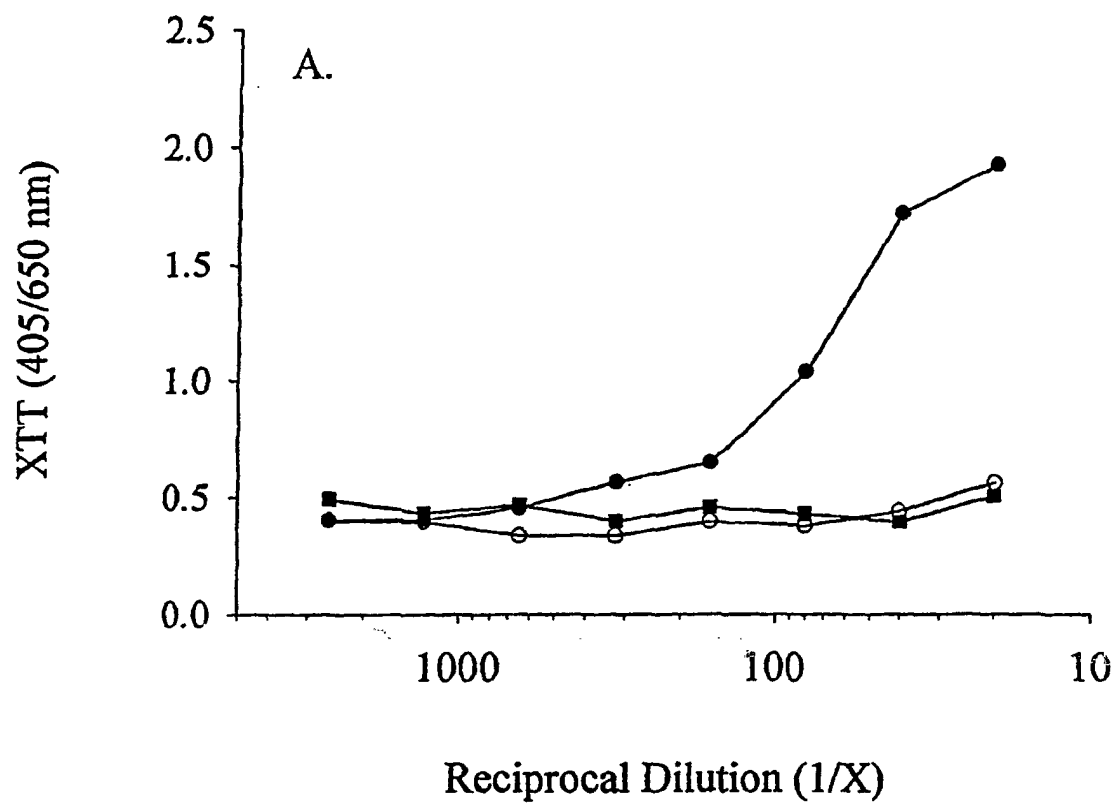


Figure 19A

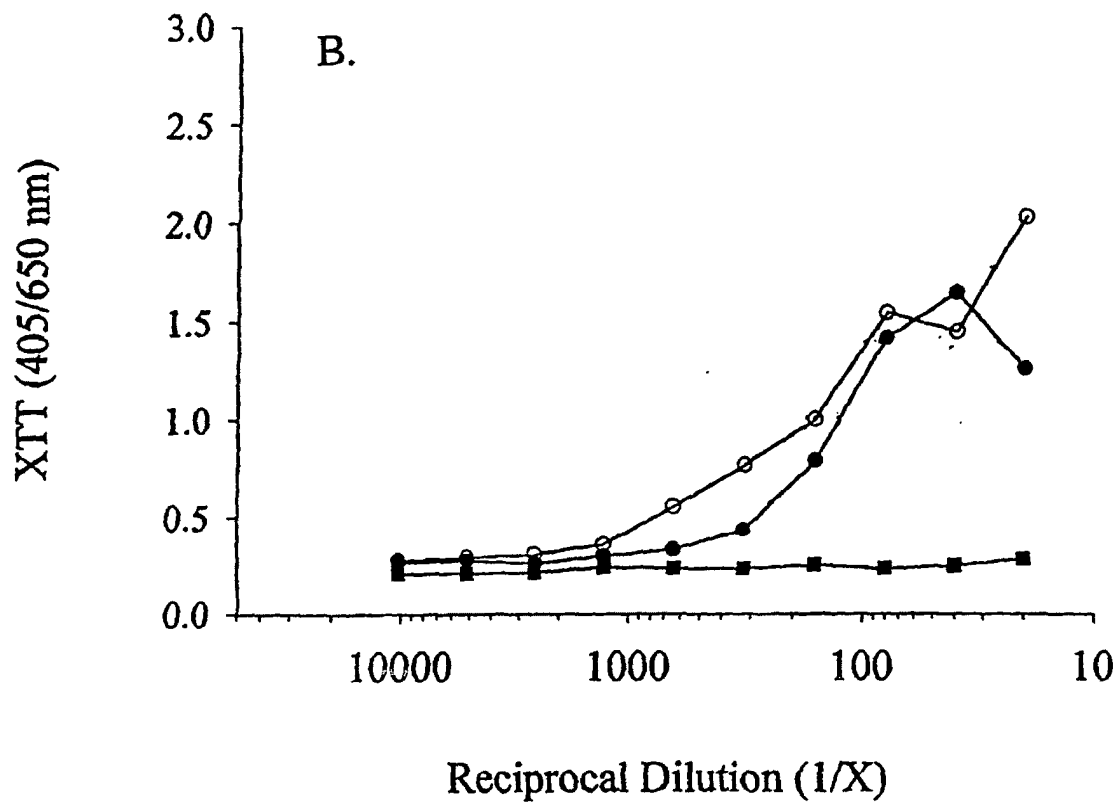
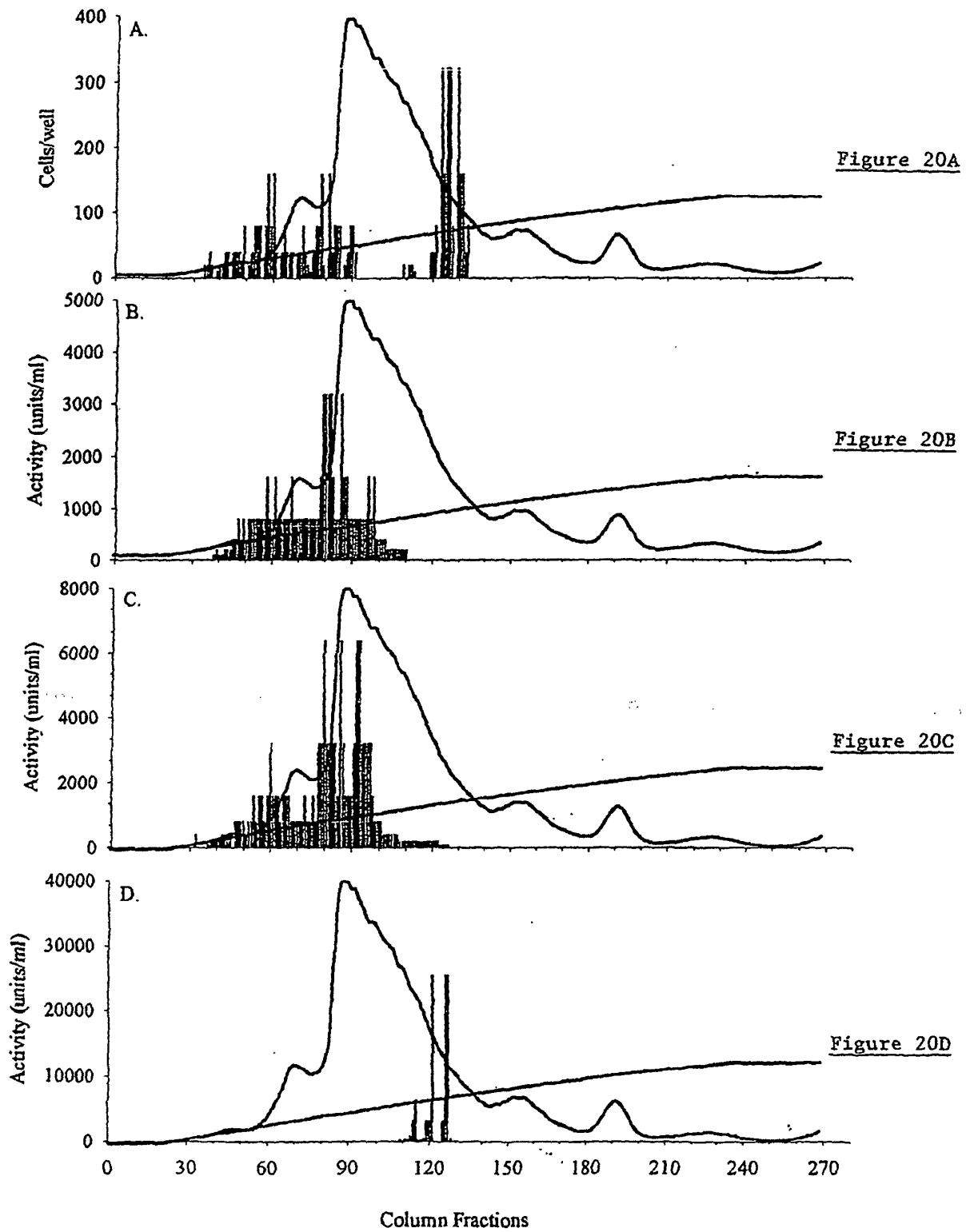
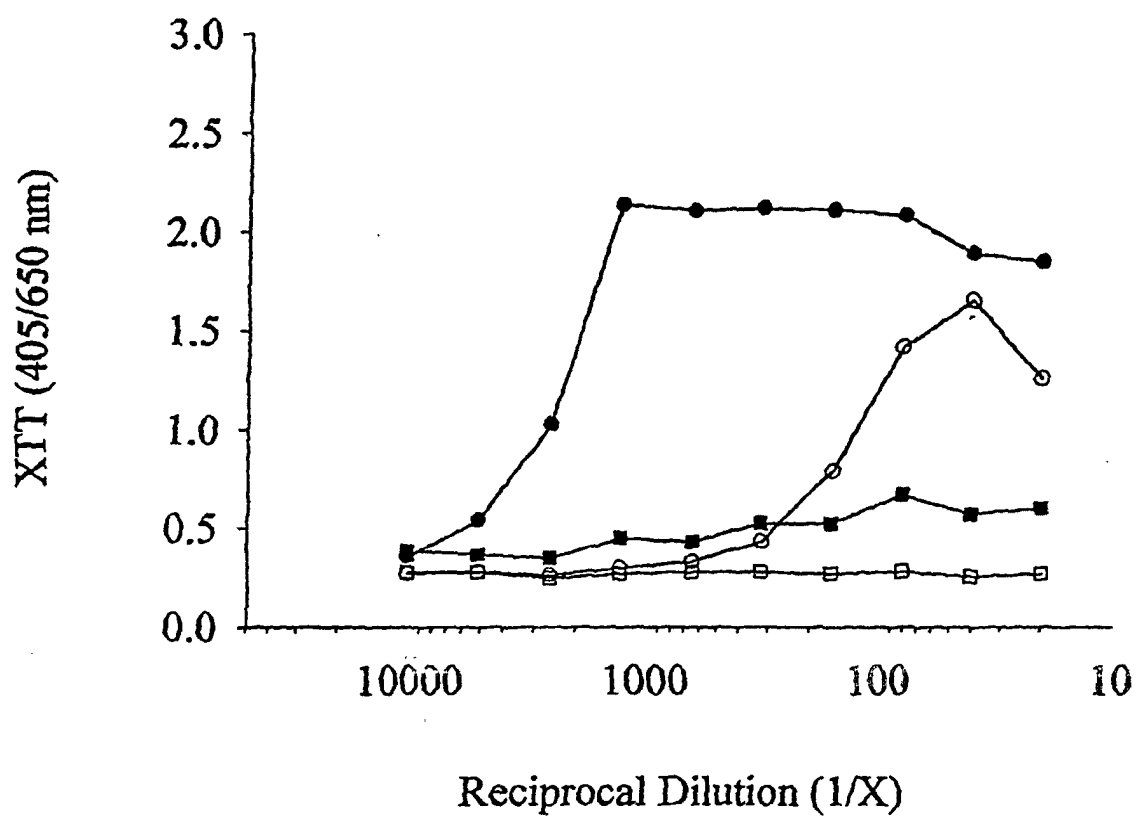


Figure 19B





Figure 21A

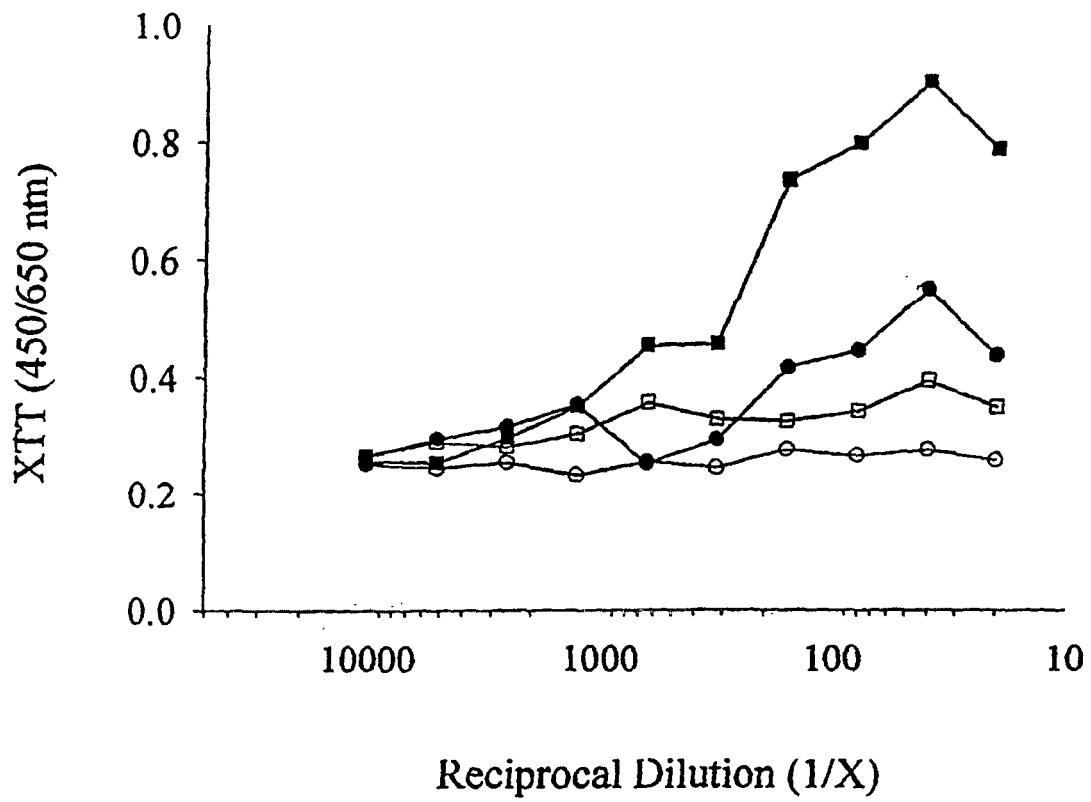


Figure 21B

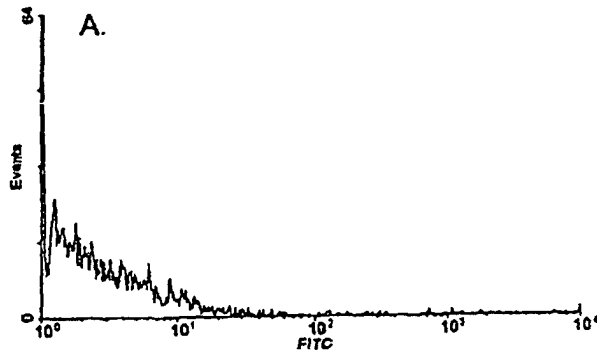


Figure 22A

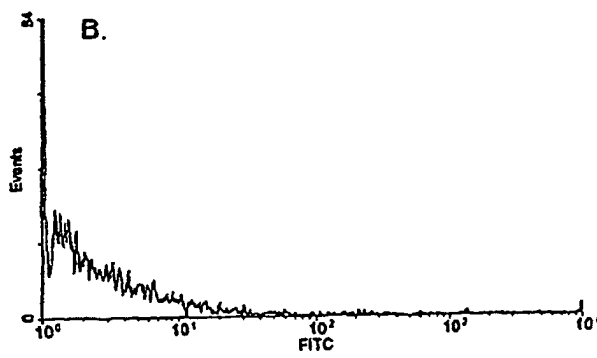


Figure 22B

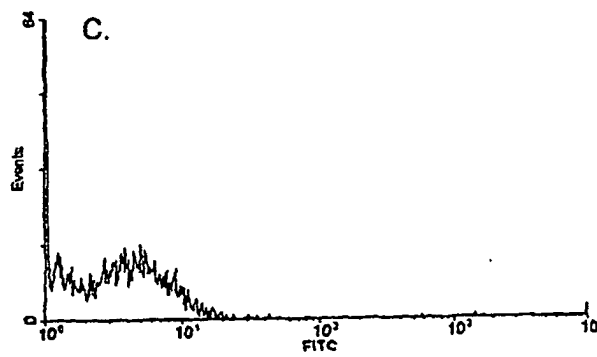


Figure 22C

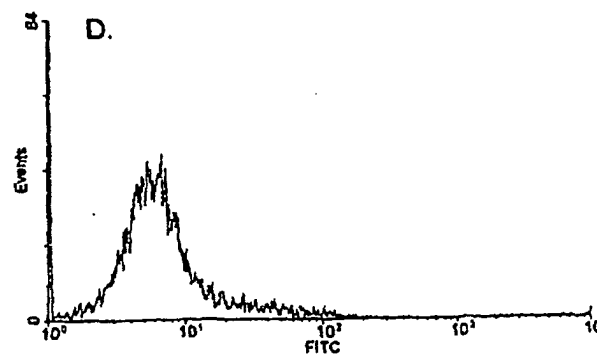
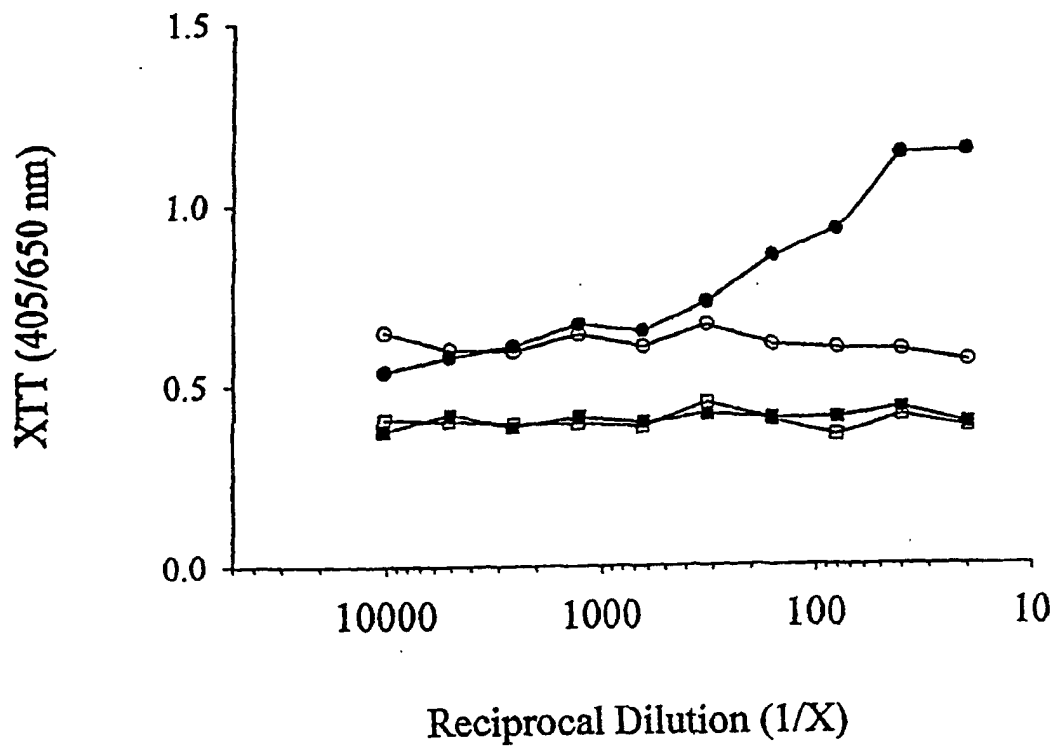


Figure 22D

Figure 23

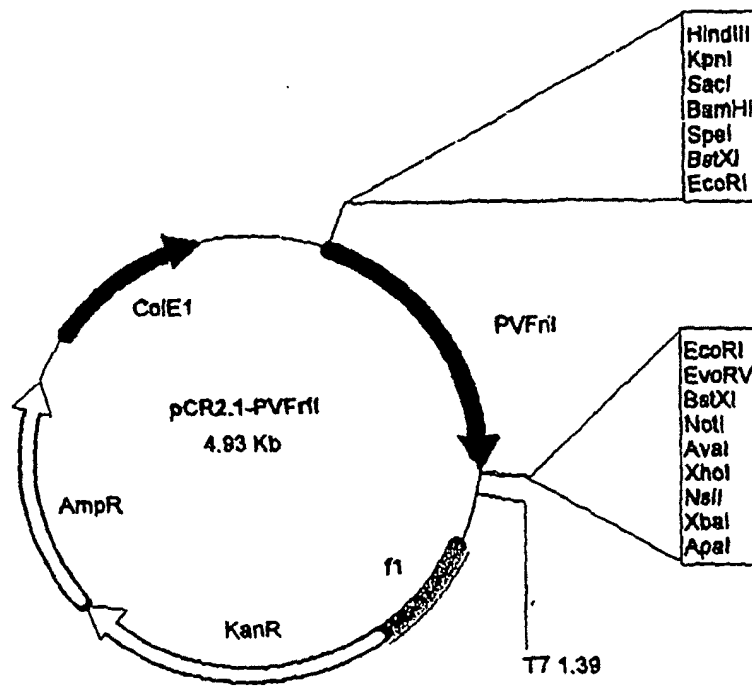


Figure 24A

DLL	1	AQSLSFSFTKFD	PNQEDLIFQGTATS	- - - -	26	
PvFRIL	1	AQSLSFNFTKFD	LDQKDLIFQGDATS	TNN -	29	
PHA-E	1	ASQTSESEFQREN	- - ETNLLIQRDATVSSKG		28	
DLL	27	- - - -	KLDSAGNPVSSS	AGRVLYSAPLR	61	
PvFRIL	30	VLQLTKLDSGGNPV	ASVGRVLE	SAPFHLW	59	
PHA-E	29	QLRLTNVNDNGEPT	LSISLGRAFY	SAPIQIW	58	
DLL	52	EDSAVLTSFDP	- - TIYIFTNYTSR	IADGL	78	
PvFRIL	60	ENSMAYSSFETN	- LTIQISTPHPHY	YAADGF	88	
PHA-E	59	DNTTGAVAASPT	SFTFNLDVPNN	SGPADGI	88	
DLL	78	AFIAPP	- DSVISYH -	GGFLGLEFPNA	AESG	105
PvFRIL	89	AFFLAPHDTV	PPNSWGKFLGLYS	NVFRNS		118
PHA-E	89	AEVLLPVGSQPKDK	- - GGLLGLFN	NNYKYDS		116
DLL	106	IAESN	- - - - -	VVAVEFDTDY	INPD	124
PvFRIL	119	PTSEN	QSFGDVNTDSR	VVAVEFDT	- - EPNA	146
PHA-E	117	NAH	- - - - -	TVAVEFDT	- - LYNNV	131
DLL	125	YGDPNYLHIGIDVNS	IRSKVTASWDWQNGK		154	
PvFRIL	147	NIDPNYRHIGIDVNS	IKSKEETARMEWQNGK		176	
PHA-E	132	HWDPKPRHIGIDVNS	IKSITKTITWDFVKGE		181	
DLL	155	IATAHISYNSVSKRL	SVTTTYYPGRGK	- PAT	183	
PvFRIL	177	TATARISYNSASKKST	VTTTFYPGMEV	- VAL	205	
PHA-E	162	NAEVLITYDISSTKLL	VASLVYPSLKTSTFV		181	
DLL	184	SYDLELHITVLP	EWVRVGLSASTGQ	- - - NIE	210	
PvFRIL	206	SHDVDLHAELP	EWVRVGLSASTGE	- - - EKO	232	
PHA-E	192	SDTVDLKSVLP	EWVIVGFTATTIGIT	KGNI	221	
DLL	211	RNTLVHSWSFTSSL	WTNVAKVG	- - - - - VAS	I	235
PvFRIL	233	KNTTISWSFTSSL	LKNNEVK	EPKEDMYIANV		262
PHA-E	222	TNDILSWSFASKL	ISDGTTS	EALN - - - LAN	F	248
DLL	236	SG	- - - - -		237	
PvFRIL	263	VRSYTWINDVLSY	ISNK		279	
PHA-E	249	ALNQIL	- - - - -		254	

Figure 24B

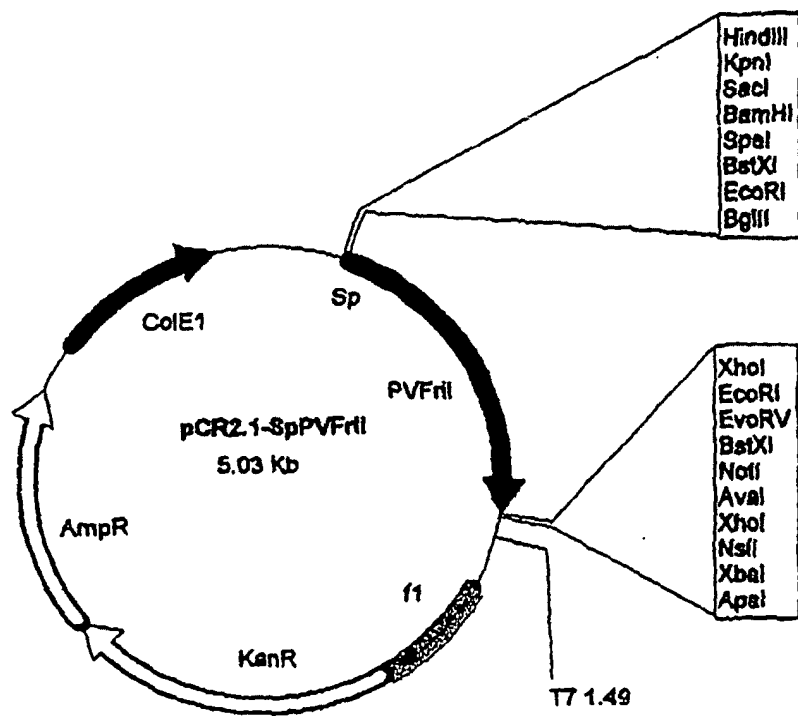


Figure 25

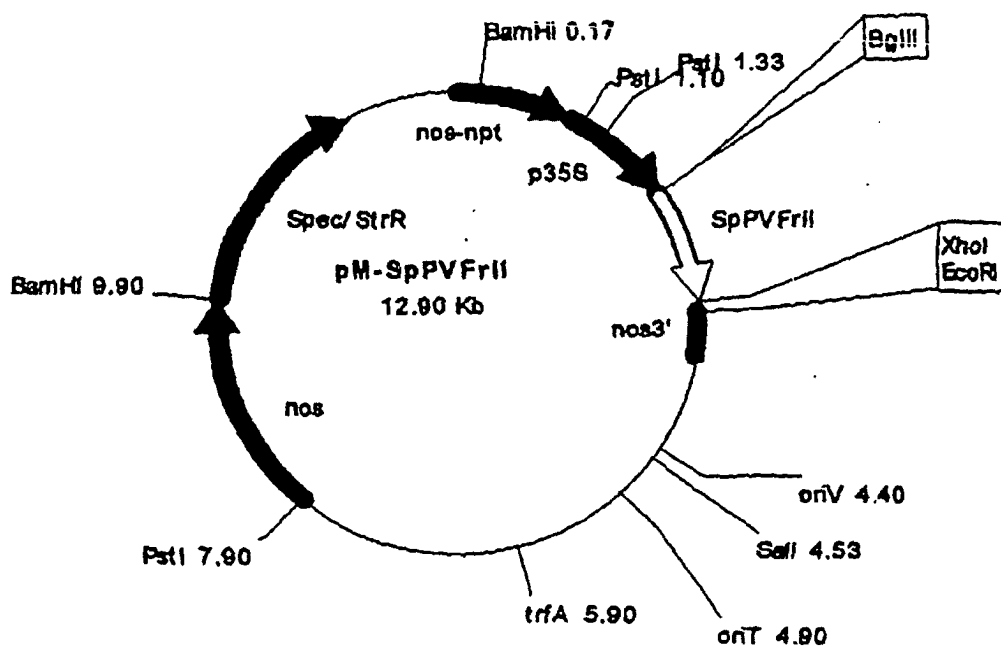


Figure 26



Figure 27A

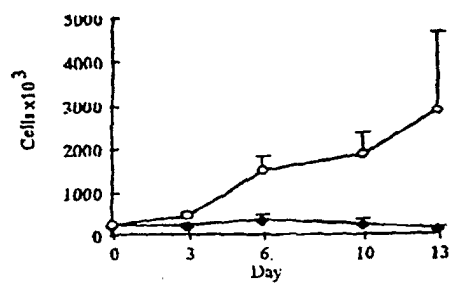


Figure 27B

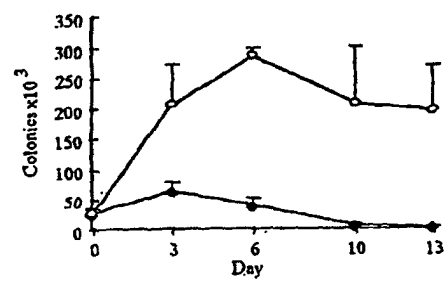


Figure 28A

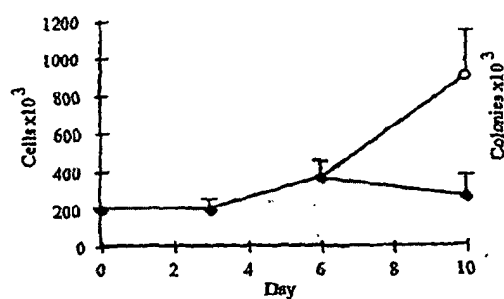


Figure 28B

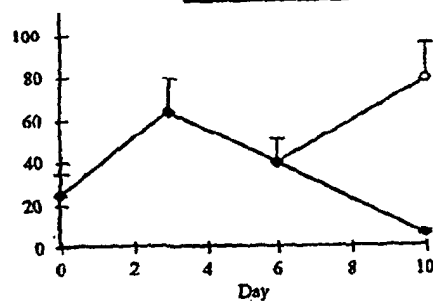


Figure 28C

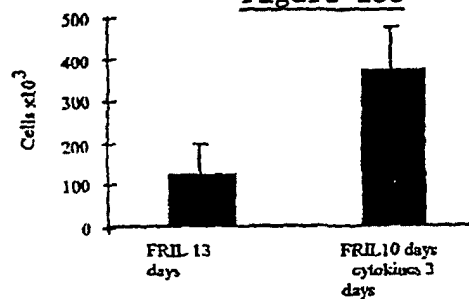
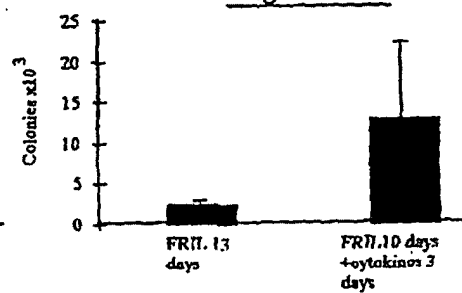
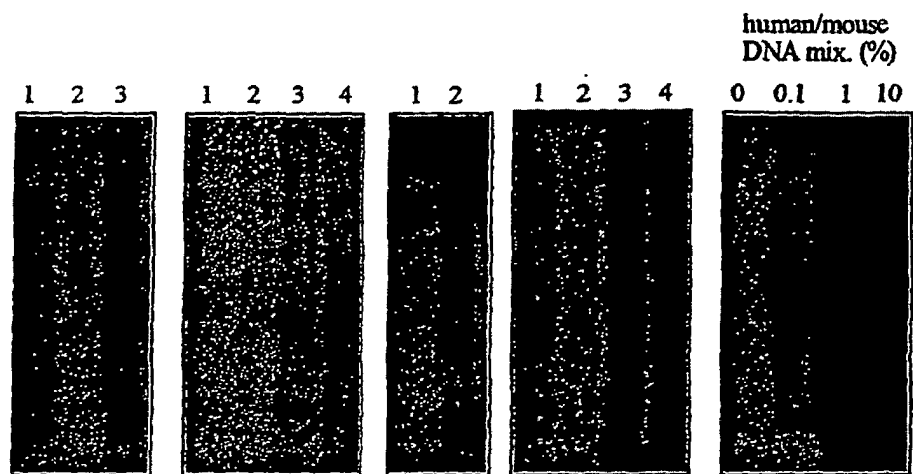


Figure 28D





Figures 29A - 29E

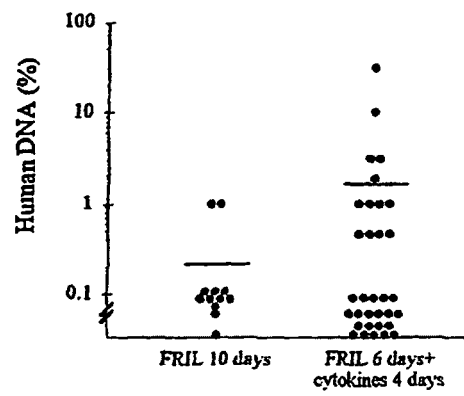


Figure 30

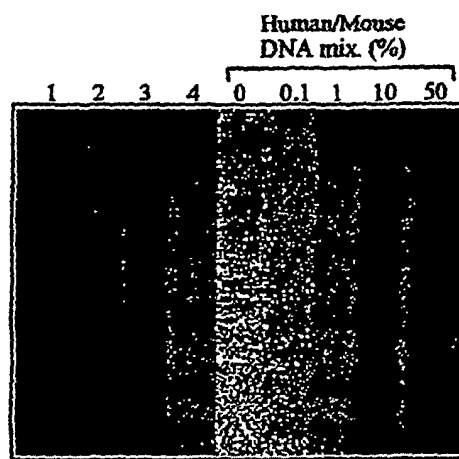


Figure 31

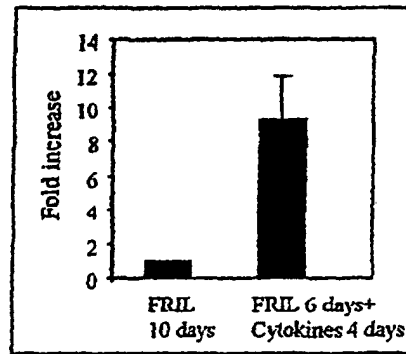
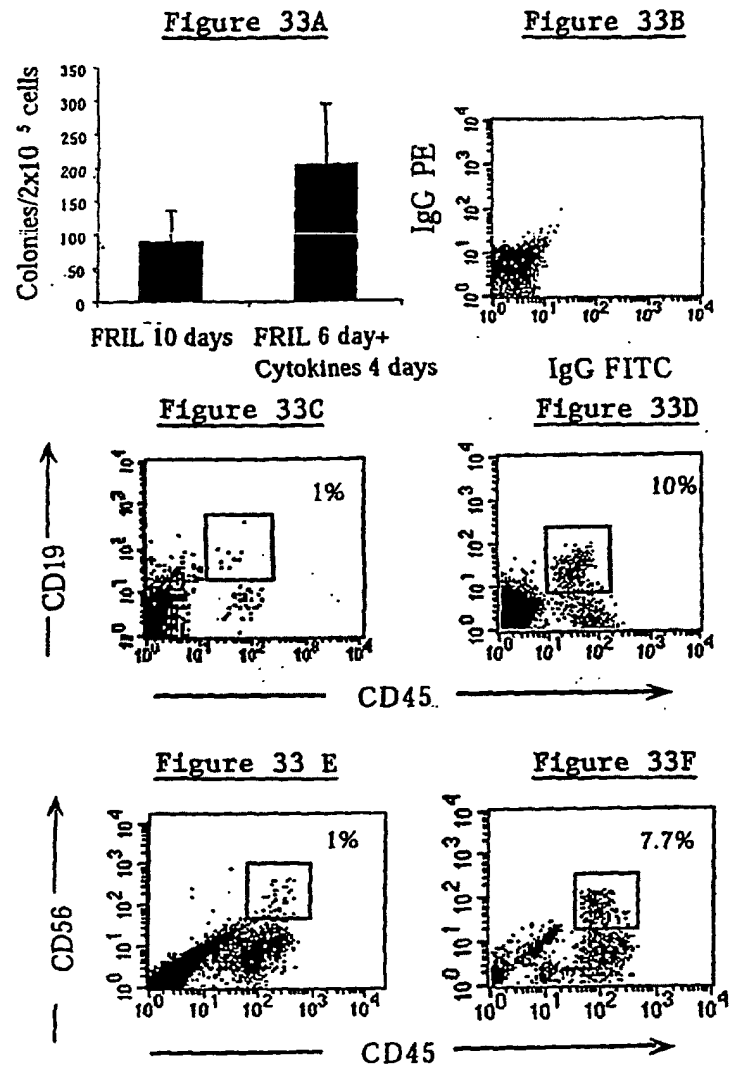


Figure 32



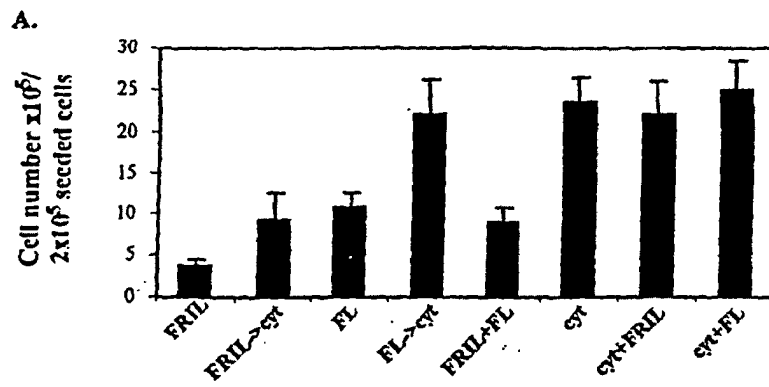


Figure 34A

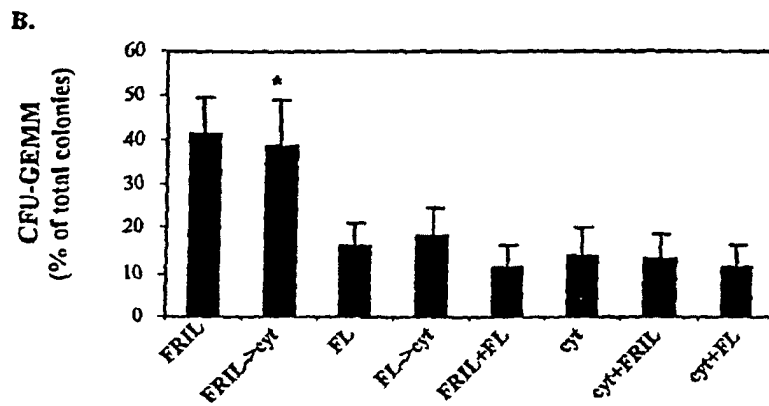


Figure 34B



Figure 35A

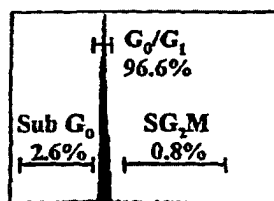


Figure 35B

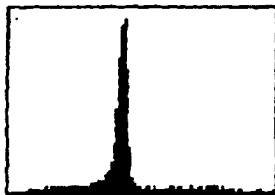


Figure 35C

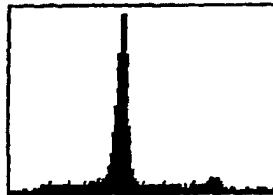


Figure 35D



Figure 35E



Figure 35F



Figure 35G

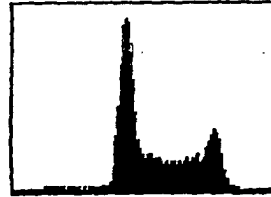


Figure 36A

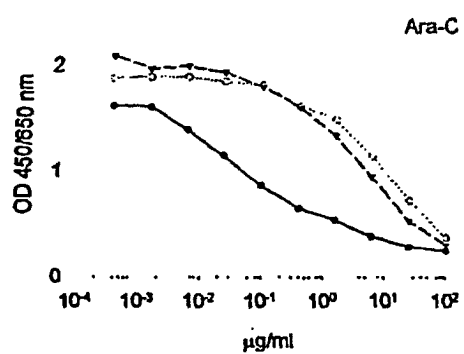


Figure 36B

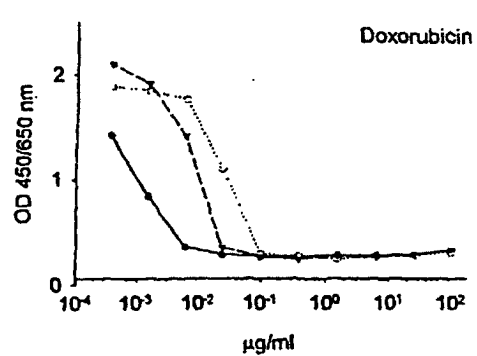
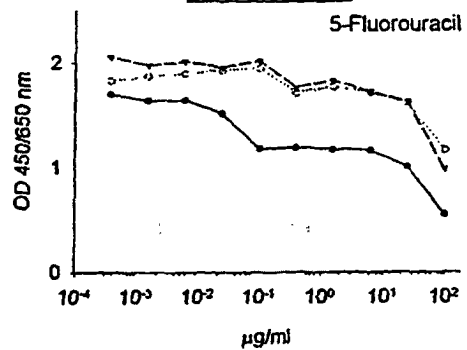
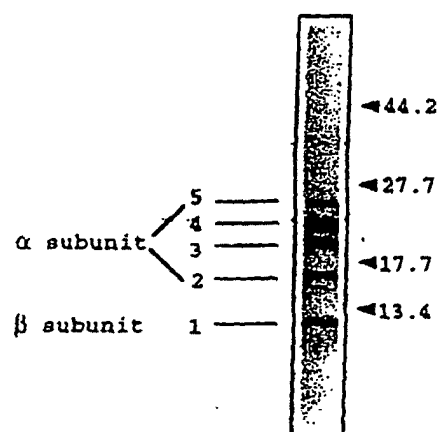


Figure 36C





Band 5 DSSTS EXQTT TKAAS SNVVA  
 Band 4 DSSTS EXQTT TKA  
 Band 3 DSSTS EXQTT TKAAS SNVVA  
 Band 2 TT TKAAS SNVVA VEFKT YLN

Band 1 AQSLSF FSPTK FDPNQ EDLIF QHATS TNNV

FIG. 1. Fractionation of purified hyacinth bean FRIL by SDS/PAGE and amino-terminal amino acid sequences of the constituent polypeptides.

Figure 37