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Differential Expression of Homing-Associated Cell Adhesion Molecule, Very Late Antigen-4 and L-Selectin in Hematopoietic Progenitor Cell Trafficking Between the Marrow and Blood

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DIFFERENTIAL EXPRESSION OF HOMING-ASSOCIATED CELL ADHESION MOLECULE, VERY LATE ANTIGEN-4 AND L-SELECTIN IN HEMATOPOIETIC PROGENITOR CELL TRAFFICKING BETWEEN THE MARROW AND BLOOD

by

CAROLINE BRIGITTE FULTZ

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Pathology
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Major Professor: William E. Janssen, Ph.D.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of variance
BC	Buffy coat
BDIS	Becton and Dickinson Immunocytometry Systems
BFU-E	Burst forming unit-erythroid lineage
BM	Bone marrow
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
CFU	Colony forming unit
CFU-E	Colony forming unit-erythroid lineage
CFU-GM	Colony forming unit-granulocyte macrophage lineages
CFU-mix	Colony forming unit-mixed lineages (erythrocytic, granulocytic and myelocytic)
CFU-S	Colony forming unit-spleen
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered salt solution
ECM	Extracellular matrix
FITC	Fluorescein isothiocyanate
FN	Fibronectin

FSC	Forward scatter fluorescence
GAG	Glycosaminoglycans
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
HCAM	Homing-associated cell adhesion molecule (CD44)
HPC	Hematopoietic progenitor cell
HSA	Human serum albumin
LTBMC	Long term bone marrow culture
LTCIC	Long term culture initiating cell
mAb	Monoclonal antibody
MACS	Magnetic activated cell sorting
MB	MACS® buffer
PB	Peripheral blood
PBS	Phosphate buffered saline
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
s.e.m.	Standard error of mean
SIP	Sample injection port
SSC	Side scatter fluorescence
VLA-4	Very late antigen-4 (CD49d)
QSC beads	Quantum simply cellular® beads

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An Abstract

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This study addresses the hypothesis that the following cell adhesion molecules (CAMs): homing-associated cell adhesion molecule (HCAM), very late antigen-4 (VLA-4) and L-selectin play a role in the trafficking of hematopoietic progenitor cells (HPCs) between the bone marrow microenvironment and the peripheral circulation. In order to ascertain differences in CAM expression based on physiologic compartment, the expression of HCAM, VLA-4 or L-selectin per $CD34^+$ myeloid progenitor cell was assessed between paired samples of blood and marrow. CAM expression was flow cytometrically quantitated in paired samples obtained from patients treated with mobilizing doses of granulocyte-colony stimulating factor (G-CSF) or from normal donors donating for allogeneic transplant. In G-CSF mobilized patients, marrow derived $CD34^+$ myeloid progenitor cells expressed more VLA-4 per cell than those in circulation. In normal donors, marrow derived myeloid progenitor cells expressed more CD34 per cell than those in circulation. To functionally demonstrate the hematopoietic potential of CAM expressing ($HCAM^+$, VLA-4^+ or L-$selectin^+$) $CD34^+$ myeloid progenitors, colony forming unit (CFU) and long term culture initiating cell (LTCIC) assays of flow cytometrically sorted normal marrow and blood $CAM^{+/-}CD34^+$ myeloid progenitors were performed. L-$selectin^+CD34^+$ myeloid progenitors formed a greater percentage of BFU-E colonies and a lower percentage of CFU-GM colonies than all other $CAM^{+/-}$ $CD34^+$ myeloid progenitors sorted from normal blood. In normal donors, $CAM^{+/-}CD34^+$ myeloid progenitors sorted from blood formed significantly more colonies per 10^6 plated cells than those derived from marrow. L-$selectin^+CD34^+$ myeloid progenitors derived from marrow contained significantly more LTCIC (per 10^6 sorted CAM^+CD34^+ myeloid

progenitors) than those expressing HCAM or VLA-4. In order to determine whether $CD34^+$ myeloid progenitors utilize VLA-4 to bind to fibronectin (FN), *in vitro* binding assays were performed. Adhesion of normal blood derived $VLA\text{-}4^+$ $CD34^+$ myeloid progenitors to FN was blocked by the addition of monoclonal antibodies against the $\alpha 4$ subunit of VLA-4. These data suggest a model of HPC trafficking, in which HPCs utilize VLA-4 to adhere to components of the bone marrow microenvironment, while HPC modulation of L-selectin affinity plays an important role in HPC homing and a less direct role in hematopoietic reconstitution.

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Date Approved: 4/25/1998

INTRODUCTION

Basics of hematopoiesis-a historical narrative

Hematopoiesis, the production and development of blood cells, is a complex process dependent upon the existence of hematopoietic stem cells. By definition, a hematopoietic stem cell is capable of self-renewal and replenishing all lineages of the hematopoietic system, a characteristic known as pluripotency. The concept of a common ancestral stem cell was first proposed almost a century ago by Artur Pappenheim (1870-1916).[1] Since that time, compelling evidence has accumulated to support the concept of a hematopoietic stem cell.[2-4] Using genetically identical mice, Lorenz et al. found that irradiated mice could be rescued from death with marrow from a non-irradiated mouse.[2] The hematopoietic reconstitution in the marrow transplanted mice was believed to be due to the transfer of either a cellular component or a humoral component within the marrow graft. To address this issue, Ford et al. transplanted marrow labeled with the T6-marker chromosome into irradiated mice. After hematopoietic reconstitution, the hematopoietic cells of the rescued mice were found to be positive for the T6-marker chromosome.[3] This study showed that a cellular component of donor origin was being transferred from the marrow graft to the transplant recipient.

Whether a single cell type or a mixed cell type was responsible for hematopoietic reconstitution post-transplantation remained unclear. By studying the splenic nodules (centers of hematopoiesis), which form in irradiated mice post-transplantation, researchers found a linear relationship between the number of normal marrow cells transplanted and the number of splenic nodules formed.[4] That is, the number of splenic colonies formed quantitatively reflected the number of stem cells or hematopoietic progenitor cells (HPCs) contained within the transplanted graft. Further study of this model system revealed that the cells of each splenic nodule were clonogenically identical. The infusion of cells from one nodule into another irradiated mouse resulted in splenic nodules of cells cytogenetically identical to the cells of the originally transplanted nodule.[5,6] Spleen colony (colony forming unit-spleen, CFU-S) assays such as these confirmed that a cellular component was being transferred in the graft and suggested that reconstitution of hematopoietic lineages could result from a single cell type.

In response to the inability to apply the CFU-S assay clinically, as well as the tediousness involved in its execution, *in vitro* colony forming unit (CFU) assays were developed.[7,8] CFU assays involve seeding cells from a stem cell source such as bone marrow into a semi-solid matrix in the presence of growth factors known to stimulate lineage-specific hematopoietic differentiation. The semi-solid matrix prohibits the migration of daughter cells away from the point of seeding of the original growth factor responsive parent cell. Thus a group of cells (a colony) represents all of the progeny of one parent cell. Only the hematopoietic cells able to respond to growth factor supplemented in the media form colonies, while the unresponsive hematopoietic cells

presumably die. For example, the addition of the growth factor erythropoietin to the culture media results in the formation of two types of erythroid colonies known as burst forming unit-erythroid (BFU-E) or colony forming unit-erythroid (CFU-E). Likewise, the addition of the growth factor granulocyte macrophage-colony stimulating factor (GM-CSF) to the culture media results in the formation of myeloid colonies known as colony forming unit-granulocyte macrophage (CFU-GM). The formation of BFU-E or CFU-E colonies *in vitro* indicates the potential of seeded cells to restore erythroid cells, while the formation of CFU-GM colonies indicate the potential of seeded cells to restore the granulocytic and monocytic myeloid lineages. Studies using CFU assays have shown that cells from the mononuclear fraction of mammalian bone marrow, peripheral blood and cord blood can give rise to myeloid lineages when cultured in the presence of lineage-specific growth factors.[9]

To observe, in an *in vitro* setting, the long-term hematopoietic potential of cells from the mononuclear fraction of mammalian bone marrow, long term bone marrow cultures (LTBMCs) were developed.[10] Subsequently, LTBMCs have been adapted such that the long-term hematopoietic potential of cells from the mononuclear fraction of peripheral blood and cord blood may be observed. LTBMCs are established by culturing nucleated marrow cells in a specifically defined media until confluent. The confluent cultures are ridden of contaminating radiation-sensitive pluripotent HPCs while sparing the stromal cells via irradiation.[11] Cells from a stem cell source to be analyzed (marrow, blood, etc.) are plated onto the irradiated stromal cultures and begin to proliferate and differentiate. During weekly half media changes, cells which are no longer bound by the

stromal elements in the LTBMC are collected and analyzed with CFU assays to determine their clonogenic potential. The formation of CFU colonies from nonadherent cells after LTBMC demonstrate the ability of cells within a stem cell source to restore myeloid lineages. Lymphoid lineage restoration may be assessed via long-term culture in media containing fetal calf serum and no exogenous steroids.[11,12] LTBMC assays performed in this manner have demonstrated that mononuclear cells derived from either bone marrow, peripheral blood or cord blood contain pluripotent hematopoietic cell types.

Recall that hematopoietic stem cells by definition are pluripotent and capable of self-renewal. Maintenance of LTBMCs plated in limiting dilutions of a stem cell source in conjunction with CFU assays, in the form of long term culture initiating cell (LTCIC) assays, enables retrospective extrapolation of the number of stem cells contained within the assayed product (e.g. mononuclear cells from marrow). The ability of cultured cells to form CFU colonies at the end of the LTBMC portion of the LTCIC assay (i.e., after 5 weeks or more of culture) demonstrates that the cells within the originally seeded product can maintain long term hematopoiesis and possess clonogenic potential indicative of a hematopoietic stem cell. [13,14]

As suggested by LTBMC and LTCIC assays, long-term hematopoiesis *in vitro* results from expansion of a single cell type which adheres to a supportive stromal layer and requires stimulation with specific hematopoietic growth factors.[15,16] Analysis of LTBMC stromal cell layers has demonstrated that stromal cells produce a variety of cytokines *in vitro* which are necessary for hematopoietic cell adherence.[17] These data

support the concept that normal hematopoiesis depends upon the presence of a pluripotent stem cell, adhesion to stroma, and a complex orchestration of growth factor production.

Hematopoietic stem cell transplant

With the advent of several successful allogeneic marrow transplants in pediatric patients with immunodeficiencies in the late 1960's, hematopoietic stem cell transplantation became a feasible clinical therapy for the treatment of hematopathologies.[18-20] At the present time, hematopoietic stem cell transplants are being applied to a variety of hematologic and nonhematologic malignancies, benign pathologies and genetically determined diseases.[16] The rationale for stem cell transplantation as a therapeutic modality for patients with malignancies is as follows: the patient is administered myeloablative (chemotherapy and/or radiation) therapy, the marrow HPCs are concomitantly destroyed and the patient is rescued with a graft containing HPCs which repopulate the marrow and restore hematopoiesis.

HPCs may be harvested from a variety of sources including marrow, peripheral blood and cord blood. Autologous stem cell transplant involves harvest of the patient's own HPCs either from marrow or apheresis of peripheral blood which are cryostored and returned after myeloablative therapy. Marrow HPCs may be harvested via aspiration of the iliac crest marrow cavity of a patient or donor under general anesthesia. Blood HPCs are typically harvested through successive phereses. Since HPCs represent a relatively

small fraction of mononuclear cells from marrow (1.5%) and blood (less than 0.5%), the overall pool of HPCs available for harvest may be increased prior to harvest via administration of growth factor therapies.[21,22] Growth factor therapies prior to HPC harvest might include, but are not limited to, treatment with hematopoietic growth factors such as granulocyte-colony stimulating factor (G-CSF) or granulocyte macrophage-colony stimulating factor (GM-CSF). A nonexhaustive list of stem cell processing procedures which may be used prior to infusion includes red blood cell salvage, T-cell depletion (allogeneic transplants only), tumor cell purging (autologous transplants only) and $CD34^+$ cell enrichment (both autologous and allogeneic transplants). The stem cell product, regardless of source, may be cryopreserved until time of transplant when it is thawed and intravenously infused into the transplant recipient.

Post-transplant hematologic recovery

Hematologic recovery or post-transplantation engraftment involves the homing of intravenously infused HPCs to the marrow microenvironment and establishment of hematopoiesis. Hematologic recovery entails both clinical and long-term hematologic engraftment. Clinical engraftment refers to the restoration of mature hematologic cells in the peripheral circulation post-transplantation. Under optimal conditions, clinical engraftment occurs within 10-14 days of intravenous infusion of a stem cell graft. Although there is not a consensus definition, clinical engraftment is most frequently

reported to have occurred on the first day that the following peripheral blood counts have been maintained for at least three consecutive days: absolute neutrophil count greater than 500 per microliter and total platelet count of 20,000 per microliter without transfusion support.[23,24] Long-term engraftment refers to the restoration of the transplant recipient's marrow stem cell population such that normal hematopoiesis is maintained for at least one year post-transplant.[23] In a recent study of patients who received bone marrow grafts transfected with a neomycin resistance marker gene, Brenner et al. was able to demonstrate that autologous marrow infusion restores long-term hematopoiesis in cancer patients for up to 18 months post-transplant.[25] Brenner's findings demonstrate that stem cell rescue in humans occurs through repopulation of the host marrow compartment with donor hematopoietic cells; however, these findings do not rule out the possibility that the patient's own stem cells surviving high-dose chemotherapy and/or radiotherapy could also be contributing to hematopoietic recovery post-transplantation.

The most common post-transplantation complications include graft-versus-host disease (allogeneic transplants only) and infection (both allogeneic and autologous transplants) followed by venoclusive disease, graft failure, and disease relapse.[25] Manipulation of several parameters lessens the likelihood of these complications. Successful opportunistic infections of the immunocompromised transplant recipient may be lessened through several pre- and post-transplantation procedures. Prior to transplant, the graft may be optimized to contain sufficient numbers of $CD34^+$ cells (a marker indicative of HPCs), approximately 1-5 x 10^6 cells per kilogram of recipient weight, and sufficient CFU-GMs (indicates potential to restore myeloid cells), approximately 1-5 x

10^4 cells per kilogram of recipient weight.[26,27] In addition, the high-dose chemotherapy regimen administered prior to transplant can affect post-transplant hematologic recovery rates.[28] The period of severe neutropenia and thrombocytopenia suffered prior to clinical engraftment may be significantly shortened through administration of growth factor therapies and specific stem cell sources. Growth factors such as G-CSF or GM-CSF are often administered post-transplant to accelerate granulocyte recovery. The mechanism by which these growth factors speed granulocyte recovery is not known but is believed to occur by stimulating CFU-GMs infused in the graft to differentiate.[29]

In the past two decades the probability of curing a patient via stem cell transplantation has improved steadily depending on the type and stage of disease.[16] Thus, hematopoietic stem cell transplantation remains a feasible clinical therapy for the acceleration of hematologic recovery after cytoreductive therapies.

The hematopoietic microenvironment and cell adhesion molecules

There is substantial evidence that the marrow microenvironment is crucial for the growth and differentiation of hematopoietic cells.[30-33] The marrow microenvironment consists of HPCs, hematopoietic cells at various stages of differentiation, non-hematopoietic cells, soluble factors such as cytokines and growth factors, and extracellular matrix (ECM). HPCs give rise to all mature blood cell types. Non-hematopoietic cells or stromal cells comprise at least four different subsets, as identified in *in vitro* marrow cultures, and include endothelial cells, macrophages,

adipocytes and pre-adipocytic fibroblasts.[32] The marrow ECM contains collagen types I, III, IV, and V; fibronectin; haemonectin; proteoglycans; hyaluronic acid and glycosaminoglycans (GAGs, such as chondroitin sulfate and heparin sulfate).[32]

In studies using long term bone marrow cultures, researchers have demonstrated the *in vitro* necessity for hematopoietic cell adherence to supportive stromal layers in conjunction with specific growth factors for maintenance of long-term hematopoiesis *in vitro*.[15,33] These findings suggest that hematopoietic cell adhesion coupled with cytokines promotes long-term hematopoiesis and further suggest that adhesion is an important step toward long term hematopoietic reconstitution. However, little is known concerning the identity and role of cell adhesion molecules which are important during hematopoiesis.

Possible roles for cell adhesion molecules (CAMs) in normal hematopoiesis and in hematopoietic reconstituion after hematopoietic stem cell transplant include peripheralization and homing of HPCs, presentation of growth factors to HPCs and regulation of mature blood cell egress from the marrow as they differentiate. Understanding the mechanisms governing the trafficking of HPCs to and from the marrow microenvironment would clarify some of these aspects of HPC physiology and prove beneficial to stem cell transplantation therapy. For example, down regulation or masking of key cell adhesion molecules involved in HPC adhesion to the marrow microenvironment might expedite HPC peripheralization. Thus understanding the peripheralization process might enable the development of more efficient and efficacious complimentary mobilization regimens than currently available. Optimization of HPC

homing to the bone marrow microenvironment post-transplantation is critical to the problem of optimizing hematologic recovery following stem cell transplant.

Hematopoietic progenitor cell peripheralization

The mechanism(s) by which hematopoietic progenitor cells are peripheralized from the marrow microenvironment into the blood is unclear. However, studies analyzing the differential surface expression of CAMs during hematopoietic cell maturation have suggested a role for these molecules in the trafficking of HPCs to and from the bone marrow microenvironment.[34,35] Of the CAMs known to mediate HPC interactions with the marrow microenvironment, including integrins and cell surface glycoproteins, the role of homing-associated cell adhesion molecule (HCAM/CD44), very late antigen-4 (VLA-4, CD49d/CD29), and L-selectin (CD62L) in cell-to-cell and cell-to-stroma interactions in the bone marrow strongly suggests that these cell adhesion molecules play an important role in HPC peripheralization.[36-44]

HCAM is involved in mature lymphocyte trafficking and is known to bind to several extracellular matrix components including collagen, hyaluronic acid and fibronectin.[36-38] *In vivo* and *in vitro* experiments have shown that the expression of VLA-4, which is known to bind fibronectin, decreases as myeloid progenitors mature. Decreased expression of VLA-4 might aid the egress of these cells into the peripheral circulation by altering fibronectin binding ability.[35] This concept is supported by *in vivo* murine and primate studies in which the blockage of VLA-4 mediated binding of HPCs

via administration of monoclonal anti-VLA-4 antibody resulted in an increase in the numbers of circulating HPCs.[39,40] These studies suggest that VLA-4 might play an important role in the peripheralization of myeloid progenitor cells. L-selectin has been shown to play a critical role in the 'homing' of mature lymphocytes to peripheral lymph nodes and is expressed on a variety of mature and immature HPCs.[41-43] In a comparative study of bone marrow and leukopheresis samples from patients who had received chemotherapy followed by granulocyte-colony stimulating factor (G-CSF) treatment, the proportion of circulating $CD34^+$ HPCs expressing L-selectin tended to be greater in leukapheresis products than in marrow.[44] These studies suggest that L-selectin might play an important role in $CD34^+$ HPC homing to the marrow microenvironment.

In spite of the many advances in the field of stem cell transplant therapy, many problems remain. Two specific problems are the quality of mobilization of hematopoietic cells into the peripheral circulation for collection and the role of stem cell homing to the marrow in post-transplant hematopoietic recovery. Understanding the role that CAMs play in HPC peripheralization and homing could lead to the development of enhanced stem cell collection protocols and post-transplant engraftment rates, respectively.

Current model of HPC peripheralization

Although the bulk of research pertaining to cell adhesion molecules on hematopoietic cells strongly suggests that HCAM, VLA-4 and L-selectin expression on

HPCs might be involved in the peripheralization of $CD34^+$ HPCs, the specific mechanisms and cell adhesion molecules involved remain unclear. In the current model for HPC peripheralization, HPC expression of HCAM could enable binding to collagens and hyaluronic acid, components of the the marrow microenvironment (Figure 1). HPCs expressing VLA-4 could bind to fibronectin, a component of the marrow microenvironment, and VCAM-1, expressed on stromal and endothelial cells. Once released into the vasculature, HPCs could home to the marrow microenvironment via the homing functions of L-selectin. Thus changes in VLA-4 and/or HCAM expression on HPCs, as a result of down-regulation or masking of cell adhesion molecule function, could result in egress from the marrow microenvironment; while up-regulation or changes in ligand binding affinity of L-selectin expressed on HPCs could result in ingress into the marrow microenvironment.

In order to determine if cell adhesion molecule modulation is involved in HPC peripheralization, the present study was designed to address the manner in which HCAM, VLA-4 and L-selectin are involved in the trafficking of hematopoietic progenitor cells between the marrow microenvironment and the peripheral circulation. First, this study illuminates the quantitative and qualitative differences in CAM (HCAM, VLA-4 or L-selectin) expression on $CD34^+$ HPCs from the blood and marrow of G-CSF mobilized patients or normal individuals. Second, the role of CAM mediated adhesion of HPCs regarding *in vitro* clonogenic potential, ability to establish long-term hematopoiesis and overall pluripotent hematopoietic stem cell content of each CAM^+CD34^+ HPC subset was determined. Third, the ability of $CD34^+$ myeloid progenitor cells to bind to fibronectin, a

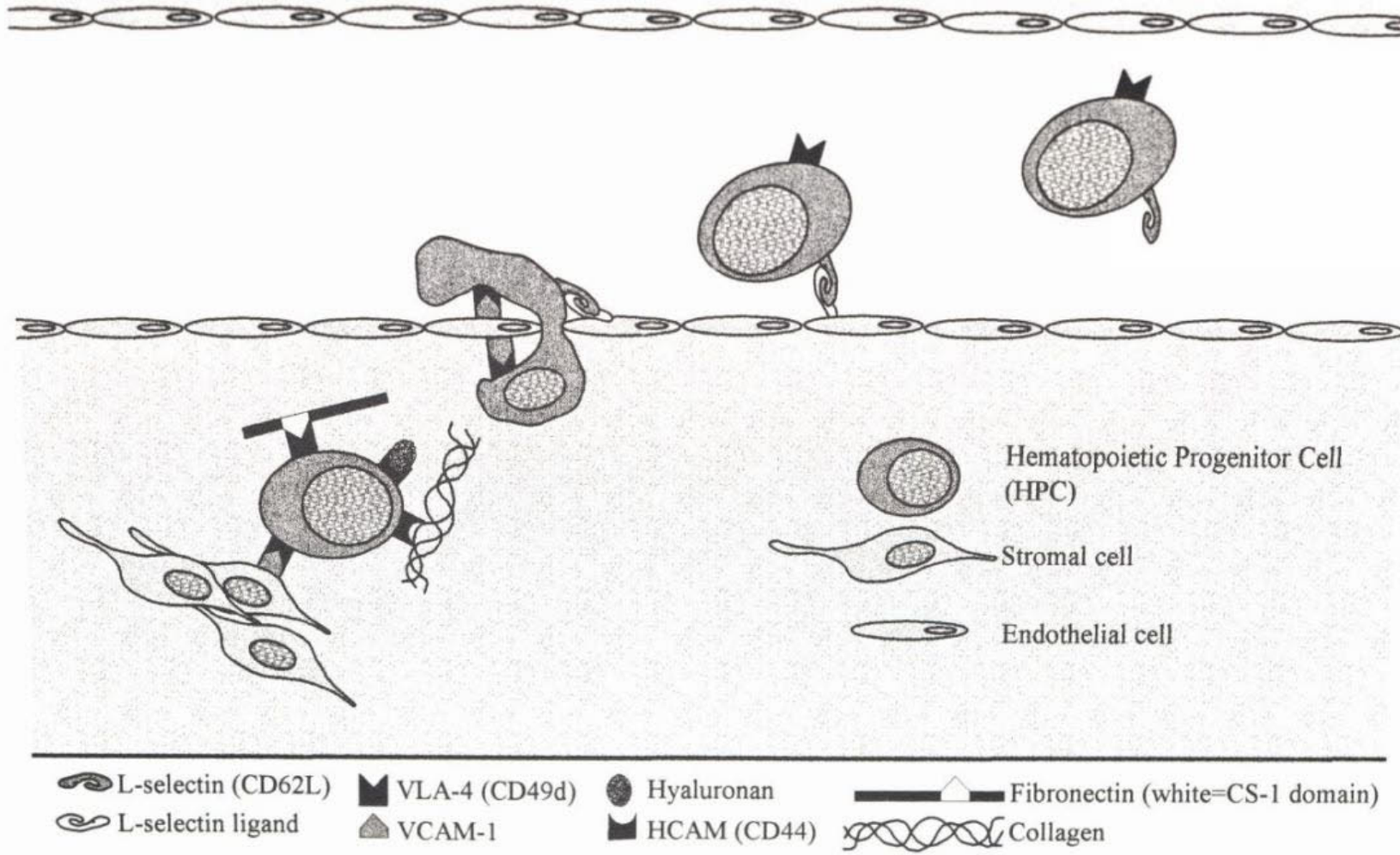


Figure 1. Current model of cell adhesion molecules in hematopoietic progenitor cell trafficking.

constituent of the bone marrow microenvironment, via the integrin VLA-4, was analyzed in *in vitro* adhesion assays. These studies provide insight into the role of cell adhesion molecules in hematopoietic progenitor cell trafficking between the marrow microenvironment and the peripheral circulation.

OBJECTIVES

Hypothesis

Modulation of the expression of the following cell adhesion molecules: homing-associated cell adhesion molecule, very late antigen-4, and L-selectin play a role in regulating the trafficking of pluripotent hematopoietic progenitor cells between the bone marrow microenvironment and the peripheral circulation.

Main objective

The main objective of this study was to test three predictions of the operating hypothesis stated above. Those predictions were 1) that differences in the surface expression of the CAM (where CAM represents the cell adhesion molecules HCAM, VLA-4 and L-selectin) would be detectable between specimens of marrow and blood; 2) that both high and low CAM expressing $CD34^+$ cells can be functionally demonstrated to be hematopoietic progenitors; and 3) that VLA-4 mediates adhesion of $CD34^+$ cells to a fibronectin matrix, a component of the hematopoietic microenvironment.

Specific aim 1

To use flow cytometric analysis to determine the qualitative and quantitative differences in cell surface expression of HCAM, VLA-4 and L-selectin on $CD34^+$ myeloid progenitors residing in the marrow as opposed to those circulating in the blood of the same individual.

1) To enable detection and differentiation of CAM expression on $CD34^+$ myeloid progenitors, a flow cytometric analysis protocol was designed.

2) To enable interpretation of qualitative and quantitative differences in cell surface CAM expression, a computer assisted analysis program was designed.

Specific aim 2

To determine if the $CAM^{+/-}CD34^+$ myeloid progenitor populations examined in Specific Aim One can be functionally demonstrated to be hematopoietic progenitors capable of establishing both short-term and long-term hematopoiesis *in vitro*.

1) To ensure that highly purified (>90%), viable populations of $CAM^{+/-}CD34^+$ myeloid progenitors (i.e., those myeloid progenitors expressing (+) or not expressing (-) HCAM, VLA-4, or L-selectin) from marrow and blood could be feasibly procured, a flow cytometric single cell sorting protocol was developed.

2) To determine the short-term hematopoietic potential of flow cytometrically sorted $CAM^{+/-}CD34^{+}$ myeloid progenitors, *in vitro* colony forming unit assays were utilized.

3) To determine the long-term hematopoietic potential and the number of stem cells contained within each sorted $CAM^{+}CD34^{+}$ myeloid progenitor population, *in vitro* long term culture initiating cell assays were utilized.

Specific aim 3

To demonstrate that VLA-4 mediates adhesion of $CD34^{+}$ cells to the hematopoietic microenvironment.

1) To observe and quantitate $CD34^{+}$ myeloid progenitor cell adhesion to fibronectin, a component of the hematopoietic microenvironment, an *in vitro* adhesion assay was developed.

2) To demonstrate that the adhesion of $CD34^{+}$ myeloid progenitors to fibronectin occurs, at least in part, via the cell adhesion molecule VLA-4, monoclonal antibodies were used to block the binding functions of the $\alpha 4$ chain of the VLA-4 heterodimer to the CS-1 binding domain of fibronectin in *in vitro* adhesion assays.

MATERIALS AND METHODS

Patient specimens

Seven female patients, ranging in age from 28 to 61 (mean=45, median=43), diagnosed with breast cancer with no prior history of marrow involvement, received five days of recombinant human granulocyte-colony stimulating factor (G-CSF, Neupogen; Amgen, Thousand Oaks, CA) at 16 μg G-CSF / kg body weight by intravenous infusion. Peripheral blood and bone marrow were collected on day 6 of G-CSF mobilization. Peripheral blood and bone marrow were collected from seven normal donors (1 male, 6 females) ranging in age from 27 to 58 (mean=37, median=34) who were being harvested for allogeneic marrow transplantation. Peripheral blood was collected immediately prior to bone marrow harvest in all cases. Informed consent was obtained from each subject according to the guidelines and with the approval of the University of South Florida Institutional Review Board.

Preparation of nucleated marrow or peripheral blood cells

Preparation of human mononuclear cell populations from bone marrow and peripheral blood was adapted from a protocol developed by Boyum in the late 1960's based on principles of density gradient centrifugation (APPENDIX 1).[45,46] Bone marrow (20 mL) and peripheral blood (approximately 17 mL) samples were collected into heparinized syringes and evacuated tubes, respectively. Each sample was diluted [1:1] with Dulbecco's phosphate buffered salt solution without calcium and magnesium (DPBS, pH=7.0; Mediatech, Herndon, VA), layered onto Polymorphprep (pH=6.8±0.5, density=1.113±0.001 g/mL, osmolarity 460±15 mOsm; NYCOMED PHARMA AS, Oslo, Norway) at 20 mL diluted sample:10 mL Polymorphprep and centrifuged at 500 x g for 30 minutes at room temperature (RT). The mononuclear and polymorphonuclear cell fractions were collected and washed with DPBS by resuspension and subsequent centrifugation at 615 x g for 5 minutes at RT. The cells were washed in magnetic activated cell sorting (MACS) Buffer (MB;[0.5% bovine serum albumin (Calbiochem-Novabiochem Corporation, La Jolla, CA) in DPBS]). Cell counts were determined using an automated hematology analyzer (Sysmex K-1000; TOA Medical Electronics Co., Ltd., Kobe, Japan). Follow-up cell counts and determination of viable cells present in a cell suspension were performed using a hemacytometer in conjunction with the trypan blue exclusion assay of cell viability.[47,48] The trypan blue dye exclusion test is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, eosin, or propidium, whereas dead cell do not. In this test, a cell

suspension is simply mixed with trypan blue and then visually examined to determine whether cells take up or exclude dye. A clear cytoplasm would indicate a viable cell whereas a blue cytoplasm would indicate a nonviable cell. Thus, a cell suspension was diluted [1:1] with trypan blue stain (0.4% trypan blue in 0.85% saline, membrane filtered, pH=7.0, osmolarity 290±340 mOsm; GibcoBRL, Life Technologies, Inc. Grand Island, N.Y.). Ten microliters of stained cell suspension were pipeted onto a hemacytometer. The hemacytometer was placed onto the stage of binocular microscope and the number of viable (clear, unstained) cells and nonviable (blue stained) cells were microscopically enumerated per 10 grids. The concentration of the cell suspension was determined from the following equation:

$$[\text{Cells/mL}] = \left(\frac{\text{total cells counted}}{\text{number grids counted}} \right) \times (\text{dilution factor}) \times 10^4.$$

The percentage of viable cells was obtained from the following equation:

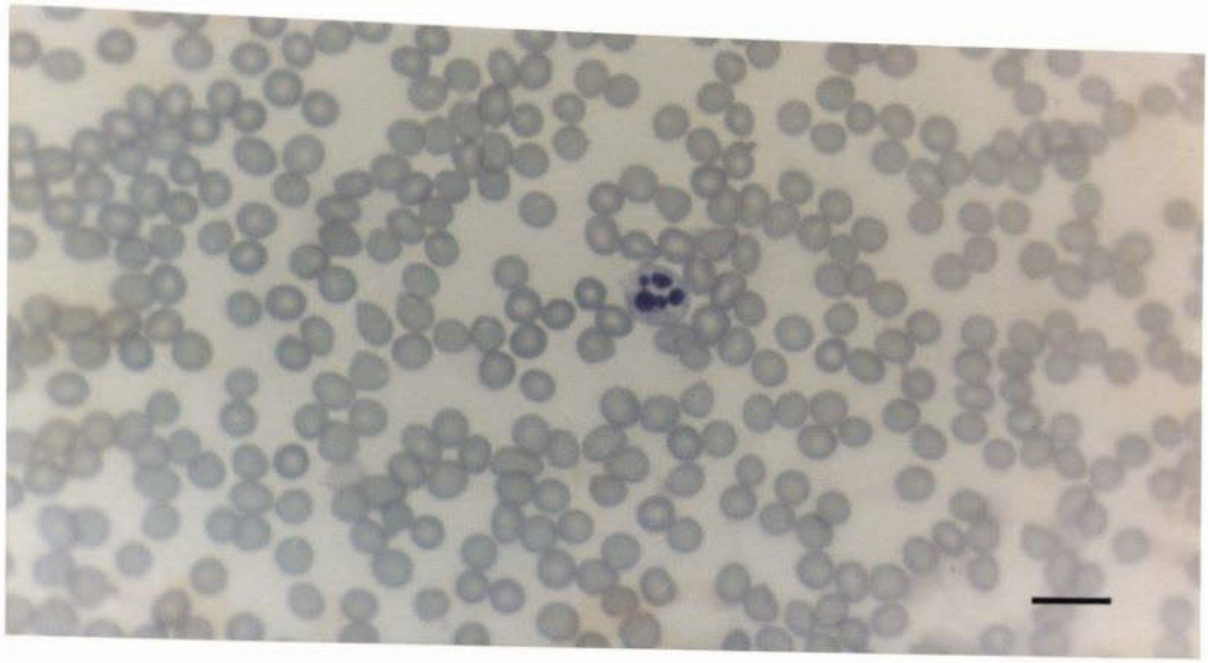
$$\% \text{ Viable cells} = \left(\frac{\text{Viable cells}}{\text{Viable cells} + \text{Nonviable cells}} \right) \times (100).$$

Cells were collected before and after centrifugation, mounted onto slides and stained with Leukostat (Fisher Scientific, Pittsburgh, PA) for morphological confirmation of density gradient enrichment of leukocytes (Figures 2 and 3.)

Preparation of nucleated peripheral blood buffy coat cells

Normal human peripheral blood was collected into bags containing 63 mL anticoagulant citrate phosphate dextrose solution (USP for collection of 450 mL blood;

A.



B.

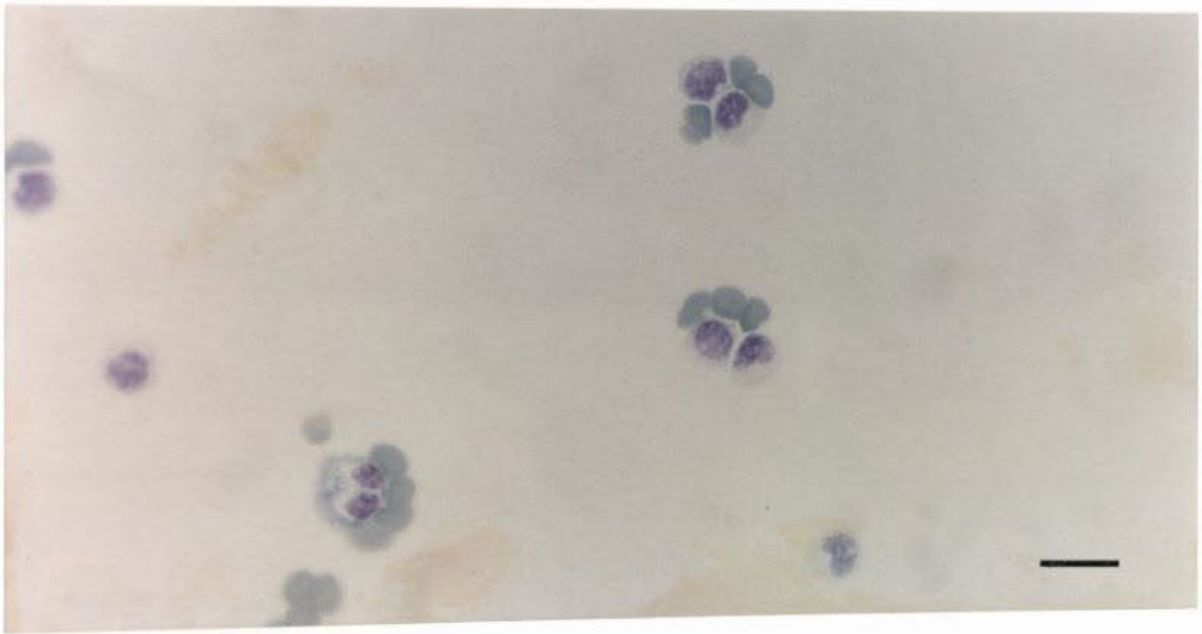
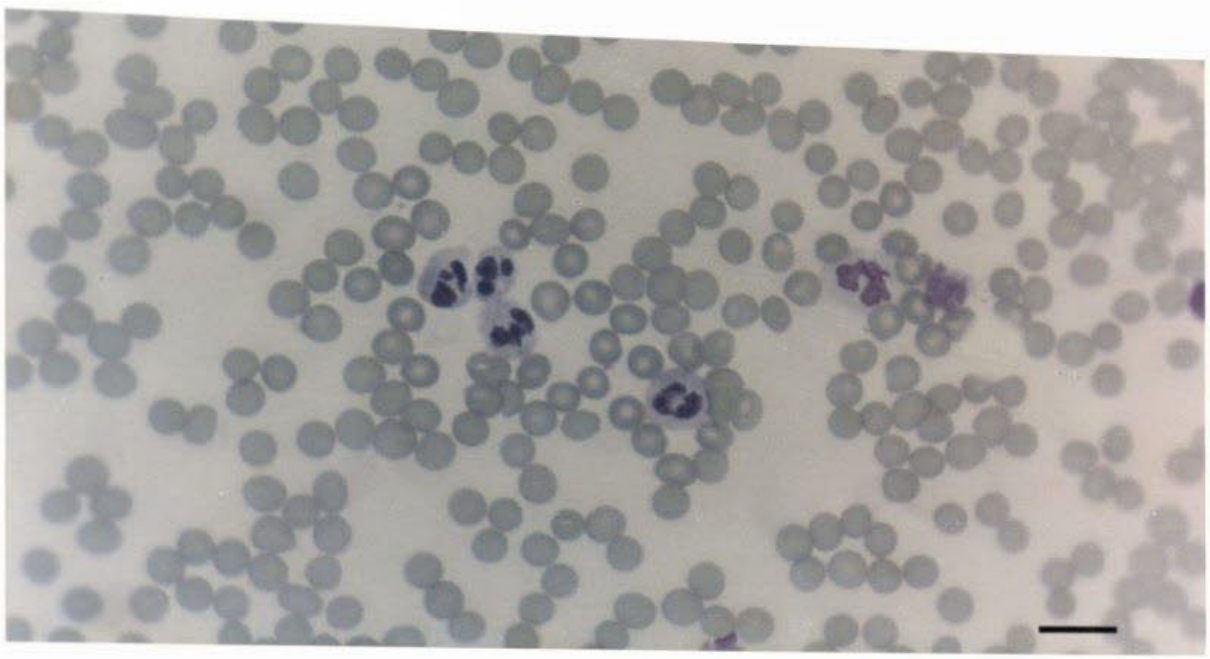


Figure 2. Morphological confirmation of density gradient leukocyte enrichment of blood specimen. Density gradient centrifugation of blood specimens was performed using Ficoll-Paque Plus in order to enrich for leukocytes. Cells were collected (A) before centrifugation (magnification 683X; bar = 15 μm) and (B) after centrifugation (magnification 597X; bar = 17 μm), mounted onto slides and stained with Leukostat.

A.



B.

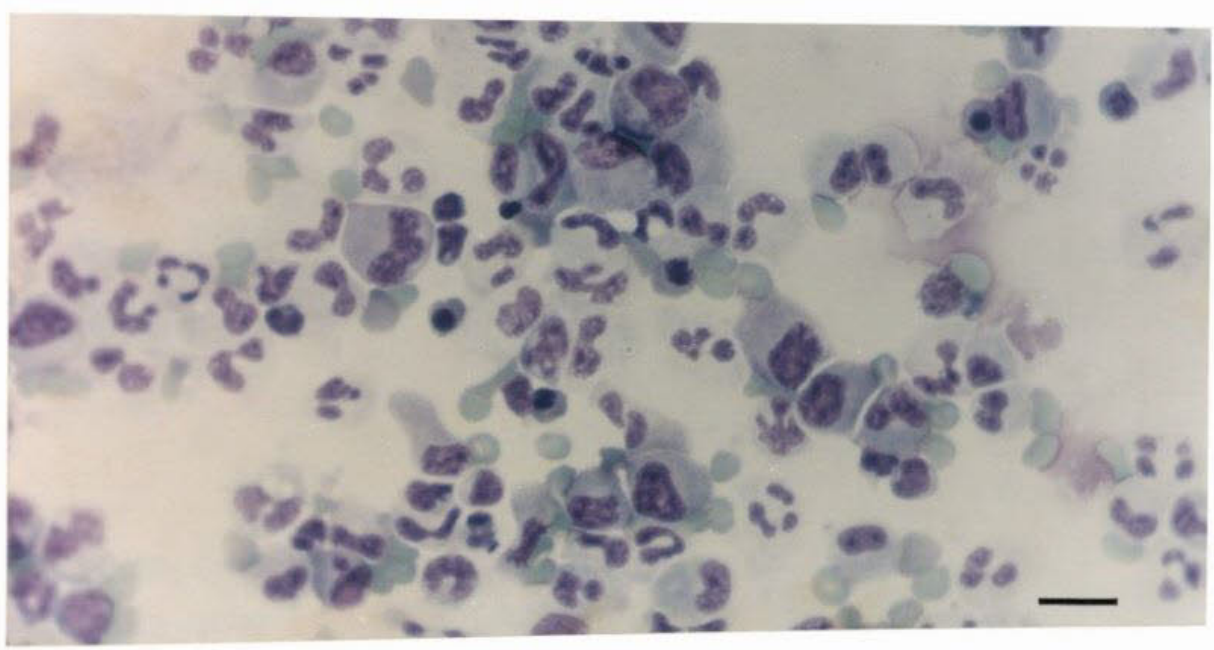


Figure 3. Morphological confirmation of density gradient leukocyte enrichment of marrow specimen. Density gradient centrifugation of marrow specimens was performed using Polymorphprep in order to enrich for leukocytes. Cells were collected (A) before and (B) after centrifugation, mounted onto slides and stained with Leukostat. Magnification = 683X. Bar = 15 μm

Appendix 2). After collection, the product was combined with 2.2 g dextrose USP, 900 mg NaCl, 750 mg mannitol USP, 27 mg adenine USP and 15.4 mEq sodium and enriched for leukocytes via centrifugation. Of the leukocyte enriched fraction (also termed buffy coat), 50 uL was diluted [1:1] with DPBS for preparation of human mononuclear cell populations as previously described.[46] Diluted buffy coat was layered onto Ficoll-Paque® Plus (pH=7.4, density=1.077±0.001 g/mL, osmolarity=300mOsm; Pharmacia Biotech AB, Uppsala, Sweden) at [25 mL diluted buffy coat:10 mL Ficoll-Paque® Plus] and centrifuged at 500 x g for 30 minutes at RT. Buffy coats were preferentially enriched for leukocytes using Ficoll-Paque® Plus because leukocyte separation using Polymorphprep (as described above for use with whole blood or marrow samples) did not result in formation of a buffy coat layer after centrifugation. The nucleated cell fraction was collected and resuspended in DPBS. In order to circumvent the clogging of the Miltenyi columns due to platelet activation, the platelet content of the nucleated cell fraction was reduced to ≤ [100 x 10^6 platelets/mL] via a *minimum* of three successive platelet reducing centrifugations at 200 x g for 8 minutes at RT using DPBS as the wash buffer after each centrifugation.[46]

Enrichment for $CD34^+$ cells

Mononuclear cells from peripheral blood, buffy coat samples and bone marrow were enriched for $CD34^+$ hematopoietic progenitor cells using a MACS CD34 Isolation Kit (Miltenyi Biotec, Auburn, CA; Figure 4). The MACS CD34 progenitor cell

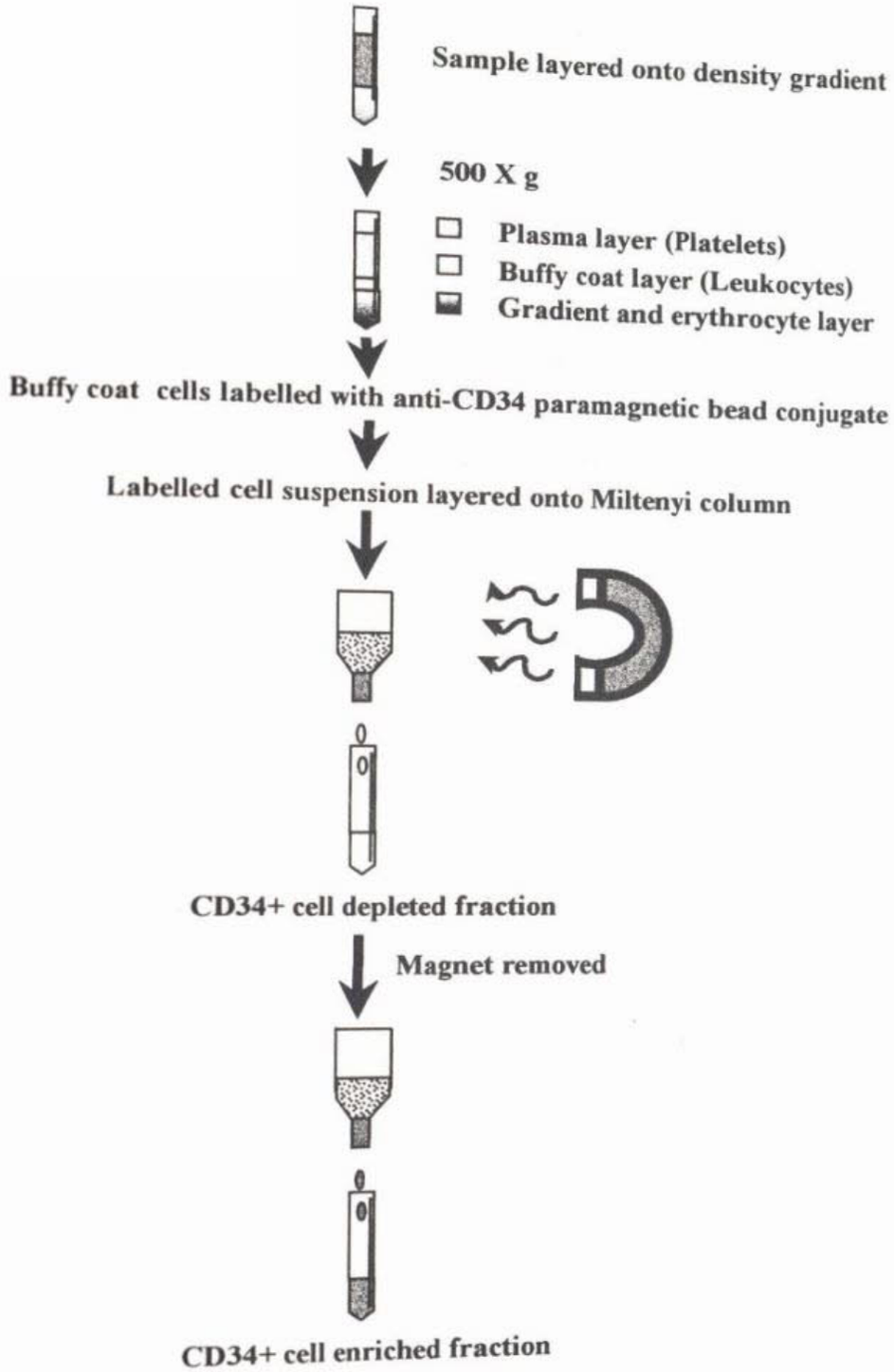


Figure 4. Enrichment of CD34+ cells from marrow or blood samples.

isolation kit is an indirect magnetic labeling system for the isolation of $CD34^+$ hematopoietic progenitor cells from a stem cell source (marrow, blood, cord blood, etc.) by positive selection of CD34 expressing cells. Mononuclear cells from a stem cell source, obtained by density gradient centrifugation using Ficoll-Paque® Plus or Polymorphprep (as previously described), are indirectly magnetically labelled using a hapten-conjugated primary monoclonal antibody and an anti-hapten secondary antibody coupled to MACS MicroBeads. The magnetically labelled cells are enriched on positive selection columns in the magnetic field of the MiniMACs or VarioMACS column.

In accordance with the MACS CD34 progenitor cell isolation kit recommended protocol, mononuclear cells from marrow, peripheral blood or buffy coats were washed in MACS Buffer (MB;[0.5% bovine serum albumin (Calbiochem-Novabiochem) in DPBS at pH 7.4]) via centrifugation at 615 x g for 5 minutes at RT. Cell counts were determined using an automated hematology analyzer (Sysmex K-1000). Cells were incubated with 100 µL Reagent A1 (Fc receptor blocking reagent; human immunoglobulin) per 1×10^8 total cells for 5 minutes at 4 °C. One hundred microliters of Reagent A2 (hapten-modified QBEND10 mAb which recognizes CD34)[49] per 1×10^8 total cells was added and incubation was carried out for an additional 15 minutes at 4°C. Cells were washed in 2.5 mL MB per 1×10^8 total cells and centrifuged at 615 x g for 5 minutes at RT. The pellet was resuspended in 400 µL MB and 100 µL Reagent B (colloidal super-paramagnetic microbeads bound to antibody which recognizes the hapten of Reagent A2; personal communication with Kevin Mills, Ph.D., Miltenyi Biotec)[50-52] per 1×10^8 total cells and incubated at 4 °C for 15 minutes. Cells were

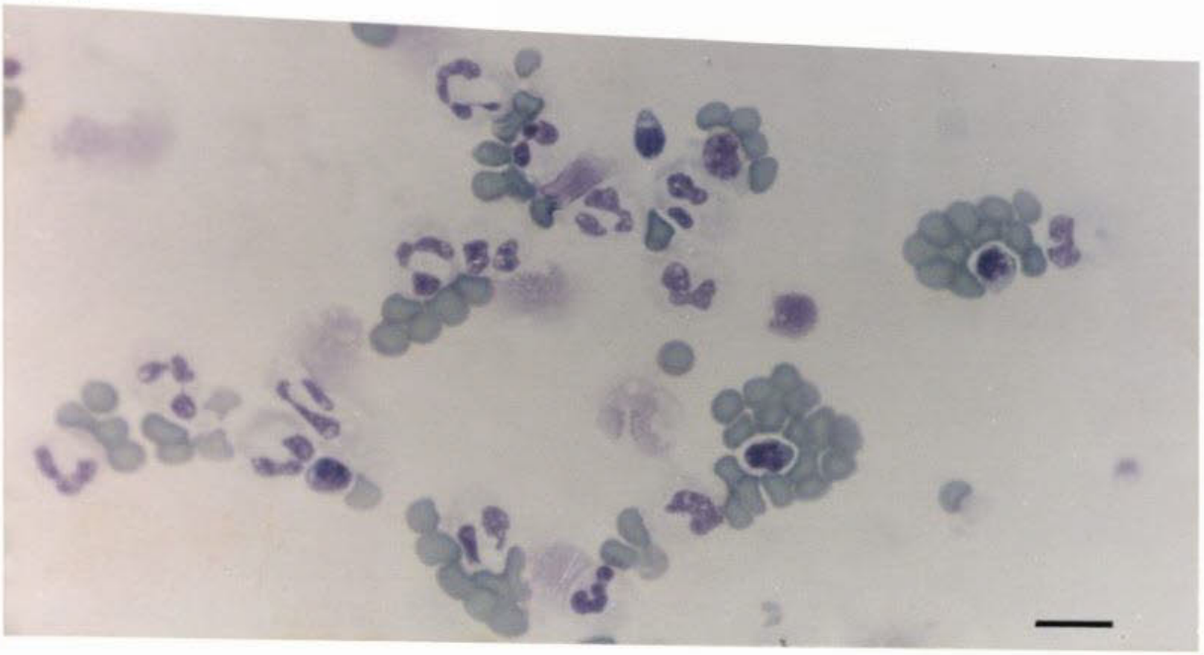
washed with MB, resuspended in MB at 500 μL per 1 x 10^8 total cells and filtered through a 30 μm nylon mesh pre-separation filter (prewashed with 1.0 mL MB; Miltenyi Biotec). The type of positive selection column chosen for $CD34^+$ hematopoietic progenitor cell enrichment depended on the number of total unseparated nucleated cells obtained after density centrifugation. The Mini-MACs Separation Column with a maximum cellular capacity of 2 x 10^8 total cells was used when the sample contained ≤ 2 x 10^8 total unseparated, nucleated cells (determined using an automated hematology analyzer after density gradient centrifugation as previously described; Sysmex K-1000). The VS^+ Vario-MACs Separation Column with a maximum cellular capacity of 2 x 10^9 total cells was used when the sample contained ≤ 2 x 10^9 total nucleated cells (based on cell counts using an automated hematology analyzer; Sysmex K-1000). The Mini-MACS column was prewashed with 1.0 mL MB while the VS^+ Vario-MACs Separation Column was prewashed with 3.0 mL MB in the presence of a magnet prior to addition of microbead labelled sample. Unbound cells were eluted by washing the column three times with 0.5 mL MB (Mini-MACS Separation Column) or with 1 mL MB (VS^+ Vario-MACs Separation Column) and were discarded (listed as $CD34^+$ cell depleted fraction in Figure 4). The bound cells ($CD34^+$ cell enriched fraction) were collected by removing the magnet and gently pushing 1 mL (Mini-MACS Separation Column) or 5 mL MB (VS^+ Vario-MACs Separation Column) through the column with a syringe plunger. $CD34^+$ cell enriched fractions of G-CSF mobilized marrow and blood contained 32±18% and 17±15% [mean ± 2 x standard error of mean (s.e.m.) %] $CD34^+$ cells, respectively, as determined by flow cytometric analysis (next section). Normal donor

marrow and blood MAC positive fractions contained 57±12% and 30±16% (mean±2 x s.e.m.%) $CD34^+$ cells, respectively, as determined by flow cytometric analysis (next section). Pooled normal buffy coat positive fractions contained 48±5% (mean±2 x s.e.m.%) $CD34^+$ cells. Cell counts were performed using an automated hematology analyzer (Sysmex K-1000) or hemacytometer as previously described. Samples of blood and marrow before and after $CD34^+$ cell enrichment were mounted onto slides and stained with Leukostat as previously described, for morphological confirmation of Miltenyi column myeloid progenitor cell enrichment of blood and marrow samples (Figures 5 and 6, Appendix 3).

Flow cytometric analysis

Flow cytometry employs instrumentation that scans single cells flowing past excitation sources in a liquid medium.[53] This technology provides rapid, quantitative, multiparameter analyses on single living (or dead) cells. Measurement of visible and fluorescent light emissions allows quantitation of antigenic, biochemical and biophysical characteristics of individual cells. Flow cytometric technology can also separate distinct subpopulations of cells on the basis of these measured characteristics, a technology called electronic cell sorting discussed in the following section. Flow cytometry experiments involve three distinct, interdependent phases (1) reagent preparation, cell preparation and cell staining with fluorescent reagents (fluorochrome conjugated mAbs); (2) processing the labelled cells with the flow cytometer and data collection of one or more parameter;

A.



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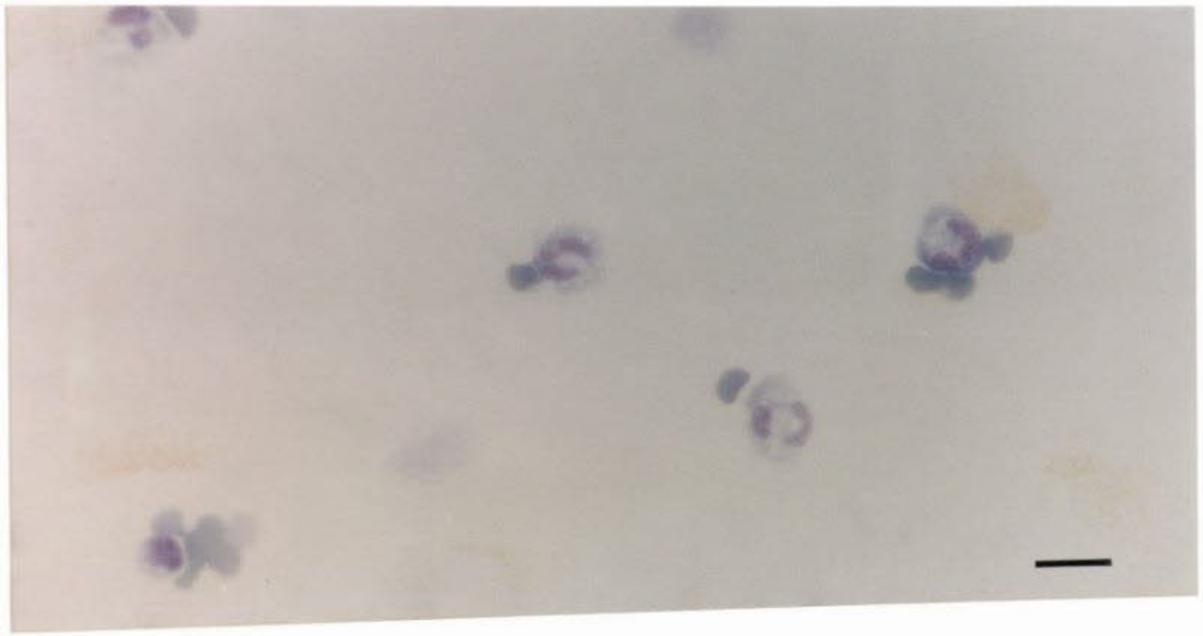
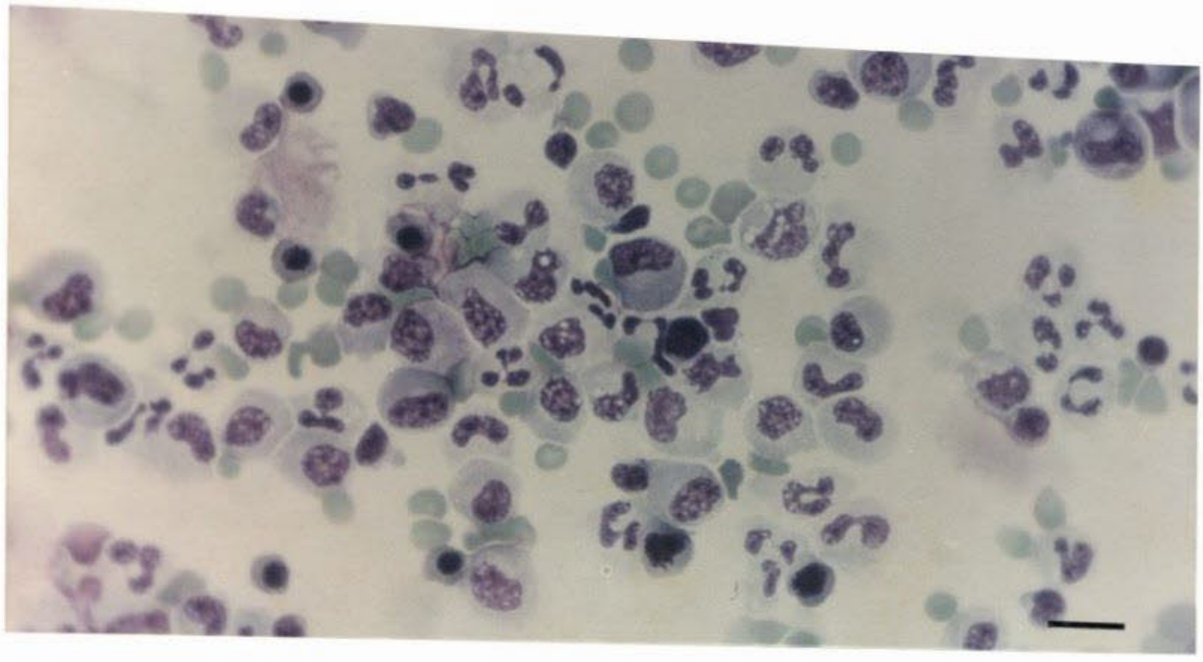


Figure 5. Morphological confirmation of Miltenyi column enrichment of blood derived myeloid progenitor cells. Leukocyte enriched blood specimens were labelled with an anti-CD34 paramagnetic bead conjugate and enriched for CD34+ cells using MACS. Cells from fractions either (A) depleted of CD34+ cells (magnification 608X; bar = 16 μm) or (B) enriched for CD34+ cells (magnification 683X; bar = 15 μm) were mounted onto slides and stained with Leukostat (Figure 4).

A.



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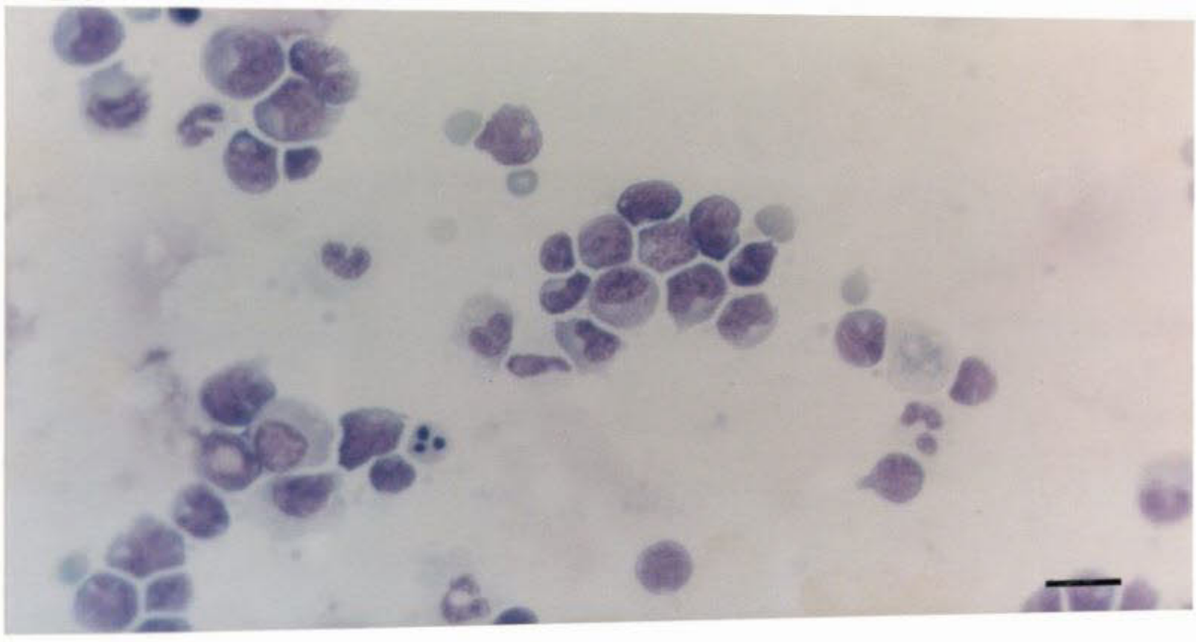


Figure 6. Morphological confirmation of Miltenyi column enrichment of marrow derived myeloid progenitor cells. Leukocyte enriched marrow specimens were labelled with an anti-CD34 paramagnetic bead conjugate and enriched for CD34+ cells using MACS. Cells from each column fraction either (A) depleted of CD34+ cells (magnification 512X; bar = 20 μm) or (B) enriched for CD34+ cells (magnification 598X; bar = 17 μm) were mounted onto slides and stained with Leukostat (Figure 4).

and (3) computer assisted analysis of flow cytometrically acquired data. FACScan and FACSCaliber flow cytometers (both from Becton Dickinson Immunocytometry Systems, San Jose, CA; BDIS) utilize single air-cooled argon lasers which emit light at 488 nm (turquoise). When a single fluorochrome labelled cell flows past the argon laser each fluorochrome emits distinct, distinguishable peak emission wavelengths as follows: fluorescein isothiocyanate at 525 nm (FITC; green), phycoerythrin at 575 nm (PE; orange-red), and peridinin chlorophyll protein above 650 nm (PerCP; red; Figure 7).[53-56] The peak emission wavelengths for each of the fluorochromes is sufficiently far enough apart that each signal can be detected by separate detectors. Thus fluorescent signal may be regarded as proportional to the amount of mAb attached to the antigenic determinants of the analyzed cell.

Samples of 1 x 10^6 $CD34^+$ cell enriched fractions or 50 µL Quantum Simply Cellular (QSC) Beads (2 X 10^6 QSC beads/mL; Flow Cytometry Standards Corporation, San Juan, PR) were labelled with three different fluorochrome-conjugated mAbs (flow cytometric fluorochrome conjugated mAb labelling summarized in Appendix 4). The samples were incubated with mAbs from Becton Dickinson Immunocytometry Systems (BDIS; San Jose, CA) unless otherwise noted. Samples were incubated for 30 minutes at 4 °C protected from light with 20 µL PE-conjugated anti-CD34 mAb (anti-HPCA-2), 10 µL PerCP-conjugated anti-CD45 (anti-HLe-1; an antigen present on all hematopoietic cells) and one of the following FITC-conjugated mAbs: 20 µL anti-CD44 (anti-HCAM/anti-Leu-44), 40 µL anti-CD49d (anti-α4 chain of VLA-4 complex; Immunotech, Westbrook, ME), or 20 µL anti-CD62L (anti-L-selectin/ anti-Leu-8).

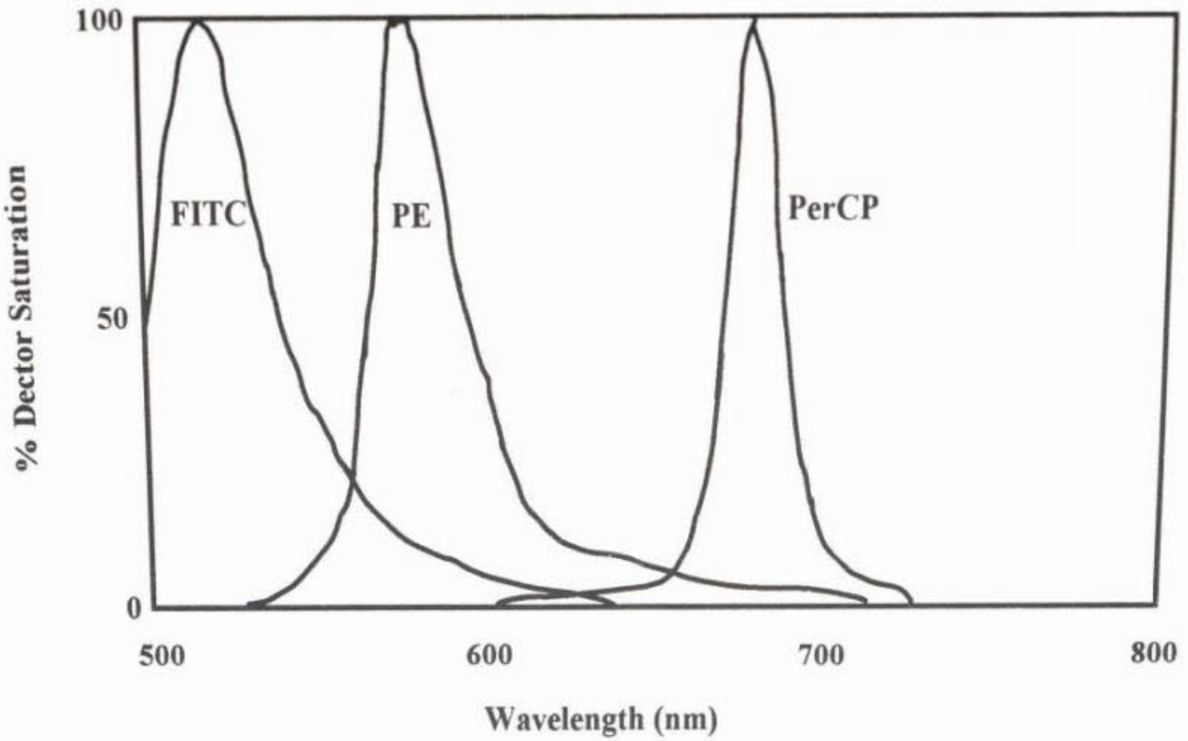


Figure 7. Emission spectra of fluorochromes: FITC, PE and PerCP. FITC=fluorescein isothiocyanate, PE=phycoerythrin, and PerCP=peridinin chlorophyll protein

Isotype-identical mouse mAbs to irrelevant antigens served as controls (IgG2a-FITC, Immunotech; IgG1-FITC and IgG1-PE). Anti-CD29 (anti-ß1 chain of VLA-4 complex; Immunotech) served as a control for recognition of the integrin VLA-4 (an $\alpha 4ß1$ heterodimer). Analysis of CD49d and CD29 expression in our samples revealed that there was no significant difference ($p>0.05$) between marrow or blood, thus confirming that our measurements of CD49d represented VLA-4 and not an unidentified $\alpha 4$ integrin. Residual red blood cells (RBCs) were lysed and the antibody-coated cells fixed by incubation at BDIS's recommended concentrations of [1×10^6 $CD34^+$ enriched cells/2 mL FACS™ Lysing Solution (BDIS)] or [1×10^5 QSC beads/ 2 mL FACS Lysing Solution] for 10 minutes at RT protected from light. RBC lysis involves the preferential lysis of RBCs by the use of a hypotonic solution (FACS™ Lysing Solution) which leaves the more robust polymorphonuclear and mononuclear cells intact. RBCs outnumber leukocytes by about a thousand to one in the peripheral circulation; thus, the aquisition of 20,000 mononuclear events without removal of RBCs would not be technologically practical.[56] After two washes with DPBS, the pellets were resuspended in 200 µL of DPBS and analyzed on a FACScan flow cytometer (BDIS). Fluorescence compensation, electronic distinction between the peak emission of each fluorochrome, was adjusted using cells labelled with either anti-CD14-FITC or anti-CD14-PE (BDIS) and an appropriate isotype-identical control (IgG1-FITC and IgG1-PE, respectively; BDIS)(Figure 8 and Appendix 4).[54] Levels of fluorescence intensity considered positive were set at regions above emissions from unlabelled cells (autofluorescence) or from cells labelled with an appropriate isotype-identical control (nonspecific mAb binding;

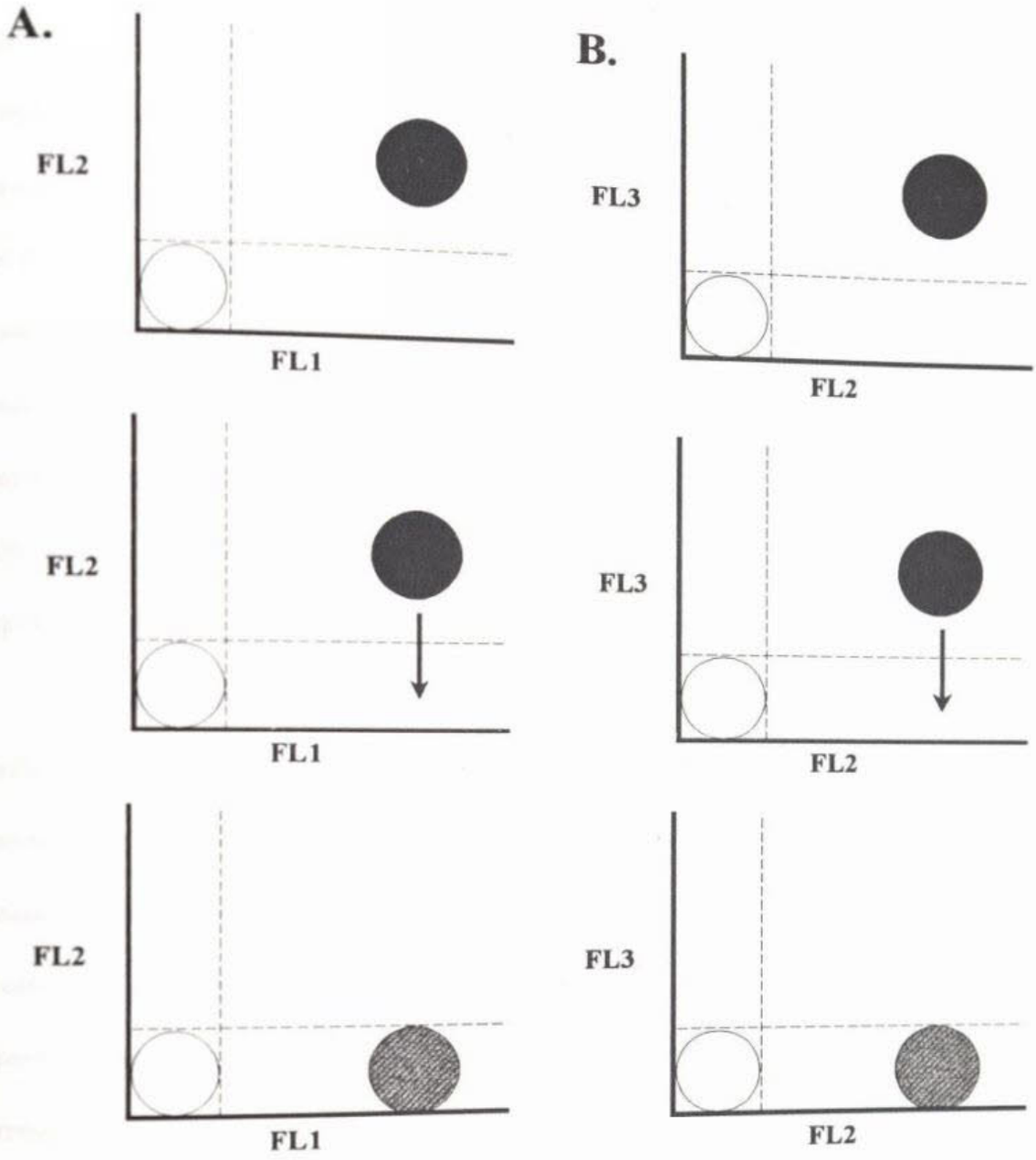


Figure 8. Fluorescence compensation. Cell populations (circles) whose emmissions spectra overlaps with another fluorochrome may be compensated (ie, false positive events removed) by directing the flow cytometer to discard fluorescence signal sensed in the inappropriate channel. When compensating between FL1 and FL2 channels, true FLl+ events whose emissions spectra overlaps into the FL2 channel (●) may be compensated (◍) by adjusting flow cytometric parameters (⟷); unlabelled cells (◯) are detected as negative events in both channels. Compensation between FL2 and FL3 channels (B) is performed as with FL1/FL2 compensation.

Figure 8). Autofluorescence can occur when cellular constituents absorb energy and emit light when illuminated by laser light. Nonspecific mAb binding can occur when Fc receptors present on the cell membrane bind the Fc portion of mAbs used for labelling. Forward scatter (FSC) events were collected on a linear scale, while side scatter (SSC) and fluorescence channel (FL1, FL2 and FL3) events were collected on a log scale. List mode files of 10,000 events per sample tube were acquired using Lysis II software in conjunction with the FACStar and FACScan flow cytometers or using CELLQuest in conjunction with the FACSCaliber flow cytometer (all programs and instruments by BDIS). Data files were analyzed offline using WinList software (Verity Software House, Topsham, ME).

The individual cell types in each sample were differentiated using a method similar to the one originally reported by Stelzer et al.[57] Analysis of SSC and CD45 fluorescence (CD45 is expressed on all human leukocytes but absent from non-hematopoietic tissues) allowed differentiation of marrow and blood into distinct regions of cell types including lymphocytes, monocytes, immature and mature granulocytes, myeloid progenitors and lymphoid progenitors (Figure 9).[57,58] Individual cell types, corresponding to SSC and CD45 fluorescence cell type regions, were sorted using a FACStar or FACSCaliber flow cytometer (BDIS) from samples of marrow and blood (consult Flow cytometric single cell sorting section). Microscope slides were prepared of each sorted cell type region using a cytocentrifuge and counterstained with Leukostat (Fisher Scientific; Figures 10 and 11). Leukocyte differentials of prepared slides enabled confirmation of the morphologic characteristics of sorted cell type regions. Leukocyte

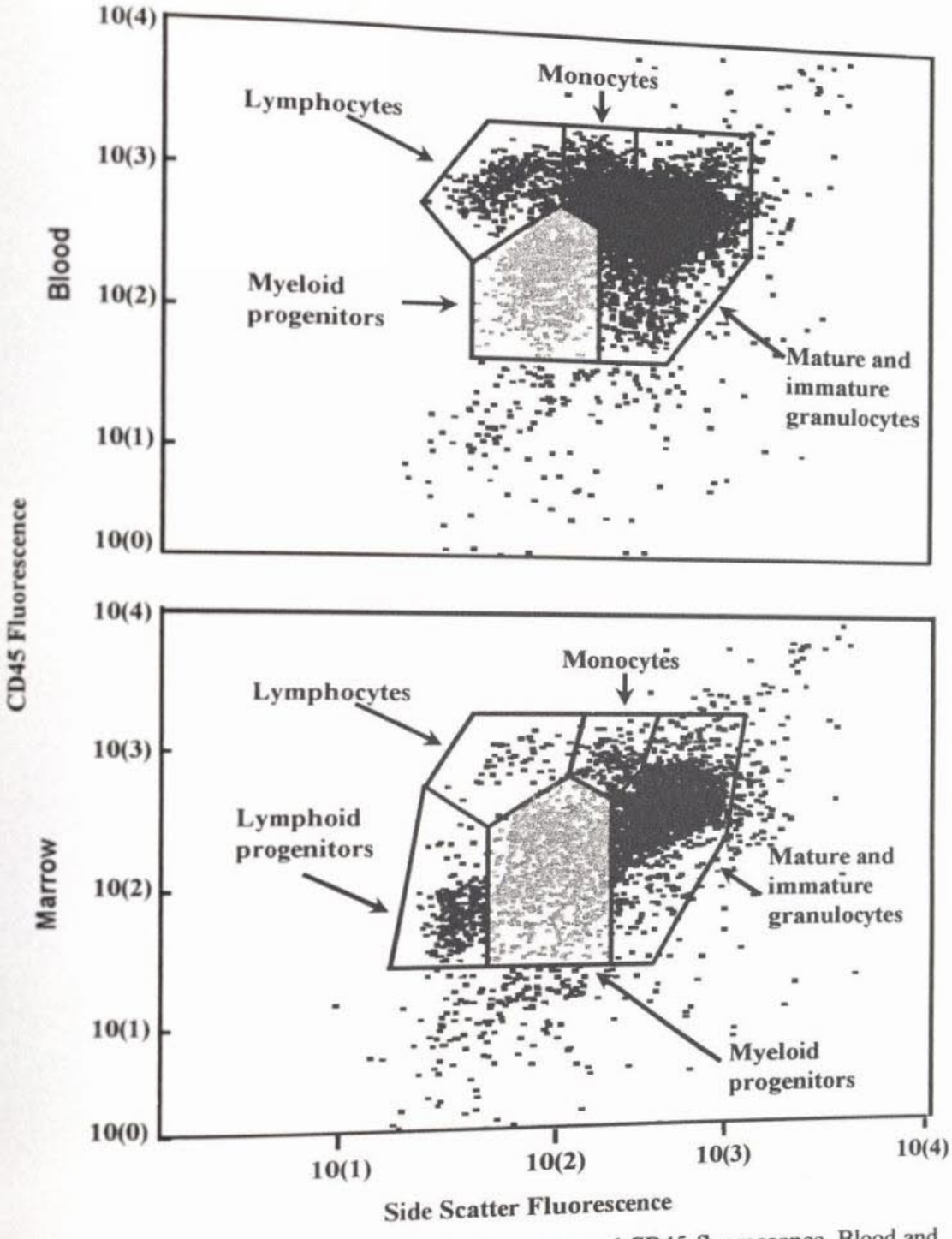
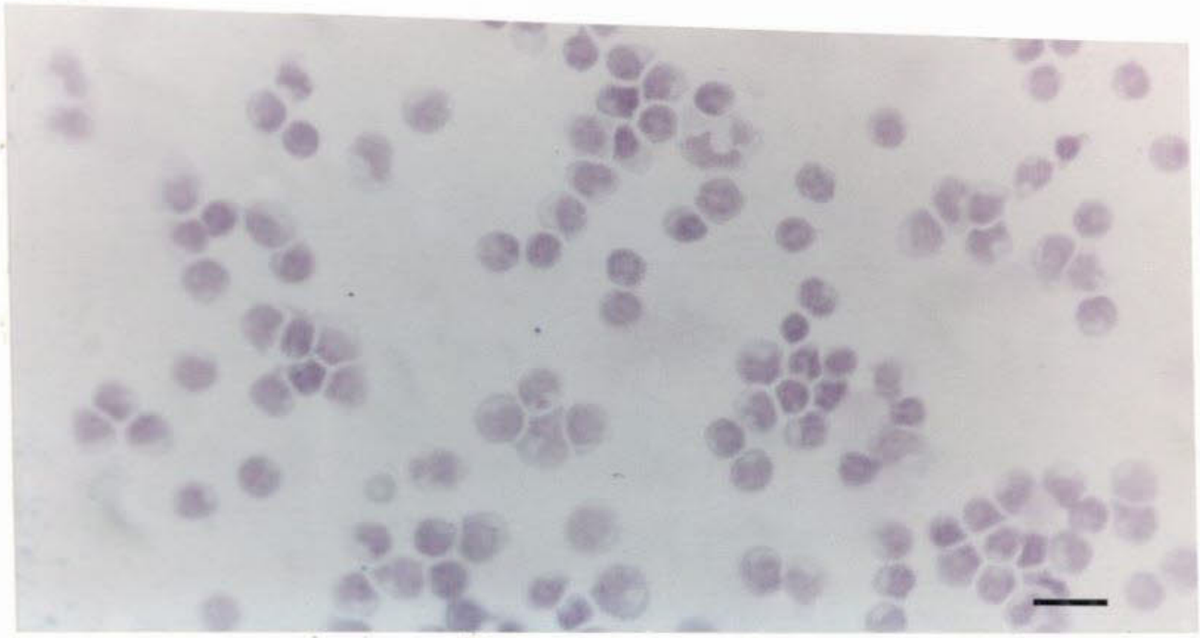


Figure 9. Flow cytometric analysis of side scatter and CD45 fluorescence. Blood and marrow samples enriched for CD34+ cells were flow cytometrically differentiated into lymphocytes, monocytes, granulocytes, lymphoid progenitors and myeloid progenitors (gray) based on side scatter and CD45 fluorescence.

A.



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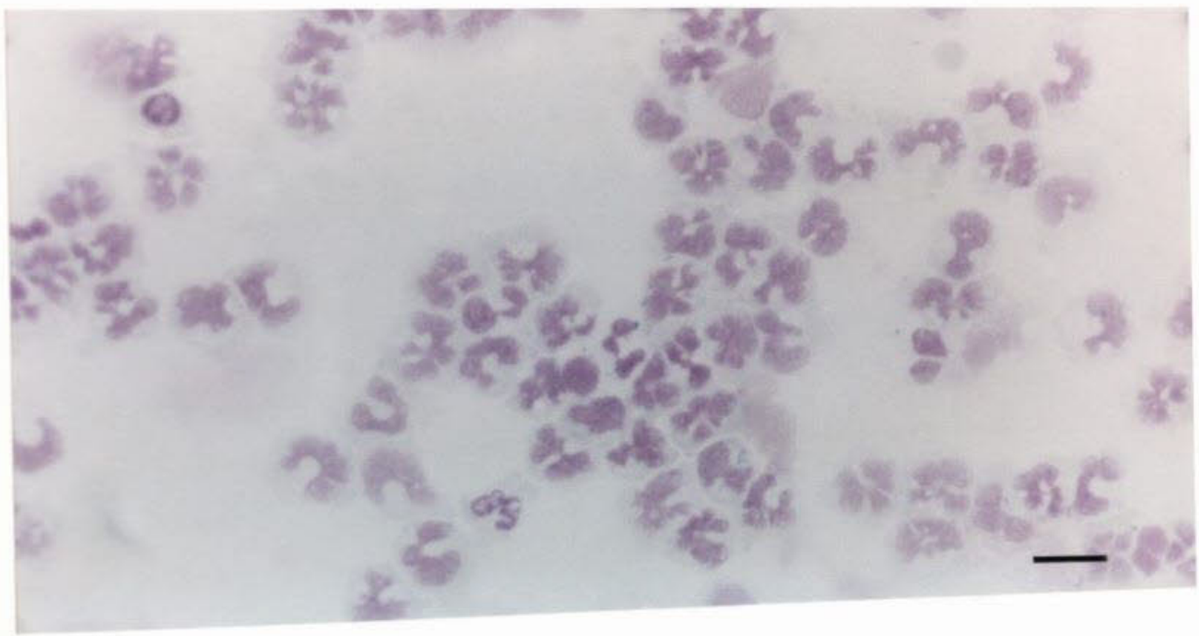
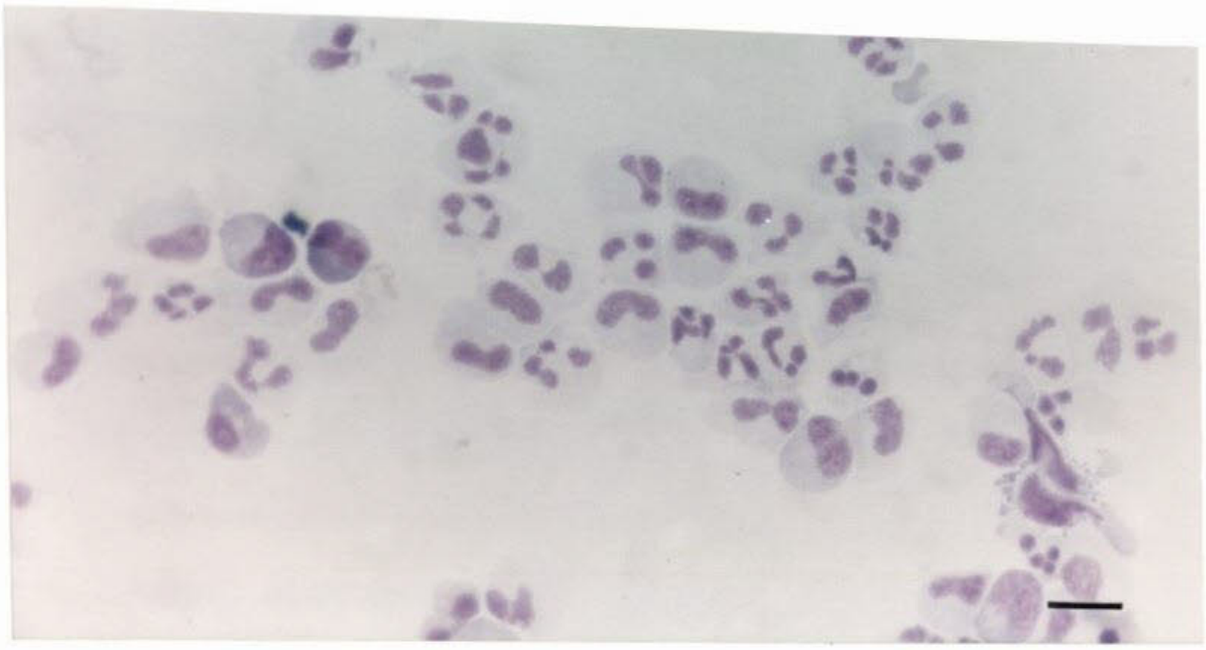


Figure 10. Morphological confirmation of sorted lymphocytes and monocytes. Marrow specimens were enriched for CD34+ cells and flow cytometrically sorted using side scatter and CD45 fluorescence based regions. Cells sorted based on the (A) lymphocyte region (magnification = 598X; bar = 17 μm) and the (B) monocyte region (magnification = 683X; bar = 15 μm) were collected, mounted onto slides and stained with Leukostat.

A.



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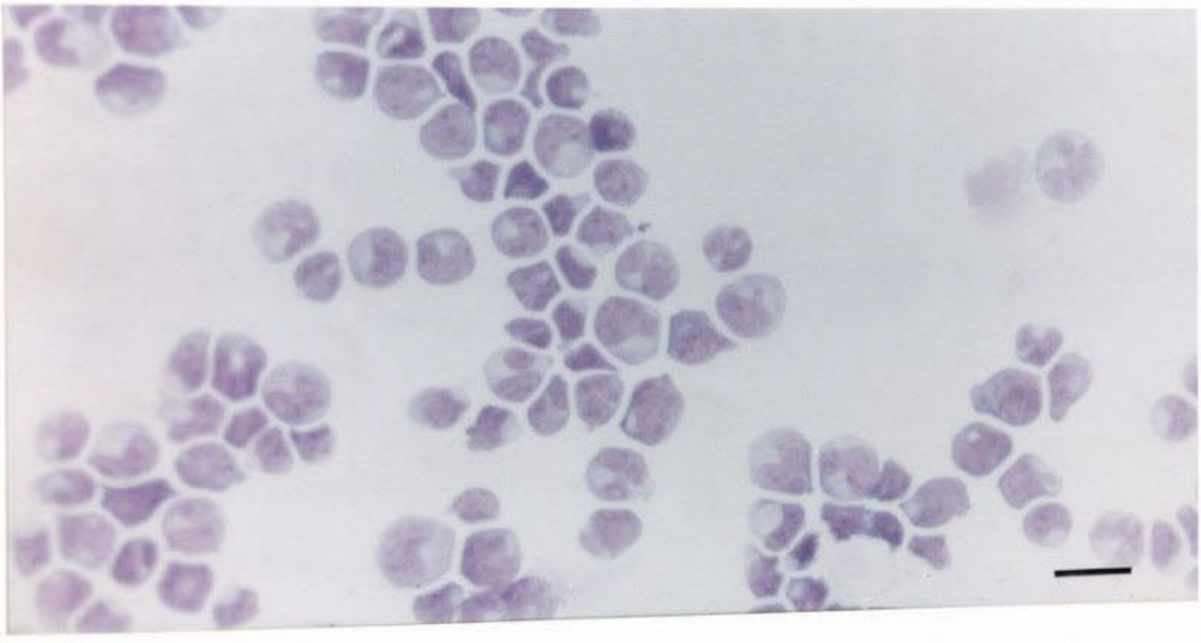


Figure 11. Morphological confirmation of sorted granulocytes and myeloid progenitors. Marrow specimens were enriched for CD34+ cells and flow cytometrically sorted using side scatter and CD45 fluorescence based regions. Cells sorted based on the (A) immature and mature granulocyte region (magnification = 598X; bar = 17 μm) and the (B) myeloid progenitor region (magnification = 683X; bar = 15 μm) were collected, mounted onto slides and stained with Leukostat.

differentials were kindly performed by Dr. Sean Farrier, a senior hematology resident with confirmation of his findings by Dr. Lynn Moscinski. Standard leukocyte differential of cytocentrifuged slide preparations include 1) scanning the entire slide to determine the most representative field to count and 2) enumeration of specific cell types until 100 total cells have been counted (2-4 fields are counted depending on the density of cells in the preparation, personal communication with Dr. Sean Farrier). A typical leukocyte differential of sorted cell type regions is listed in Table One.

The number of $CD34^+$ cells in the myeloid progenitor population was determined by subtracting the PE false positive events of the isotype control from the $CD34^+$ events in the myeloid progenitor gated population of each specimen. The percentage of $CD34^+$ cells within each sample was determined by dividing the total number of $CD34^+$ cells by the total number of cellular events as gated on SSC and CD45 fluorescence based regions in each specimen (Figures 9 and 12). The percentage of $CD34^+$ cells within the myeloid progenitor population of each specimen was determined by dividing the number of $CD34^+$ events in the myeloid progenitor gated population by the total number of events in the myeloid progenitor gated population. The total number of CAM^+CD34^+ cells in the myeloid progenitor population was determined by subtracting the FITC/PE false positive events of the isotype control from the number of CAM^+CD34^+ events in the myeloid progenitor gated population of each specimen. The percentage of CAM^+ cells in the $CD34^+$ myeloid progenitor population was determined by dividing the number of CAM^+CD34^+ events in the myeloid progenitor gated population by the number of $CD34^+$ events in the myeloid progenitor gated population. The mean channel fluorescence for

Table One. Leukocyte differential of sorted cell type regions. On the basis of side scatter versus CD45 fluorescence individual lymphocytes, monocytes, granulocytes and myeloid progenitors were flow cytometrically sorted according to cell type regions described by Stelzer et al.[57] Leukocyte differentials of Leukostat stained slides of flow cytometrically sorted cell type regions are listed as a percentage of 100 cells counted per slide. Leukocyte differential of a representative sorting experiment of normal marrow is shown.

Cell Type Observed	Cell Type Region Sorted (Based on Side Scatter versus CD45 Fluorescence)			
	Lymphocytes	Monocytes	Mature & Immature Granulocytes	Myeloid Progenitors
Myeloblast				52
Promyelocyte			3	30
Myelocyte, neutrophilic			3	7
Metamyelocyte, neutrophilic		1	15	2
Band neutrophil		1	20	
Segmented neutrophil		5	51	
Eosinophils (including young forms)			2	
Basophils (including young forms)				
Monocytes		90	3	
Lymphocytes	100	3	3	6
Plasmacytes				3
Pronormoblasts & Basophilic normoblasts				
Polychromatic normoblasts and Orthochromatic normoblasts				

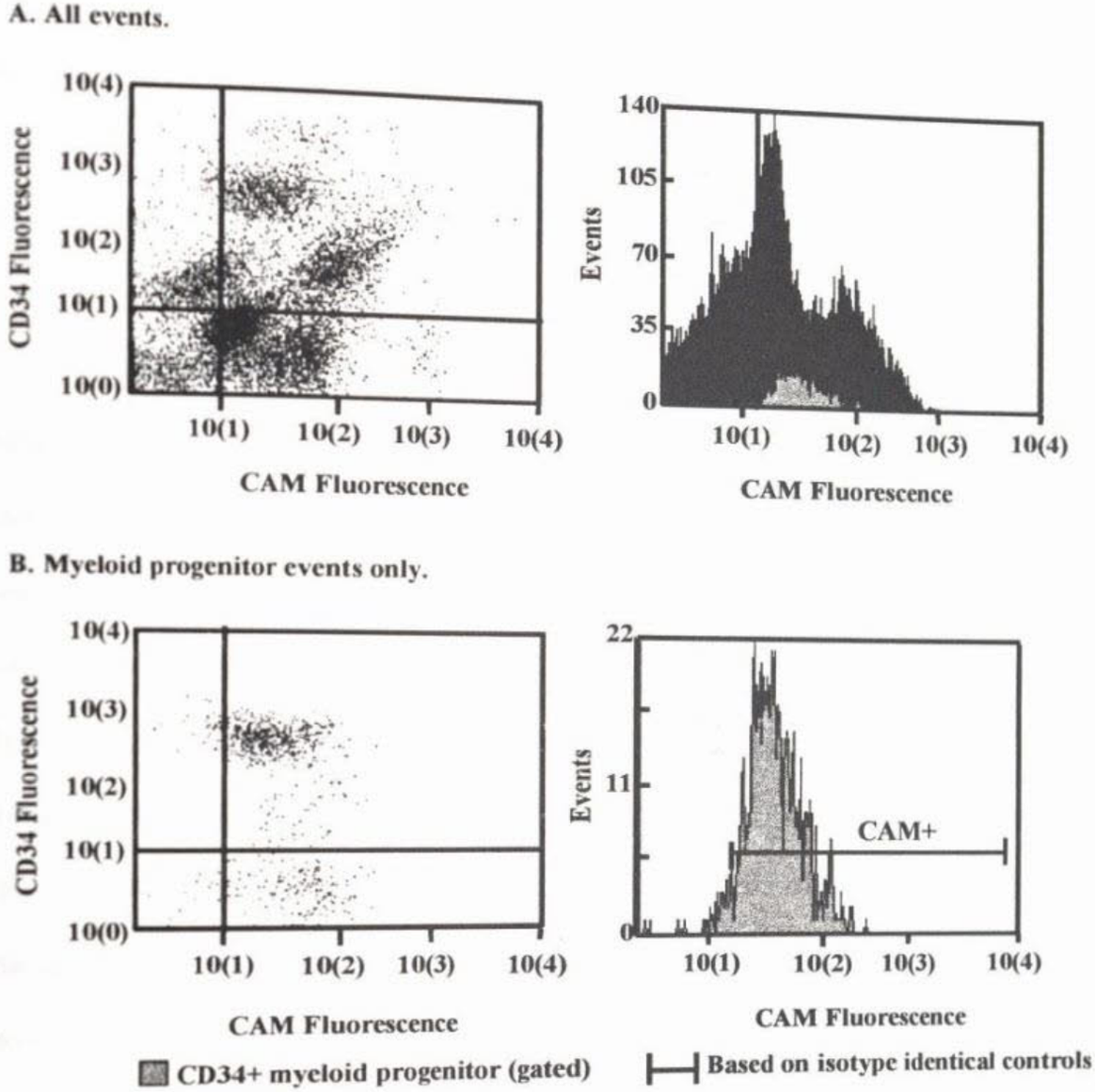


Figure 12. Flow cytometric analysis of cell adhesion molecule expression on CD34+ myeloid progenitors. Samples enriched for CD34+ cells were labelled with mAbs against CD34, CD45 and one of the CAMs under investigation. Myeloid progenitors were distinguished from all events (A) on the basis of side scatter and CD45 fluorescence and gated (Figure 9). Gated myeloid progenitor events (B) were then analyzed on the basis of CAM and CD34 fluorescence in order to determine the percentage of CD34+ myeloid progenitors coexpressing CAMs. Gated CD34+ myeloid progenitor cells (gray) were analyzed to determine their peak channel fluorescence such that CAM expression per CD34+ myeloid progenitor could be quantitated. Analysis of marrow derived HCAM+CD34+ myeloid progenitors is depicted.

each CAM-FITC conjugate within the $CD34^+$ myeloid progenitor population was determined and used to extrapolate the number of Abc units per $CD34^+$ myeloid progenitor as described in the following section.

Use of QSC beads to quantitate number of antibodies bound per cell

QSC beads were provided from the manufacturer in a mixture of five types of beads, each with a defined number of antibody binding sites or antibody binding capacity (Abc) units per bead. The QSC bead mixture was labelled and analyzed just as the cells were labelled; that is, using the same isotype and quantity of mAb, incubation conditions, wash conditions and acquistion conditions used to label the blood and marrow samples. The mean peak channel fluorescence for each of the five beads was determined (Figure 13) and plotted against the each QSC bead's Abc units or against the natural log (ln) of each QSC bead's Abc units (Figure 14). Linear regression analysis of these standard curves generated a best fit line from which the number of Abc units per $CD34^+$ myeloid progenitor cells from blood or marrow was determined (Figure 14). Analysis and graphics of standard curves of peak channel fluorescence versus antibody binding capacity were created for each antibody using Lotus1-2-3 version 4 (Lotus Development Corporation, Cambridge, MA).

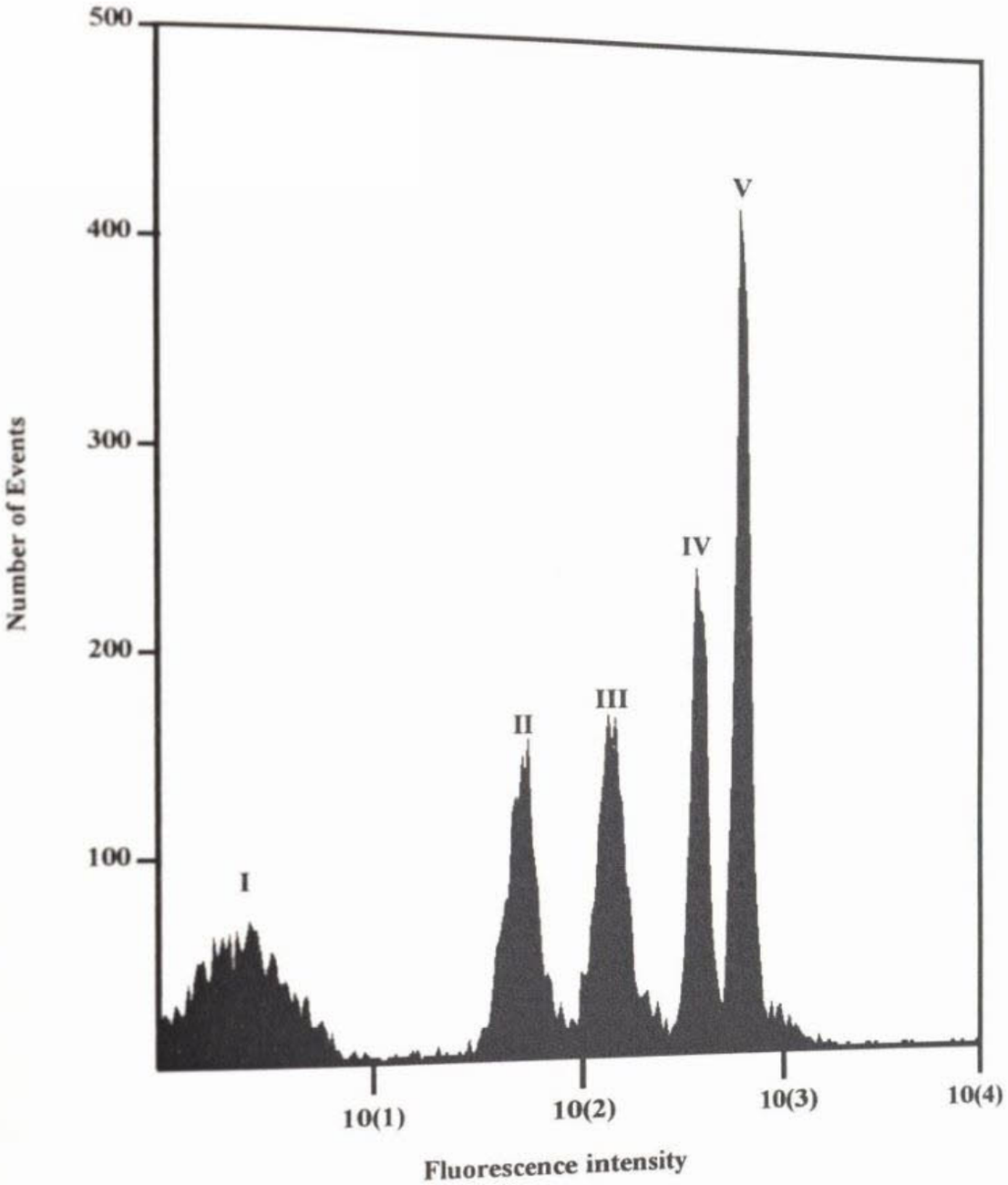


Figure 13. Determination of mean peak channel fluorescence of QSC bead standards. Number of binding sites per QSC bead: I=0; II=6,841; III=16,438; IV=51,927 and V=81,191. Representative experiment depicted.

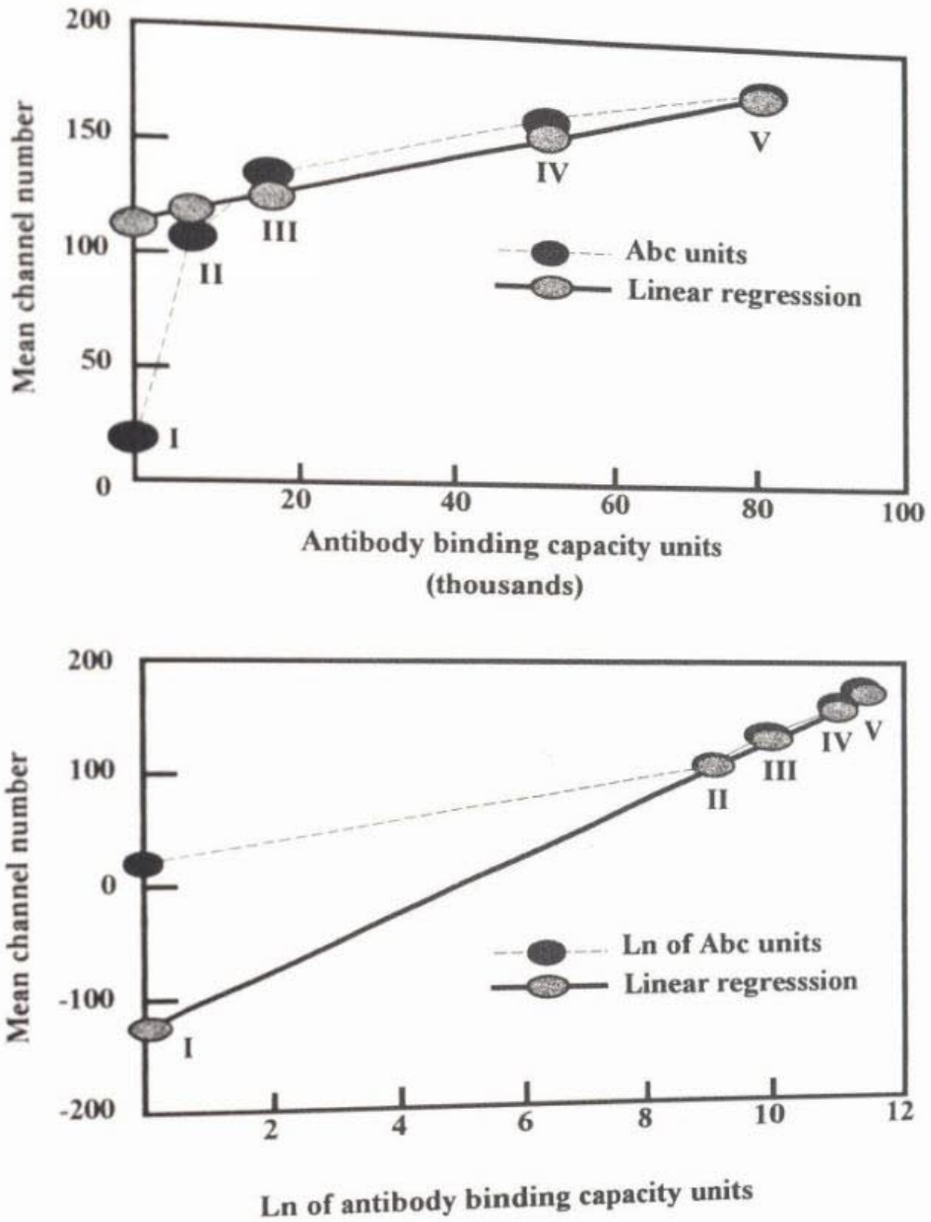


Figure 14. Standard curve of antibody binding capacity units of QSC beads. Representative experiment depicted. Roman numerals correspond with QSC bead number.

Flow cytometric single cell sorting

All processing of blood and marrow samples was performed in a sterile manner in preparation for sterile sorting and subsequent *in vitro* assays. Prior to sterile sorting with the FACSCaliber flow cytometer, the instrument was thoroughly washed with sterile, filtered 70% ethanol. Each of the samples to be analyzed were collected as data files on the flow cytometer (described in the flow cytometric analysis section). Random sort gates were drawn, sterile filtered 70% ethanol was placed on the sample injection port (SIP), the sheath fluid reservoir was replaced with a reservoir containing sterile filtered 70% ethanol and three sterile 50 mL conical tubes tubes were placed onto the collection tube ports. The instrument was directed to sort until each of the three collection tubes had filled to approximately 37 mL per tube, which took approximately 9 minutes per collection tube. The collection tubes containing 70% ethanol were discarded and replaced with three additional sterile 50 mL conical tubes. The reservoir containing 70% ethanol was replaced with a reservoir containing sterile, filtered phosphate puffer (pH 7.4; Appendix 5) and sterile DPBS was placed onto the SIP. The instrument was directed to sort until each of the three collection tubes had filled to approximately 37 mL per tube, as described above. Once the FACSCaliber had been sterilized in this manner, the sort sample was placed onto the SIP. Aquisition of the sort sample on FSC versus SSC fluorescence enabled region 1 to be drawn about small, viable, single myeloid progenitor cells within the marrow or blood sample (Figure 15). This region was based on light scatter patterns dictated by SSC versus CD45 fluorescence as previously

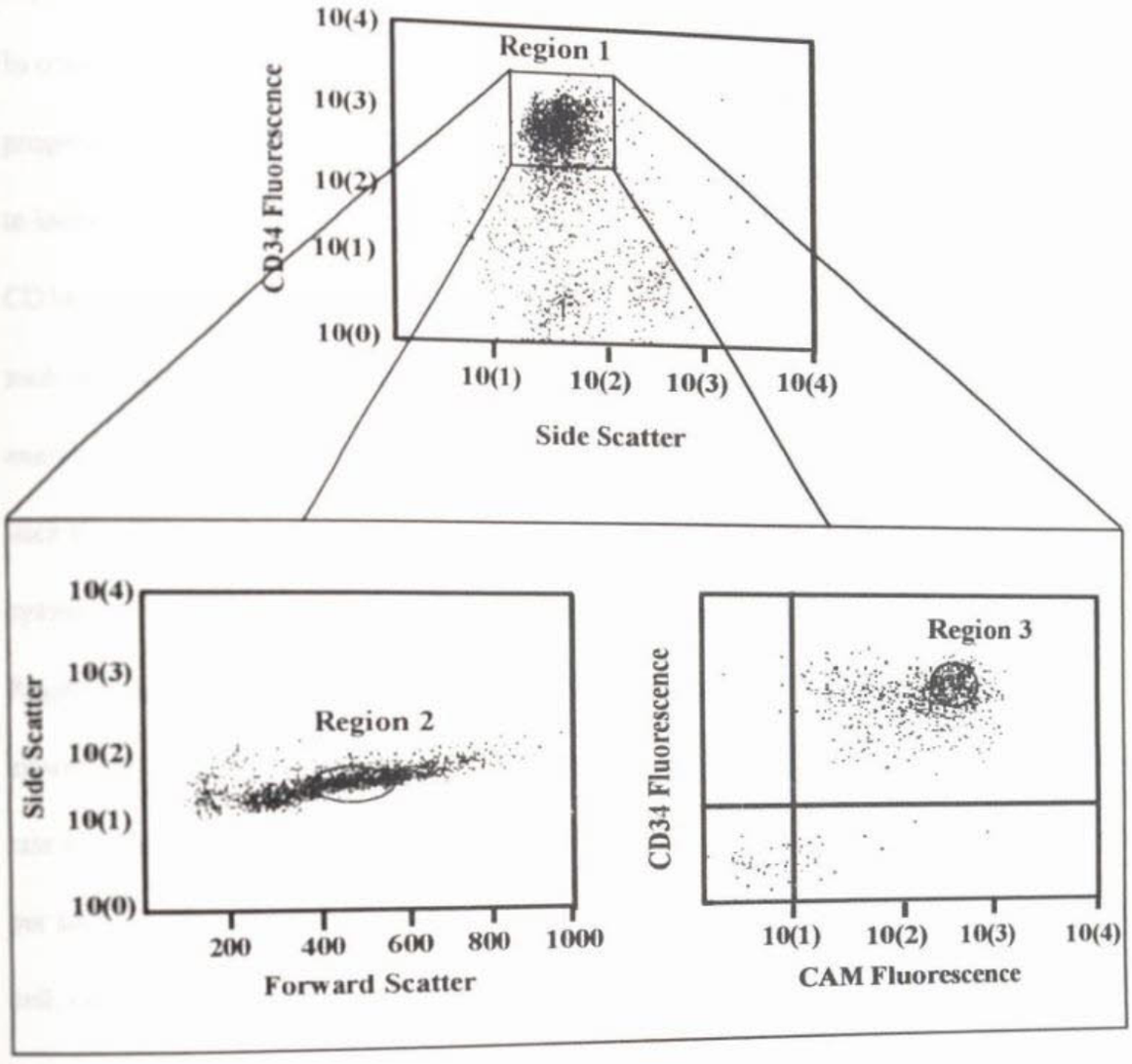


Figure 15. Flow cytometric sorting regions and gates. Pooled buffy coat samples were enriched for CD34+ cells and labelled with fluorochrome conjugated mAbs for flow cytometric analysis and single cell sorting. Labelled cells were analyzed on the basis of side scatter and CD34 expression. Small CD34+ cells (Region 1) were gated and analyzed on the basis of forward scatter versus side scatter such that Region 2 could be drawn around myeloid progenitors. Region 2 was gated and analyzed on the basis of CAM versus CD34 fluorescence such that Region 3 could be drawn around CAM+CD34+ events. The flow cytometer was directed to sort only those events which satisfied the criteria of Region 2 (myeloid progenitors of low cellular complexity) and Region 3 (cells expressing both CAM and CD34).

described (Figure 9). The logic of the FACSCaliber flow cytometer sorting software requires that the first region in the sort gating be based on FSC versus SSC fluorescence. In order to determine where the region inclusive of target cells ($CAM^{+/-}CD34^{+}$ myeloid progenitors) should be drawn in FSC versus SSC fluorescence plots, Region 1 was drawn to include viable, single $CD34^{+}$ myeloid progenitor cells on the basis of SSC versus CD34 florescence. Region 1 was gated and analyzed on the basis of FSC versus SSC such that region 2 could be drawn around myeloid progenitors. Region 2 was gated and analyzed on the basis of CAM (HCAM, VLA-4 or L-selectin) versus CD34 fluorescence such that Region 3 could be drawn around $CAM^{+}CD34^{+}$ events (Figure 15). The flow cytometer was then directed to sort only those events which satisfied the criteria of Region 2 (myeloid progenitors of low cellular complexity) and Region 3 (cells expressing both CAM and CD34; Figure 15). The target cells were sorted at a threshold rate of approximately 1500 events per second and a sort rate of approximately 15 events per second. The FACSCaliber possesses the following three distinct sort modes: single cell, recovery and exclusion (Figure 16). The sort mode with the highest purity of recovered cells is the single cell mode followed by the exclusion and recovery modes, respectively. The sort mode with the highest yield (but lowest purity) is the recovery mode, followed by the exclusion and single cell modes, respectively. By sacrificing recovery in the pursuit of the highest post sort purities possible, the single cell mode was used for all sterile, FACSCaliber assisted sorts. These sorting parameters enabled the maximum number of target cells to be sorted in the minimum amount of time (on average 60 minutes per sort). Post-sort analysis was performed by flow cytometrically

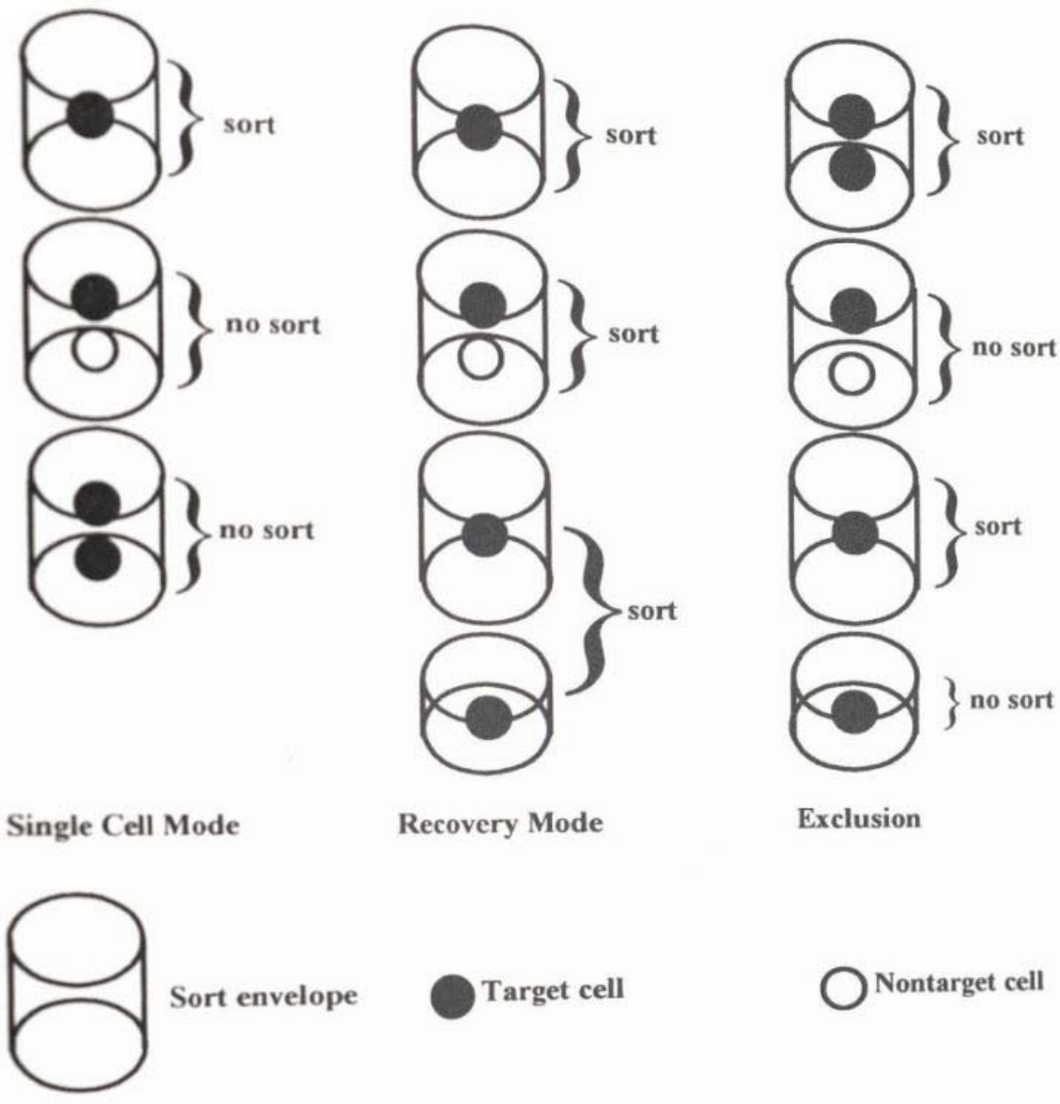


Figure 16. How envelopes were sorted for each sort mode. In Single Cell Mode, a sort occurred whenever a single target cell was identified in the sort envelope. Envelopes containing more than one cell, even if a target cell, were not sorted. In Recovery Mode, a sort occurred whenever a sort envelope contained a target cell, even if a nontarget cell was also in the envelope or a target cell was just outside the sort envelope. In Exclusion Mode, a sort occurred only when a target cell was identified within the defined sort envelope, no attempts were made to capture target cells outside the sort envelope.

analyzing 200 μL of sorted sample prior to further processing (i.e., performance of CFU or LTCIC assays). If a post-sort purity of ≥90% was not obtained, then further processing of the specimen was aborted. Microscope slides were prepared of pre-sort and post-sort specimens ($CD34^+$ enriched fraction and CAM^+CD34^+ or CAM^-CD34^+ myeloid progenitors, respectively) using a cytocentrifuge and counterstained with Leukostat (Fisher Scientific) as previously described (Figure 17). Leukocyte differentials of prepared slides enabled confirmation of post-sort purities (kindly performed by Dr. Sean Farrier as previously described). A typical leukocyte differential of pre- and post-sort $CAM^{+/-}CD34^+$ myeloid progenitor is listed in Table Two.

Colony forming unit assay

To assess short-term hematopoietic potential, sorted CAM^+CD34^+ myeloid progenitor cells from normal marrow or pooled buffy coats were plated in triplicate into $35mm^2$ petri dishes containing 1 mL Methocult™H4434 per dish (Methocult; StemCell Technologies Inc., Vancouver, B.C., Canada). Methocult is a "complete" methlycellulose medium containing recombinant human (rh) cytokines necessary for colony assays of human cells [0.9% methylcellulose, 30% fetal bovine serum, 1% bovine serum albumin, 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, 50 ng/mL rh stem cell factor, 10 ng/mL rh GM-CSF and 10 ng/mL IL-3, 3 units/mL rh erythropoietin in 70% Iscove's DMEM]. Each dish received 1 mL of cell suspension [500 sorted cells in 0.1 mL Iscove's media (Mediatech)/mL

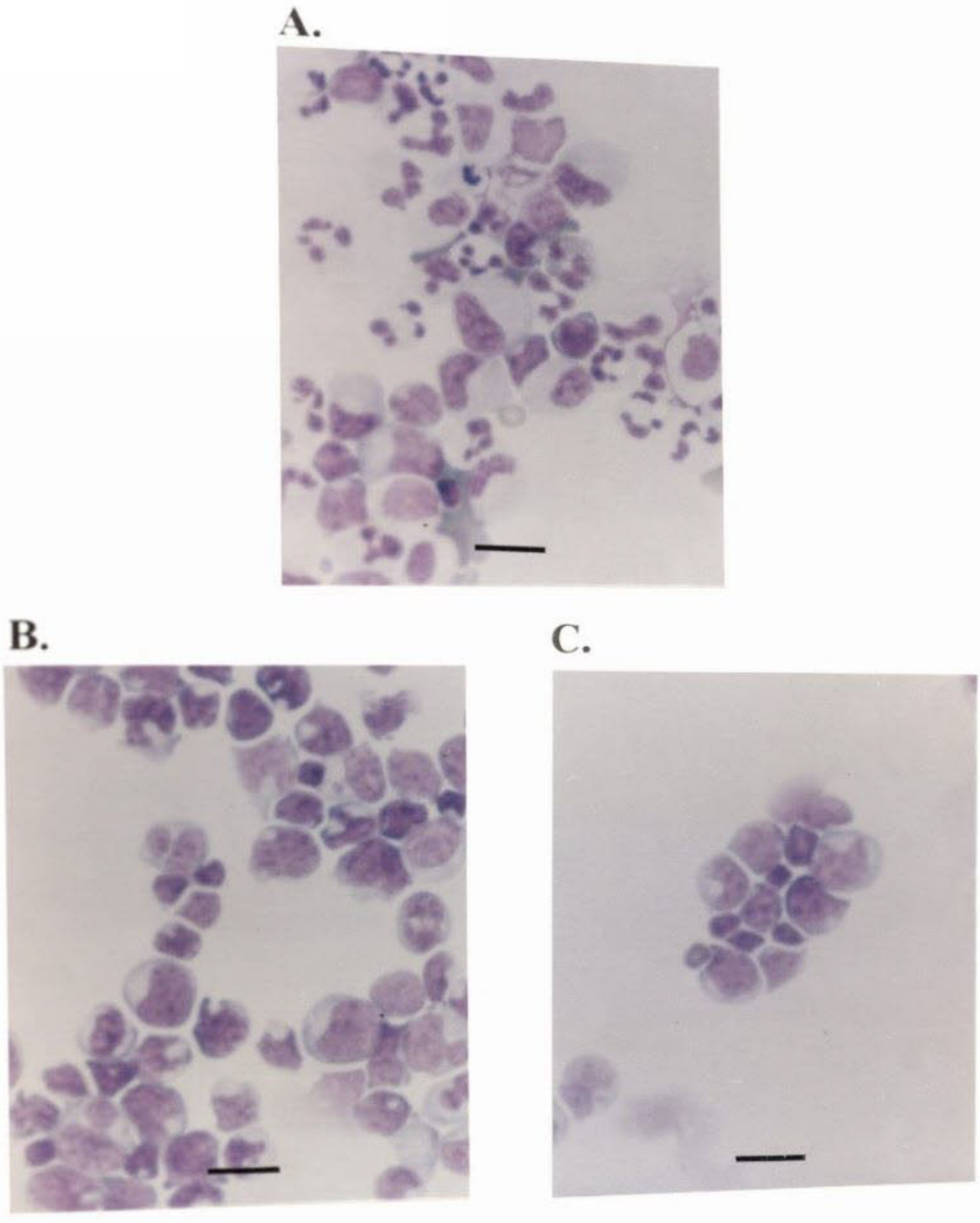


Figure 17. Morphological confirmation of myeloid progenitor sort purity. (A) Samples were enriched for CD34+ cells using MACS (magnification 683X; bar = 15 μm). (B) CD34+ myeloid progenitors expressing (magnification 640X, bar = 16 μm) or (C) not expressing (magnification 512X; bar = 20 μm) the cell adhesion molecule of interest were flow cytometrically sorted. Sort of VLA-4+CD34+ myeloid progenitors derived from marrow shown.

Table Two. Leukocyte differential of pre- and post-sort $CD34^+$ myeloid progenitor cell preparations. Leukocyte differentials of Leukostat stained slides of $CD34^+$ cell enriched fraction (pre-sort) and flow cytometrically sorted $CAM^{+/-}CD34^+$ myeloid progenitors (post-sort) were reported as a percentage of 100 cells counted per slide. A leukocyte differential of a single cell sort from marrow targeted at obtaining highly purified post-sort specimens of $VLA\text{-}4^+CD34^+$ and $VLA\text{-}4^-CD34^+$ myeloid progenitors is presented.

Cell Type Observed	Pre-Sort	Post-Sort	
	$CD34^+$ Cell Enriched Fraction	$VLA\text{-}4^+CD34^+$ Myeloid Progenitors	$VLA\text{-}4^-CD34^+$ Myeloid Progenitors
Myeloblast	2	98	69
Promyelocyte	4		15
Myelocyte, neutrophilic	6		3
Metamyelocyte, neutrophilic	9		
Band neutrophil	13		
Segmented neutrophil	50		
Eosinophils (including young forms)	1		
Basophils (including young forms)			
Monocytes	5		3
Lymphocytes	6	2	10
Plasmacytes			
Pronormoblasts & Basophilic normoblasts	2		
Polychromatic normoblasts and Orthochromatic normoblasts	2		

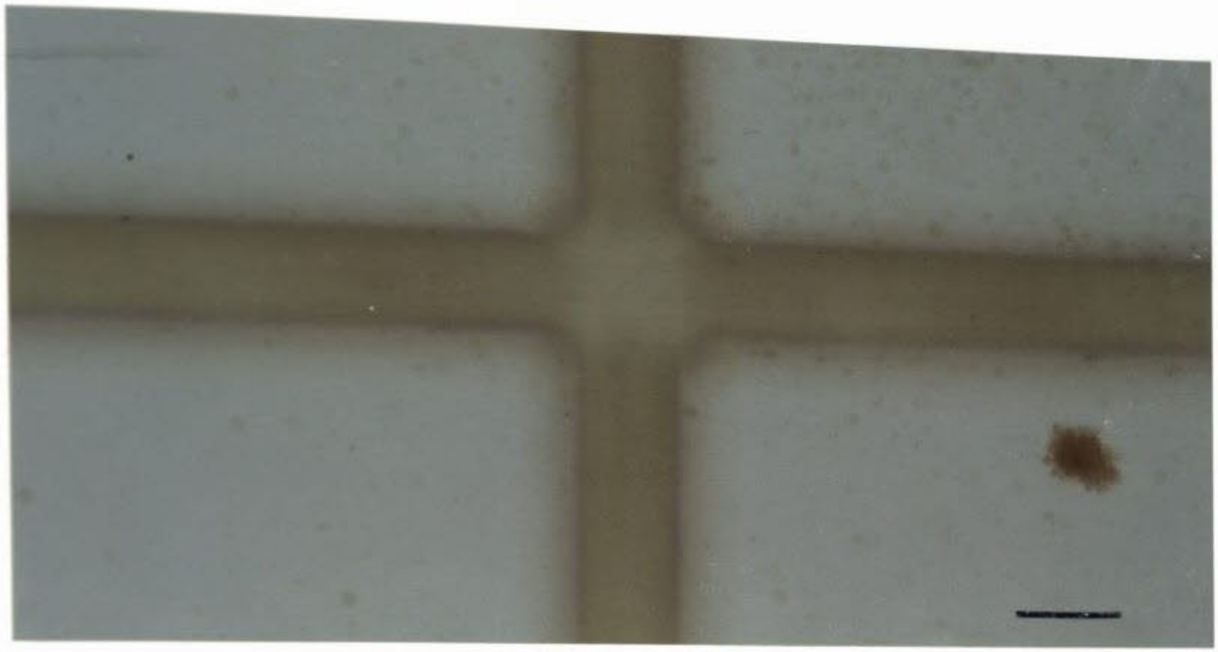
Methocult]. After 14 days of incubation at 37 °C in a humidified atmosphere of 5% CO_2:95% air, colonies were scored and typed as CFU-E, BFU-E, CFU-GM and CFU-mix based on an atlas of human hematopoietic colonies developed by Eaves et al. (Figures 18 and 19).[59]

Using the culture conditions outlined above, progenitors differentiate as they divid with the result that after a finite and predictable incubation (i.e. 14 days) all cells will have reached maturity and the colony will not increase in size any further.[59] This feature of colony development allowed different stages of progenitor development to be distinguished according to the sizes of colony they ultimately generated. Thus the bigger the colony, the greater the proliferative capacity and the more primitive the original progenitor from which it arose.[59]

CFU-E refers to those colonies which give rise to the smallest (less than 0.5 mm), most rapidly maturing erythroid colonies.[59] In the context of the CFU assays presented herein, a colony was scored as a CFU-E colony 1) when it consisted of 1-2 clusters containing up to a maximum of ~100-200 erythroblasts and 2) when each cluster contained a minimum of 8 erythroblasts (Figure 18A). Erythroblasts are readily discernible in methycellulose by their distinct reddish-orange hue due to their hemoglobin content (Figure 18A).[59]

BFU-E refers to the immediate precursors of CFU-E.[59] In order for a colony to be scored as a BFU-E, it had to contain at least 3 erythroblast clusters with each cluster consisting of ~100-500 hemoglobinized erythroblasts or a single colony >0.5 mm in size, usually presenting with a "fried egg" morphology (Figure 18B).[59]

A.



B.

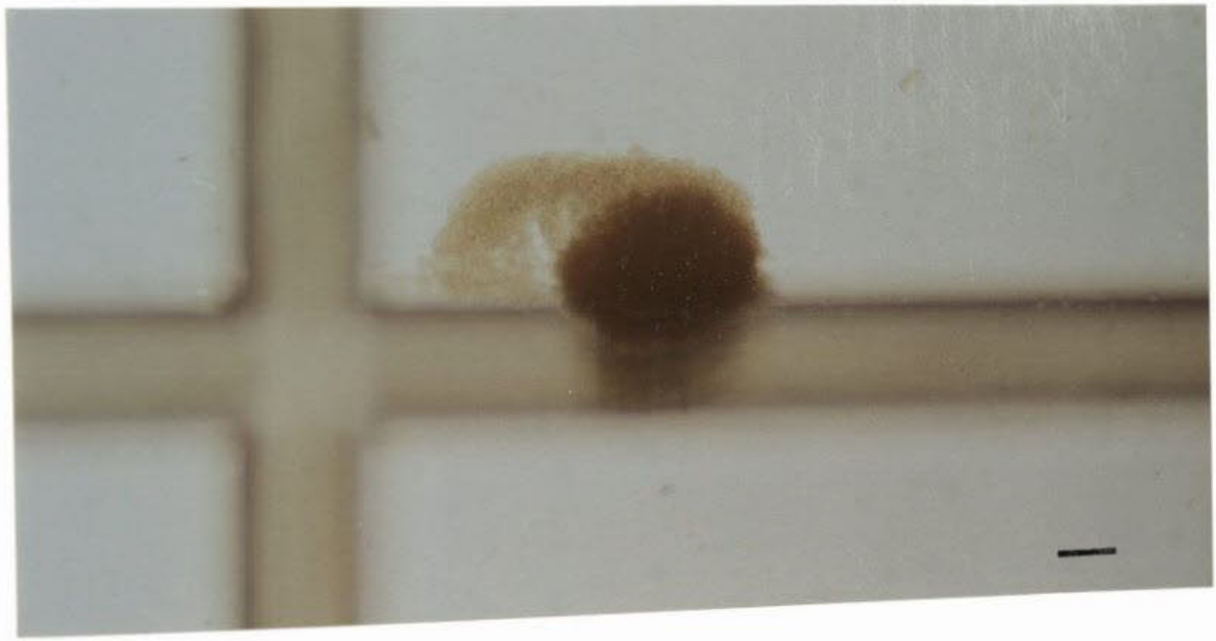


Figure 18. Microscopic characteristics of CFU-E and BFU-E colony types. Sorted CAM+/-CD34+ myeloid progenitors were plated into colony forming units assays.[59] Colonies were scored according to standards set forth by Eaves et al. as (A) CFU-E colonies (magnification 68X) or (B) BFU-E colonies (magnification 41X). Bar = 200 μm.

A.

B.

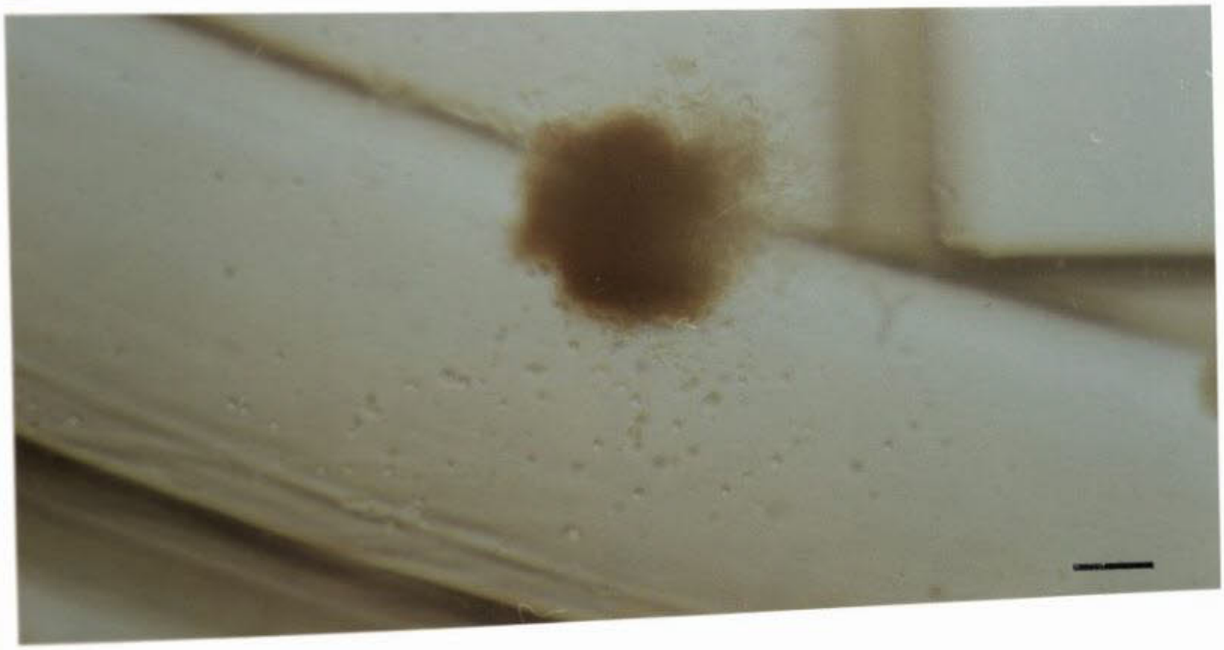


Figure 19. Microscopic characteristics of CFU-GM and CFU-mix colony types. Sorted CAM+/-CD34+ myeloid progenitors were plated into colony forming units assays. Colonies were scored according to standards set forth by Eaves et al.[59] as (A) CFU-GM colonies (magnification 54X) or (B) CFU-mix colonies (magnification 49X). Bar = 200 μm.

CFU-GM colonies were scored on the basis of their ability to produce colonies of granulocytes and macrophages. A colony was scored as a CFU-GM colony when it contained a minimum of 20 granulocytes and/or macrophages (Figure 19A).[59] Typically these colonies showed a relatively homogenous morphology often with a more concentrated central core of cells surrounded by a less dense halo of cells (Figure 19A).[59]

CFU-mix colonies refer to colonies which resulted from progenitor cells that gave rise to colonies containing multiple lineages of cell. CFU-mix colonies are sometimes refered to as CFU-GEMM (colony forming unit-granulocyte, erythroid, macrophage, megakaryocyte) to indicate their multiple lineage potential.[59] CFU-mix colonies were scored when three distinct cell lineage types could be visualized by focusing up and down under high power (Figure 19B).[59]

Preparation of stromal cell layers

Stromal cell layers for long term culture initiating cell (LTCIC) assays were prepared from cells trapped on filters designed to remove cell clumps and other debris from harvested marrow samples. Salvage of these filters (they are normally discarded as waste) after a bone marrow harvest from a normal individual donating for allogeneic transplant enabled normal human bone marrow stromal cells to be obtained. Filters were flushed with DPBS to collect trapped cells. To deplete the RBC content, collected cells were layered onto Ficoll-Paque® Plus at [25 mL diluted sample:10 mL Ficoll-Paque® Plus] and centrifuged at 500 x g for 30 minutes at RT according to a modified version of

Boyum's method.[45] The light density cells were washed three times with DPBS via centrifugation at 615 x g for 5 minutes at RT. The resultant cells were admixed in LTBMC medium [10^{-6} M hydrocortisone, 12.5% horse serum (GibcoBRL) and 12.5% fetal bovine serum (GibcoBRL) in alpha-minimum essential medium (alpha-MEM; Mediatech)] at a final cell concentration of [10^6 cells/mL LTBMC media] and were aliquoted at 5 mL per 25 cm^2 flask to be seeded. The cultures are incubated at 33°C in a humidified atmosphere of 5% CO_2:95% air. After three weeks of culture with weekly complete medium changes, the cells were trypsinized, pooled, washed, and re-plated at a ratio of two new flasks per one original flask; thus a stromal feeder layer consisting of normal human stromal cells was established.

Long term culture initiating cell assay

Long term culture initiating cell (LTCIC) assays, developed by Sutherland et al., were established in order to measure the number of stem cells with long term *in vivo* repopulating potential within sorted populations of CAM^+CD34^+ myeloid progenitors (Figure 20).[13] LTCIC assays enable quantitation of human LTCIC content based on an assessment of the number of clonogenic cells present after 5-8 weeks *in vitro*. Clonogenic cell output depends on the presence of a stromal cell feeder layer and is linearly related to the input cell number over a wide range of cell concentrations. Limiting dilution analysis of clonogenic cell output enables quantitation of LTCICs or

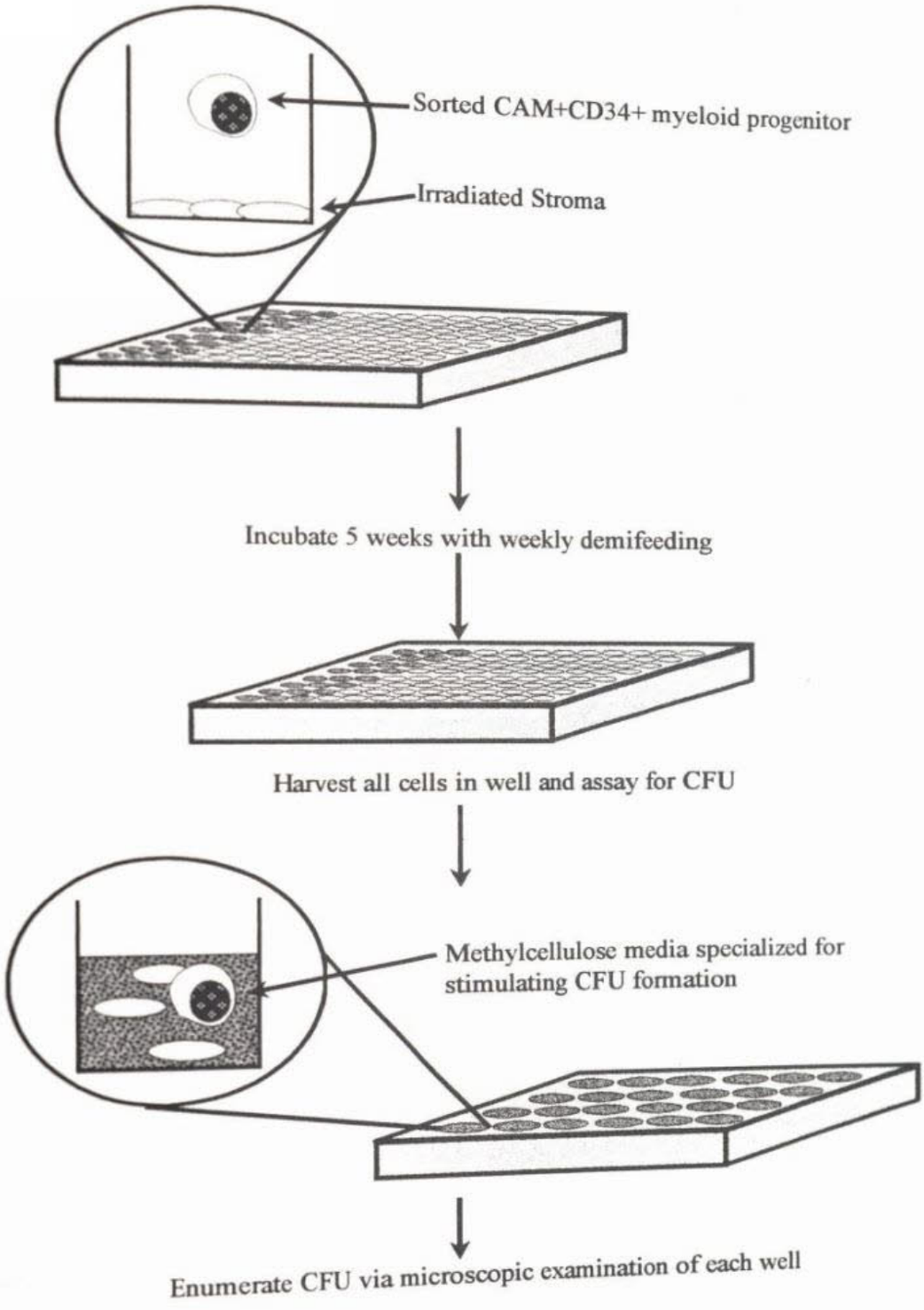


Figure 20. Long term culture initiating cell assay.

stem cell content within the input cell populations.[13] In the LTCIC assays presented herein, the input cell population was sorted populations of CAM^+CD34^+ myeloid progenitors.

Trypsinized normal human stromal cell layers from established LTBMCs (see preparation of stromal cell layers section) were irradiated at 20 Gy (destroys radiation sensitive hematopoietic stem cells while stromal cells remain functionally intact) and plated into 96 well flat-bottom microtiter trays (Fisher Scientific) at 6×10^4 cells per well. Alternatively, M2-10B4 cells (Batch F-12495; American Type Culture Collection, Rockville, MD) were used as the stromal cell layer. M2-10B4 cells are a murine bone marrow stromal cell line capable of maintaining long term human hematopoiesis *in vitro* as effectively as human marrow derived stromal layers.[60] M2-10B4 cells were irradiated (80 Gy) and plated into 96 well flat-bottom microtiter trays at 3×10^4 cells per well. The trays were incubated for at least 24 hrs at 33 °C in a humidified atmosphere of 5% CO_2:95% air to allow adherence of stromal cells. Cells to be assayed (sorted CAM^+CD34^+ myeloid progenitor cells) were plated in LTBMC media in dilutions of 3, 10, 30, 100, 300 and 1000 cells/well in replicates of 24 wells per dilution. An additional plate of stromal cells received LTBMC media alone and served as a negative control. The cultures were maintained for five weeks at 33 °C in a humidified atmosphere of 5% CO_2:95% air with weekly demifeeding by half volume complete LTBMC media changes. At week five of culture, cells in each well were harvested via trypinsinization and plated into CFU assays. Since no significant difference ($p>0.05$) in LTCIC content has been demonstrated in determined LTCIC content between five and eight week

cultures, five weeks of culture was chosen.[13] On day 14 of the CFU assay portion of the LTCIC assay, wells were scored as either positive (colonies observable) or negative (no observable colonies).

The number of stem cells present per input cell was determined using Poisson statistics which states that if the average number of long term culture initiating cells plated per well equals one, then the probability of no positive wells is

$$p(0) = [(e^{-1})(10)]/\ 0! = (1/e)(1) = 1/e = 0.37$$

Thus the probability of finding ≥ 1 positive well (i.e. the probablity of not finding 0 positive wells) = 1-0.37 = 0.63; that is, 63% of the wells plated will be positive when one LTCIC is plated per well. By plotting the number of cells plated per well by the percentage of positive wells, the number of cells corresponding to 63% positive wells was interpolated as the number of cells containing one LTCIC (Figure 21). The number of LTCICs per sorted cell plated was then converted to LTCIC/10^6 sorted cells for all comparative analyses.

VLA-4 adhesion assay

The adhesion of sorted populations of VLA-4^+CD34^+ myeloid progenitors to immobilized fibronectin (FN) was measured using a modified version of Shimizu et al.'s adhesion assay.[61,62] Typically static adhesion assays involve five steps (1) immobilization of target ligand to microtiter wells, (2) addition of ^{51}Cr-labelled target cells at 4°C in the presence or absence of activating agents, (3) initiation of adhesion by

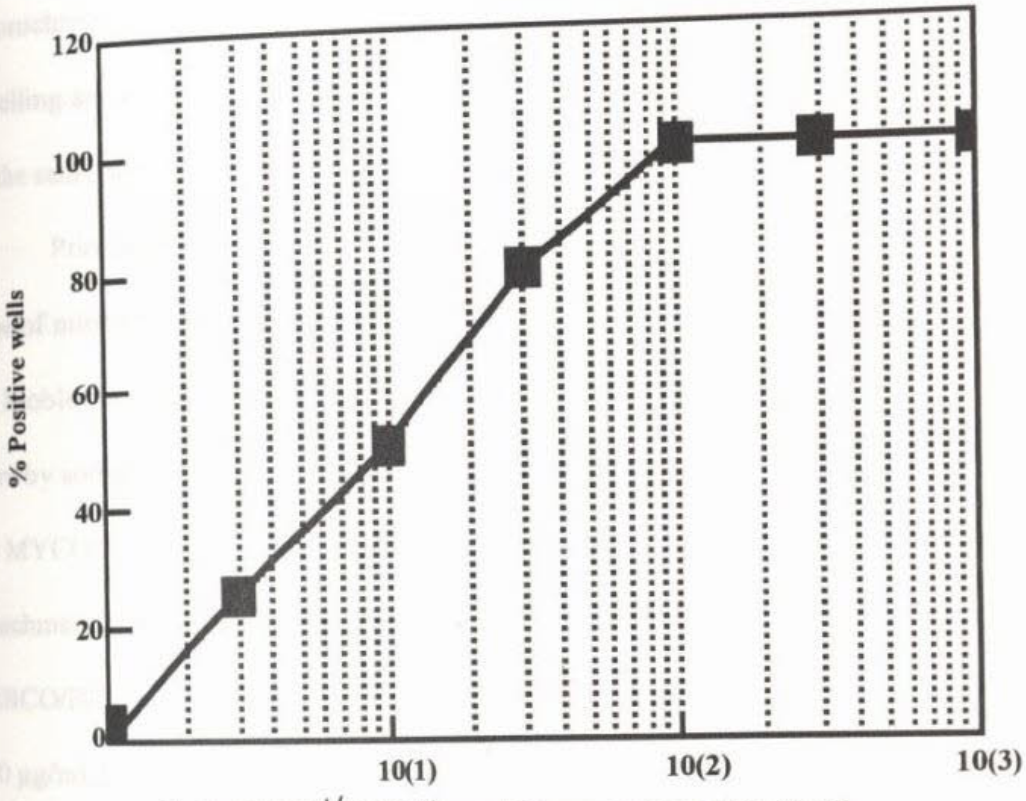


Figure 21. Determination of number LTCIC per 10^6 sorted $CAM^{+/-}CD34^+$ myeloid progenitor cells. Myeloid progenitors were flow cytometrically sorted from either pooled buffy coats or hematopoietically normal marrow. These highly purified cell populations (post-sort purity >90% in all cases) were plated in limiting dilutions ranging from 3 to 1000 cells/well (replicates of 24 per dilution) in accordance with performance of the LTCIC assay (Figure 20). According to Poisson statistics, the number of sorted myeloid progenitors corresponding with 63% positive wells (i.e., wells containing at least 1 colony) reflect the number of sorted myeloid progenitors containing one LTCIC. Using the above *representative* experiment as an example, 63% of the wells were positive when 19 sorted myeloid progenitor cells were plated per well. In other words, there were 52,632 LTCIC per 10^6 $CAM^{+/-}CD34^+$ myeloid progenitor cells sorted.

increasing temperature to 37°C, (4) washing away nonadherent cells, and (5) determining % adherent cells by lysing them and measuring ^{51}Cr γ emissions. A nonradioactive adhesion assay was developed in which cells were labelled with a fluorochrome conjugated mAb and analyzed flow cytometrically instead of using ^{51}Cr labelling and a scintillation counter. The remaining steps of both assays were carried out in the same manner.

Prior to execution of the VLA-4 adhesion assay, the concentration of FN per well, type of microtiter plate, and incubation duration was optimized using protocols suggested by Mobley et al. and described in detail herein.[62] Human plasma fibronectin (FN; >95% pure by sodium dodecylsulfate polyacrylamide gel electrophoresis, mycoplasm negative by MYCOTECT™ System and DNA staining, functional performance verified by cell attachment and spreading assays using BHK-21 cells performed by manufacturere; GIBCO/BRL, Grand Island, NY) was sequentially diluted at [2 mg/mL], [200 µg/mL], [20 µg/mL], [2 µg/mL], [200 ng/mL], and [20 ng/mL]. Fifty microliters of each dilution was pipeted into seven rows of either a 96-well tissue culture-treated microtiter plate (Costar Corporation, Cambridge, MA) or a 96-well ELISA-treated microtiter plate (Nunc, Naperville, IL) with the eighth row left empty for use as negative control wells. Final FN concentrations per well were 100 µg/well, 10 µg/well, 1 µg/well, 100 ng/well, 10 ng/well, and 1 ng/well. Plates were covered and incubated for 1 hour at RT. Unbound ligand was washed from wells via several successive washes with 200 µL PBS/HSA buffer [PBS with Ca^{2+} and Mg^{2+}, (v/v) 0.5% human serum albumin U.S.P, pH=7.2 (Therapeutic Alpha Corporation, Los Angeles, CA)]. Remaining unbound

protein-binding sites were blocked by incubation with PBS/BSA buffer [PBS with Ca^{2+} & Mg^{2+} and 2.5% (w/v) bovine serum albumin, pH=7.2] for 2 hours at 37°C. The wells were washed with 200 µL PBS/HSA and incubated with 50 µL of PBS/HSA buffer ≥ 30 minutes at 4°C. Pooled buffy coat samples enriched for $CD34^+$ hematopoietic progenitor cells were resuspended in PBS/HSA buffer at a concentration of [10^3 cells/µL]. A small fraction (200 µL) of $CD34^+$ HPCs was reserved for flow cytometric analysis of viable VLA-4^+CD34^+ myeloid progenitor cell content as previously described. Cells were plated in triplicate onto FN-coated and blank (not FN-coated) wells at 50 µL/well (50,000 cells/well). Cell adhesion to immobilized FN was initiated by raising the temperature to 37°C for 10, 20, 30, 40, 50, 60, and 120 minutes or overnight in a humidified atmosphere of 5% CO_2:95% air (optimization of incubation time). Nonadherent cells (i.e. free in the solution) were collected via three gentle successive washes with 200 µL PBS/HSA buffer per well and pooled. Adherent cells were harvested via trypsinization. Viable nonadherent and adherent cells per well were enumerated via the trypan blue exclusion assay. Nonadherent and adherent cells from each well were labelled for flow cytometric analysis of VLA-4, CD34 and CD45 expression as previously described.

The percentage of pre-binding assay sample (originally seeded cell preparation of 50,000 cells/well), nonadherent cells (free in solution after initiation of adhesion), and adherent cells (bound after initiation of adhesion) which were viable VLA-4^+CD34^+ myeloid progenitors was determined flow cytometrically as previously described. The number of viable cells within the pre-binding assay, nonadherent cell and adherent cell samples were quantitated via the trypan blue exclusion assay. The absolute number of

viable VLA-4$^+$CD34$^+$ myeloid progenitors contained within the pre-binding assay, nonadherent cell and adherent cell samples was determined from the following equation:

VLA-4$^+$CD34$^+$ myeloid progenitors = (%VLA-4$^+$CD34$^+$ myeloid progenitors)(0.01)(total cells)

The percentage of viable VLA-4$^+$CD34$^+$ myeloid progenitors adhering to fibronectin was calculated from the following equation:

$$\left(\frac{\text{Viable Adherent VLA-4}^+\text{CD34}^+\text{ myeloid progenitors}}{\text{Viable VLA-4}^+\text{CD34}^+\text{ myeloid progenitors (pre-adhesion assay)}}\right) \times 100 = \text{\% viable adherent VLA-4}^+\text{CD34}^+\text{ myeloid progenitors}$$

The percentage of viable nonadherent VLA-4$^+$CD34$^+$ myeloid progenitors was calculated from the following equation:

$$\left(\frac{\text{Viable Nonadherent VLA-4}^+\text{CD34}^+\text{ myeloid progenitors}}{\text{Viable VLA-4}^+\text{CD34}^+\text{ myeloid progenitors (pre-adhesion assay)}}\right) \times 100 = \text{\% viable nonadherent VLA-4}^+\text{CD34}^+\text{ myeloid progenitors}$$

The percentage of viable nonadherent VLA-4$^+$CD34$^+$ myeloid progenitors was calculated only during the optimization adhesion assay experiments to demonstrate the ability to track the location of VLA-4$^+$CD34$^+$ myeloid progenitors as either free in solution or bound to FN.

The optimal parameters determined for the adhesion assay (n=3) were as follows: FN concentration of 1 µg/well, 96-well ELISA-treated microtiter plate (Nunc), and incubation at 37 °C for 1 hour (Figure 22). In all of the adhesion assays described herein, the same lot of FN and NUNC plates were used to minimize alterations in adhesion due to batch-to-batch differences in FN or plate preparations.

FN [20 ng/µL PBS with Ca^{2+} and Mg^{2+}] was dispensed at 50 µL/well into 96 well plates (Nunc) with one row of wells left empty to serve as negative controls. The plates

were incubated for one hour at RT. Unbound FN was removed by several successive washes with PBS/HSA buffer. Protein-binding sites in ligand-coated wells and wells receiving no ligand were blocked by incubation with PBS/BSA buffer for 2 hours at 37°C. The wells were washed with 200 µL PBS/HSA and incubated with 50 µL of PBS/HSA buffer ≥ 30 minutes at 4 °C. Pooled buffy coat samples were enriched for $CD34^+$ myeloid progenitor cells (Figure 4). A small fraction of cells was reserved for flow cytometric analysis of VLA-4^+CD34^+ or $CD34^+$ myeloid progenitor cell content as previously described. Cells were resuspended in PBS/HSA buffer at a concentration of [10^3 cells/µL]. Treatment groups were plated in replicates of four at 50 µL/well (50,000 cells/well). Group one was plated onto blank, non-coated wells (served as nonspecific binding controls). All remaining groups were plated onto FN coated wells. Group two was plated in PBS/HSA buffer alone. Group three received 6 µL/ well mAb (anti-CD49d-FITC; Immunotech) against the α4 chain of the VLA-4 α4ß1 heterodimer. Group four received an immunotype identical mAb (IgG2a-FITC; Immunotech) which does not bind either chain of the VLA-4 heterodimer (served as an isotype identical, nonspecific binding control for mAbs). Cell adhesion to the immobilized FN was initiated by raising the temperature to 37°C for 60 minutes in a humidified atmosphere of 5% CO_2:95% air. The number of viable $CD34^+$ cells not adhering to fibronectin was determined by the trypan blue exclusion assay as previously described. The number of viable $CD34^+$ myeloid progenitor cells binding FN per well was determined as described above. Adherent cells were harvested via trypsinization, labelled for flow cytometric

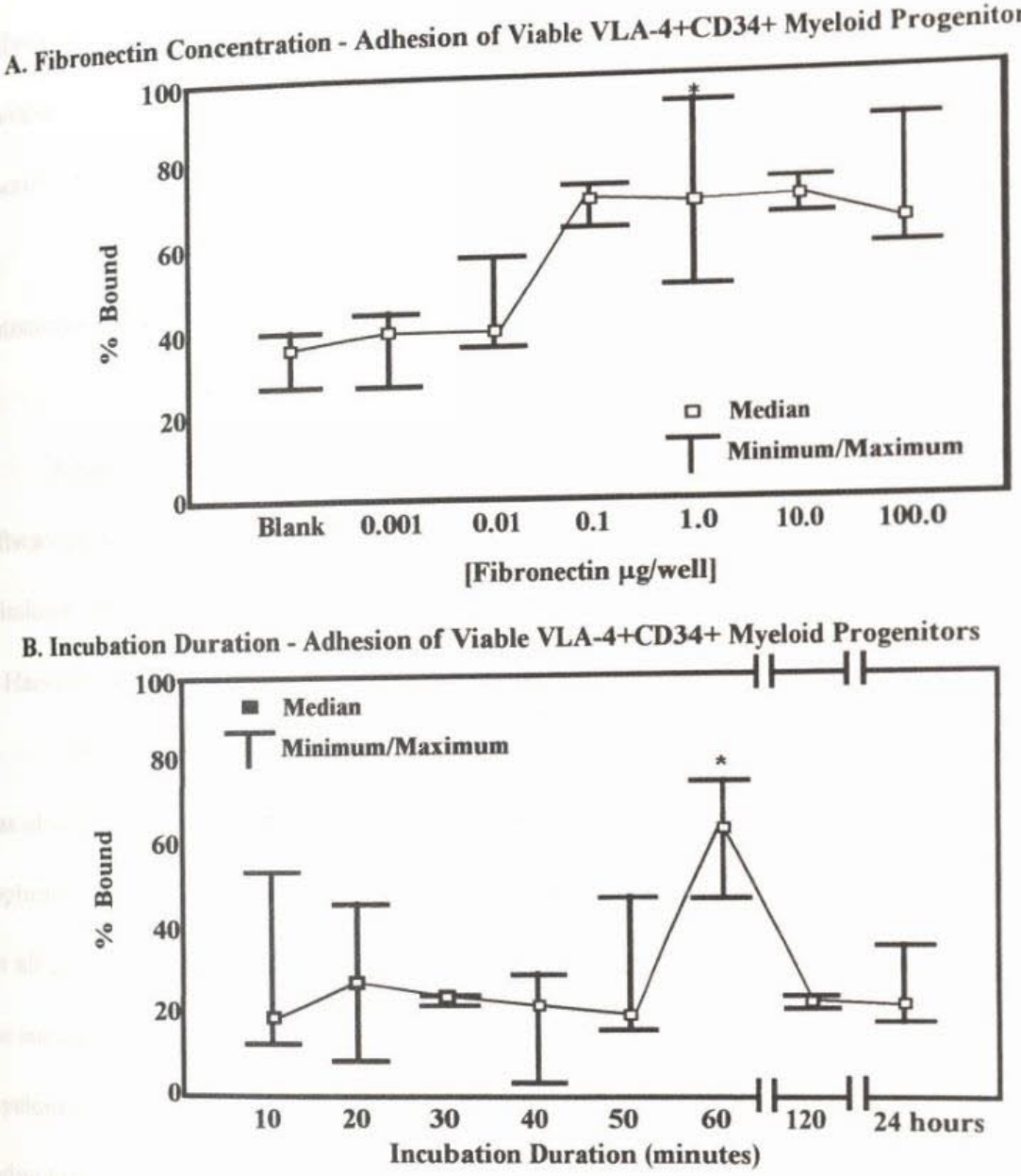


Figure 22. Optimization of adhesion assay. To determine the optimal concentration of fibronectin (FN), adhesion assays (n=3) of pooled buffy coat samples enriched for CD34+ cells were performed using concentrations of FN ranging from 0.001 to 10 µg FN/well (A). On average the highest percentage of VLA-4+CD34+ myeloid progenitors bound to FN concentrations > 0.01 µg FN/well (A). A concentration of 1 µg FN/well was chosen from the plateau phase of the graph (indicated by asterisk) for all adhesion assays. To determine the optimal incubation time, adhesion assays (n=3) of pooled buffy coat samples enriched for CD34+ cells were performed at incubations ranging from 10 minutes to 24 hours (B). The greatest percentage of VLA-4+CD34+ myeloid progenitor cells bound after 1 hr of incubation (indicated by asterisk).

analysis of VLA-4, CD34 and CD45 expression as previously described. The percentage of viable adherent VLA-4$^+$CD34$^+$ or CD34$^+$ myeloid progenitors was determined as described above.

Statistical analysis

All statistical analyses and graphics were generated using one of the following software programs, alone or in combination as dictated by the application: Statistica for Windows (Statsoft, Tulsa, OK), Lotus1-2-3 version 4 (Lotus Development Corporation), or Harvard Graphics version 3.0 (Software Publishing Corporation, Santa Clara, CA).

The following discussion of statistical tests, their applications and assumptions was obtained from the Statistica for Windows owner's manual supplied with the application software. The Student's t-test for dependent samples (paired T-test) was used for all comparative analyses of paired marrow and blood samples. This statistic enables the comparison of means among variables (e.g. mean CAM expression per CD34$^+$ myeloid progenitor between blood and marrow) measured in the same sample (e.g. same individual's blood and marrow). The null hypothesis of the paired t-test states that there is no diffeence between the means of the measured variable. For example, the null hypothesis of the paired marrow and blood samples states that there is no difference between the mean expression of VLA-4 per myeloid progenitor collected from the marrow and those collected from the circulating blood. If the null hypothesis were true, then any measured differences (mean VLA-4 expression per myeloid progenitor cell)

would most likely be attributable to measurement errors which would be normally distributed around a mean of zero. If sufficient evidence for rejecting the null hypothesis were found (i.e., $p \leq 0.05$), then any measured differences between the two sample population means would most likely be attributable to true differences in the population means. Thus a p-value of ≤ 0.05 would indicate that, at least 95% of the time, differences observed in the experiment reflect true differences in CAM or CD34 expression between blood and marrow myeloid progenitors. The paired t-test assumes that the variables under analysis are normally distributed and homogeneity of variance. In order to test for normality, the Shapiro-Wilks W test was performed on each variable prior to paired t-test analysis. This statistic is the preferred test of normality as compared with other tests of normality, such as the Kolmogorov-Smirnov test. In both the Shapiro-Wilks and Kolmogorov-Smirnov test the null hypothesis states that the respective distribution is normal; thus, a significant W or D statistic, respectively, would lead to rejection of the null hypothesis that the variable is normally distributed. In order to test for homogeneity of variance, the Levene Test was performed on each variable prior to paired t-test analysis. In this test, analysis of variance for each dependent (measured) variable is performed on the absolute deviations of the values from the respective group means. The null hypothesis of the Levene test states that the variability between variables is homogeneous. If a significant p-value was obtained (i.e., $p \leq 0.05$), then the null hypothesis of homogeneity of variance was rejected.

Analysis of CFU assays (subtypes of colonies formed, total colonies formed), LTCIC assays (LTCIC content of sorted subsets) and binding assays, was performed

using analysis of variance (ANOVA) for multiple measures. In general, the purpose of ANOVA is to test for significant differences between sample means; however, as the name of this test implies, this test actually compares the variances of each sample. Under the null hypothesis, the variance estimated based on within-group variability should be about the same as the variance due to between-groups variabilty. For example, the variability of the total number colonies formed/10^6 cells plated (within-group variability) should be about the same as the variability between the total number colonies formed/10^6 sorted cells of marrow and blood (between-groups variabilty). Rejection or failure to reject the null hypothesis of the ANOVA test follows the same criteria as previously described for the Student's t-test; that is, a significant F statistic (≤ 0.05) would lead to rejection of the null hypothesis with 95% confidence that the differences measured in the experiment reflect true population differences. Assumptions of the ANOVA test include normal distribution and homogeneity of variance of the dependent (measured) variables. In general, ANOVA analysis is robust against violations of the assumption of normality and homogeneity of variance when 1) $N \geq 10$ and 2) the means of each group do not correlate with the standard deviations across groups, respectively. Normality and homogeneity of variance for each dependent variable was tested using the Shapiro-Wilks and Levene test, respectively, as previously described.

After obtaining a significant F statistic (≤ 0.05) from ANOVA, it was important to determine which groups (variables) were specifically different. Performance of a series of t-tests to compare all possible pairs of means would capitalize on chance, meaning that reported probabilities would actually overestimate the statistical significances of mean

differences (Type I error). Post hoc comparison techniques specifically take into account the fact that more than two samples were taken. In most post hoc comparisons, the means of all variables are sorted into ascending order. For each pair of means, the probability under the null hypothesis of obtaining differences between means of this magnitude (or greater) given the respective number of samples is then calculated. Finally, the actual probabilties based on the distribution of the calculated (studentized) range of statistics is calculated and reported. For analysis of specific effects between variables deemed significantly different via ANOVA analysis, Newman-Keuls Test and Critical Ranges was utilized. The Newman-Keuls Test, like ANOVA, is based on the studentized range statistic. Computationally, the means were sorted into ascending order and the probability under the null hypothesis (no differences between means in the population) of obtaining differences between means of this (or greater) magnitude, given the respective number of samples, was determined.

In those analyses in which the assumptions of the parametric tests (paired t-test, ANOVA, etc.) were violated (e.g. one or more of the variables was not normally distributed), the nonparametric equivalent to the parametric test was utilized for statistical analysis. The Wilcoxon matched pairs test is a nonparametric alternative to the paired t-test (t-test for dependent samples). The Wilcoxon test assumes that the variables under examination were measured on a scale which allows rank ordering of the observations based on each variable and that allows rank ordering of the differences between variables. The obtained p-value is interpreted just as that of the paired samples t-test. The Kruskal-Wallis ANOVA by ranks is a nonparametric alternative to ANOVA.

This test assumes that the variable under consideration is continuous and was measured on at least an ordinal scale. This test assesses the hypothesis that the different samples in the comparison were drawn from the same distribution or from distributions with the same median. If a significant difference was found using the Kruskal-Wallis ANOVA, the Mann-Whitney U test, a nonparametric alternative to the t-test was used. In order to circumvent the committance of Type I error the Bonferroni method was employed to determine an acceptable p-value. In this technique, the acceptable p-value of 0.05 was divided by the number of comparisons being performed with the Mann-Whitney U test.

In summary, when the measured variable was normally distributed the parametric test dictated by the experiment design (t-tests, ANOVA, or Newman-Keuls test) was used. When the measured variable was not normally distributed the nonparametric tests were applied (Mann-Whitney U test, Kruskall Wallis ANOVA by ranks, or Wilcoxon matched pairs test) as dictated by the design of the experiment.

RESULTS

Specific aim 1: differential expression of CAMs between marrow and blood

Distribution of CAM expressing cells within the CD34$^+$ myeloid progenitor populations of marrow and blood. Nucleated cell fractions of paired marrow and blood specimens from either granulocyte-colony stimulating factor (G-CSF) mobilized patients or normal donors were enriched for CD34$^+$ cells (Figure 4). The paired specimens were labeled with monoclonal antibodies (mAb) against CD34, CD45 and one of the following cell adhesion molecules (CAMs) under study: homing-associated cell adhesion molecule (HCAM), very late antigen (VLA-4) or L-selectin (Appendix 4). The individual cell types in each sample were differentiated using a method similar to the one originally reported by Stelzer et al. (Figure 9).[57] The myeloid progenitor population was gated and analyzed for the coexpression of each CAM (HCAM, VLA-4 or L-selectin) with CD34 (Figure 10).

No significant difference ($p>0.05$) was detected between the percentage of CD34$^+$ myeloid progenitors coexpressing HCAM or L-selectin (paired t-test analysis) between normal marrow and blood (Figure 23A). Marrow derived myeloid progenitors from normal donors expressed a significantly higher ($p=0.017$;Wilcoxon Matched Pairs Test)

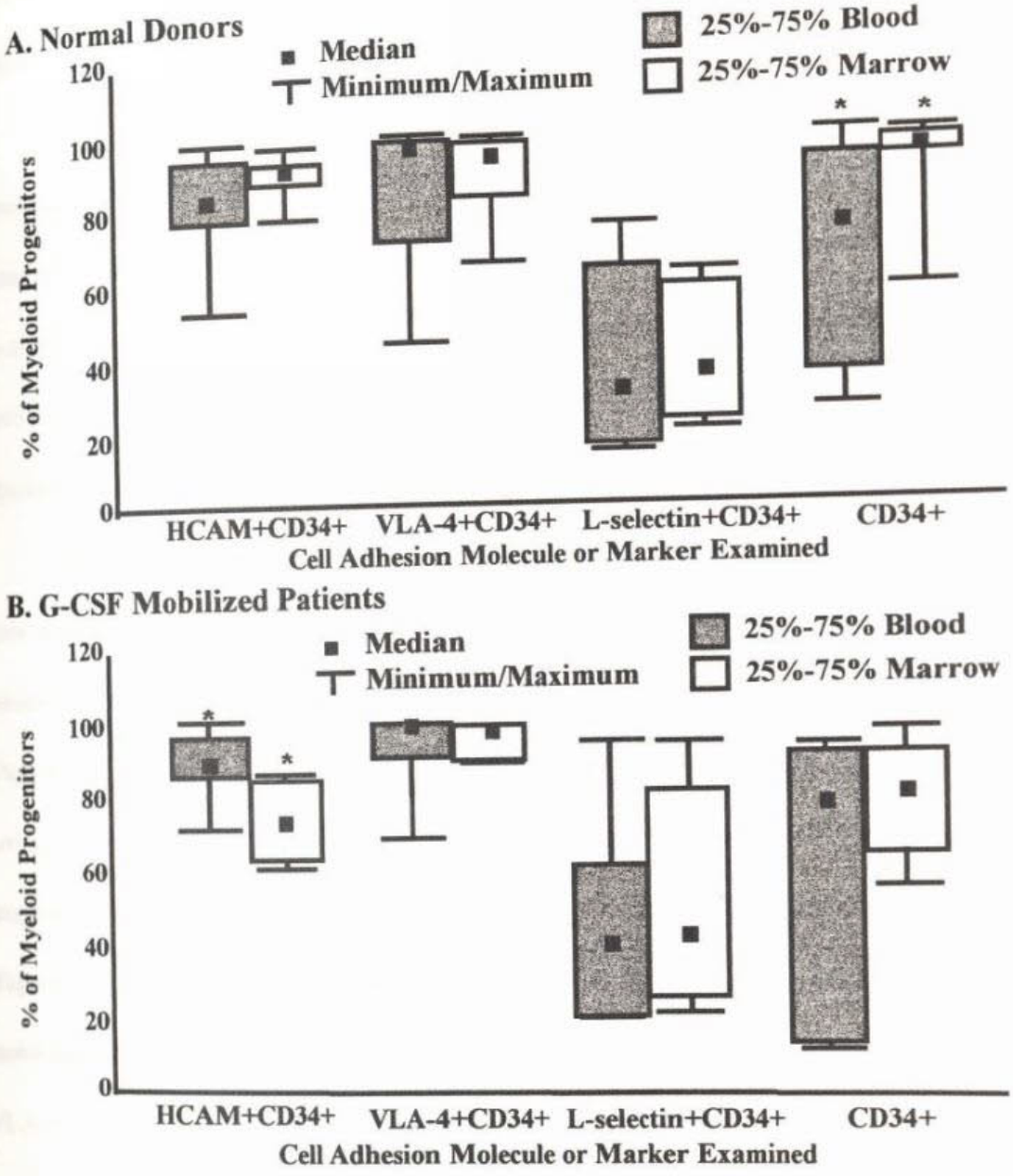


Figure 23. Qualitative expression of HCAM, VLA-4, L-selectin and CD34 on myeloid progenitors between marrow and blood samples. Paired samples of marrow and blood from either normal donors (n=7) or patients mobilized with G-CSF (n=7) were enriched for CD34+ cells. Blood (gray) and marrow (white) myeloid progenitors were examined flow cytometrically for their coexpression of cell adhesion molecules: HCAM, VLA-4 or L-selectin with CD34; or their expression of CD34. In normal donors (A), the percentage of marrow derived myeloid progenitors expressing CD34 was significantly greater ($p=0.017$) than on circulating myeloid progenitors. In G-CSF primed patients (B), the percentage of CD34+ myeloid progenitors coexpressing HCAM was significantly lower ($p=0.014$) on myeloid progenitors residing in the marrow than those in circulation. Significant differences are indicated with asterisks.

percentage (mean±2 x standard error of mean (s.e.m.); 91±2%) of CD34 than their circulating counterparts (68±11%; Figure 23A).

In G-CSF mobilized patients, no significant difference ($p>0.05$) was found in the percentage of $CD34^+$ myeloid progenitor cells coexpressing VLA-4, L-selectin or CD34 between marrow and blood (Figure 23B). However, the percentage (mean±2 x s.e.m.%) of $CD34^+$ myeloid progenitors coexpressing HCAM was significantly lower ($p=0.014$; paired t-test) on those residing in the marrow (74±8%) than those in circulation (89±7%; Figure 23B).

No significant difference ($p>0.05$) was detected between blood specimens derived from normal donors and those derived from G-CSF mobilized patients with regard to the percentage of myeloid progenitors expressing HCAM, VLA-4, L-selectin and CD34 (Figure 24). The percentage of marrow derived $CD34^+$ myeloid progenitors coexpressing HCAM was significantly higher ($p=0.002$; Student's t-test) on myeloid progenitors from normal donors than on those from G-CSF mobilized patients (Figure 25). No other significant differences between normal donor and G-CSF mobilized patient marrow derived myeloid progenitors in terms of their coexpression of VLA-4 or L-selectin with CD34; or expression of CD34 alone (Figure 25).

CAM expression per $CD34^+$ myeloid progenitor cell. To quantitate the expression of CAM or CD34 per $CD34^+$ myeloid progenitor cell, the mean peak channel fluorescence of each marker within the $CD34^+$ myeloid progenitor gated population was determined (Figure 12). The mean channel fluorescence was compared with the QSC

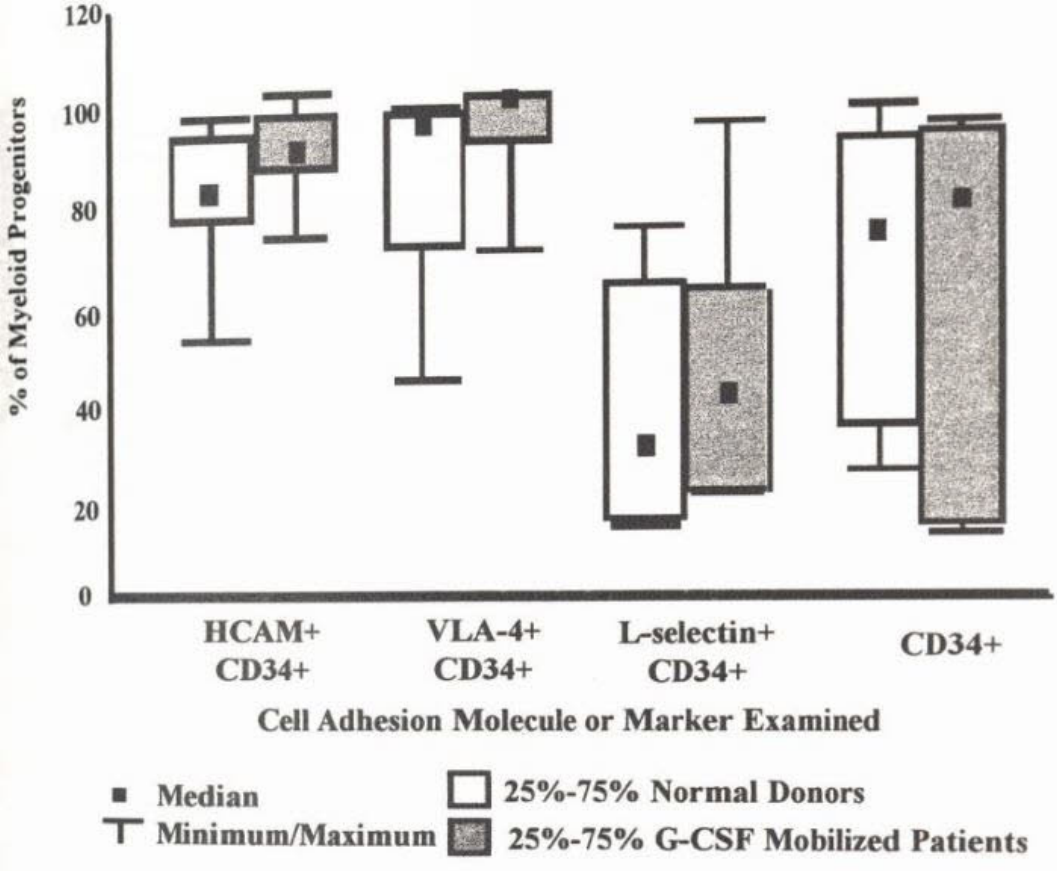


Figure 24. Comparison of blood specimens derived from normal donors and G-CSF mobilized patients for qualitative differences in myeloid progenitor cell expression of HCAM, VLA-4, L-selectin and CD34. Blood samples obtained from normal donors (n=7; white) or patients mobilized with G-CSF (n=7; gray) were enriched for CD34+ cells (same data set as presented in Figure 21). Myeloid progenitors were examined flow cytometrically for their coexpression of cell adhesion molecules: HCAM, VLA-4 or L-selectin with CD34; or their expression of CD34. No significant difference ($p>0.05$) was detected between blood specimens derived from normal donors and those derived from G-CSF mobilized patients with regard to the percentage of myeloid progenitors expressing HCAM, VLA-4, L-selectin and CD34.

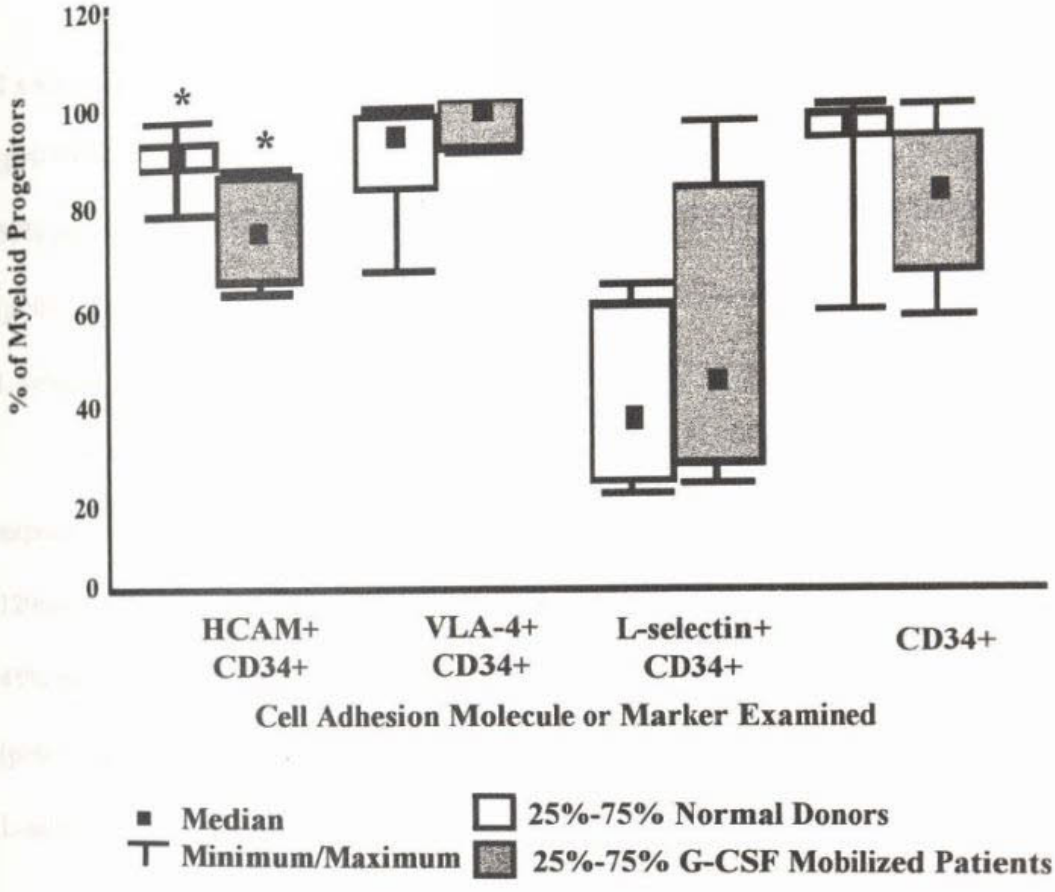


Figure 25. Comparison of marrow specimens derived from normal donors and G-CSF mobilized patients for qualitative differences in myeloid progenitor cell expression of HCAM, VLA-4, L-selectin and CD34. Marrow samples obtained from normal donors (n=7; white) or patients mobilized with G-CSF (n=7; gray) were enriched for CD34+ cells (same data set as presented in Figure 21). Myeloid progenitors were examined flow cytometrically for their coexpression of cell adhesion molecules: HCAM, VLA-4 or L-selectin with CD34; or their expression of CD34. The percentage of marrow derived CD34+ myeloid progenitors coexpressing HCAM was significantly higher ($p=0.002$) on myeloid progenitors from normal donors than on those from G-CSF mobilized patients. Significant differences indicated by asterisks.

bead linear regression standard curves generated for each experiment and the antibody binding capacity (Abc) units per $CD34^+$ myeloid progenitor was extrapolated (Figure 14).

The expression of CD34 per myeloid progenitor cell (mean Abc units ± 2 x s.e.m.) of normal donor marrow (32113±10681) was significantly greater ($p=0.0036$; paired t-test) than those in circulation (13640±8976), an average decrease of 58% per circulating $CD34^+$ myeloid progenitor (Figure 26A). No significant difference ($p>0.05$) between normal donor marrow and blood was detected in HCAM, VLA-4 or L-selectin expression per $CD34^+$ myeloid progenitor cell (Figure 26A).

In G-CSF mobilized patients, marrow derived $CD34^+$ myeloid progenitor cells expressed significantly greater ($p=0.0007$) VLA-4 per cell (mean Abc units ± 2 x s.e.m.; 12966±3482) than those circulating in the blood (7097±3523), an average decrease of 45% per circulating $CD34^+$ myeloid progenitor (Figure 26B). No significant difference ($p>0.05$) between G-CSF mobilized marrow and blood was detected in HCAM, L-selectin or CD34 expression per $CD34^+$ myeloid progenitor cell (Figure 26B).

CD34+ myeloid progenitors expressed significantly more ($p=0.032$; Student's t-test) L-selectin per cell than those derived from G-CSF mobilized patients (Figure 27). No significant difference ($p>0.05$) in the expression of HCAM or L-selectin and CD34; or CD34 alone per blood derived myeloid progenitor cells was detected (Figure 27). No significant difference ($p>0.05$) was detected between marrow specimens derived from

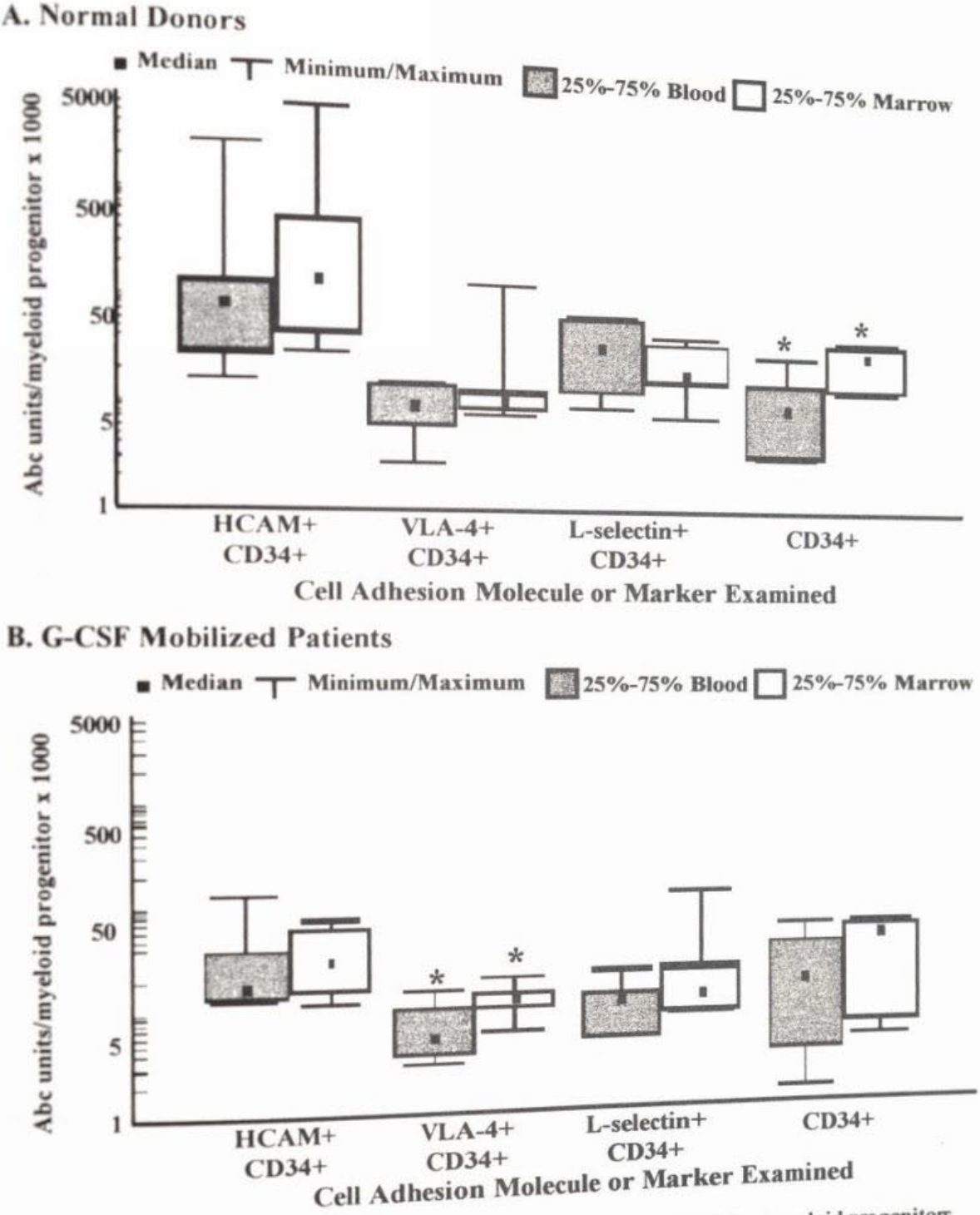


Figure 26. Quantitative expression of HCAM, VLA-4, L-selectin and CD34 on myeloid progenitors. In normal donors (A), myeloid progenitor expression of CD34, as reflected by antibody binding capcity (Abc) units, was significantly higher (p=0.0036) on those residing in the marrow (white) than on those in circulation (gray). In G-CSF primed patients (B), circulating CD34+ myeloid progenitors expressed significantly less (p=0.0007) VLA-4 per cell than those residing in the marrow. Significant differences indicated by asterisks.

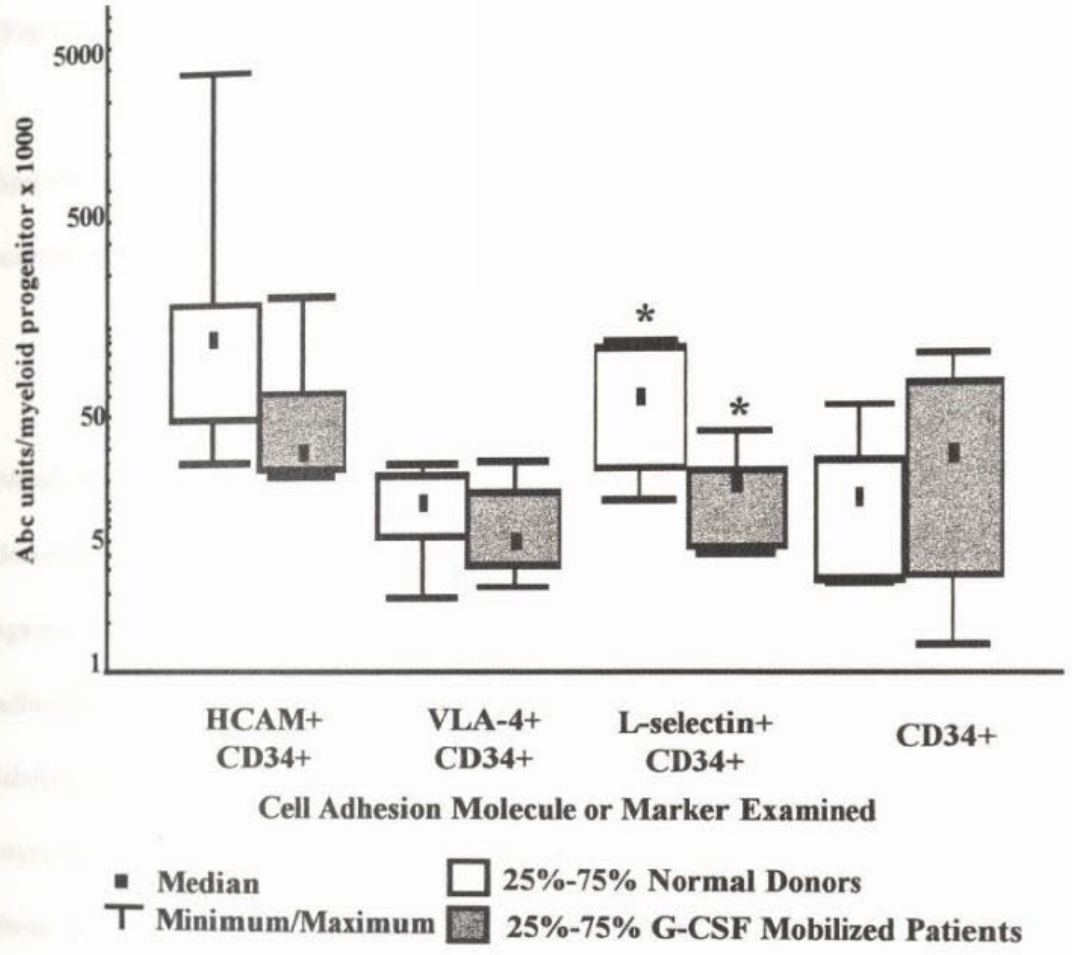


Figure 27. Comparison of blood specimens derived from normal donors and G-CSF mobilized patients for quantitative differences in myeloid progenitor cell expression of HCAM, VLA-4, L-selectin and CD34. Blood samples obtained from normal donors (n=7; white) or patients mobilized with G-CSF (n=7; gray) were enriched for CD34+ cells (same data set as presented in Figure 26). Myeloid progenitors were examined flow cytometrically for their coexpression of cell adhesion molecules: HCAM, VLA-4 or L-selectin with CD34; or their expression of CD34. CD34+ myeloid progenitors from normal donors expressed significantly more ($p=0.032$) L-selectin per cell than those derived from G-CSF mobilized patients. Significant differences indicated by asterisks.

normal donors and those derived from G-CSF mobilized patients with regard to per cell expression of HCAM, VLA-4 or L-selectin with CD34; or CD34 expression alone (Figure 28).

Specific aim 2: clonogenic potential and long term culture initiating cell content of marrow and blood

CFU assays of CAM$^{+/-}$CD34^{+} myeloid progenitor populations from marrow and blood. Nucleated cell fractions of marrow or pooled buffy coat specimens from normal donors were enriched for CD34^{+} cells (Figure 4). The specimen was labelled with mAb against CD34 and one of the following cell adhesion molecules: homing-associated cell adhesion molecule (HCAM), very late antigen (VLA-4) or L-selectin (Appendix 4). The labelled sample was analyzed flow cytometrically for the presence of CAM$^{+/-}$CD34^{+} myeloid progenitors (Figures 9 and 12). The flow cytometer was directed to sort only those cells which displayed the following criteria: low side scatter (indicating small size and minimal cellular complexicity), CD34^{+} and CAM^{+} (Figure 15). Only samples whose post-sort purity was $\geq$90% (as determined by flow cytometric analysis of sorted samples) were used to plate CFU assays as summarized in Table Three. After 14 days of culture, the CFU cultures were examined and the number of BFU-E, CFU-E, CFU-mix and CFU GM colonies were enumerated via microscopic inspection (Figures 18 and 19).

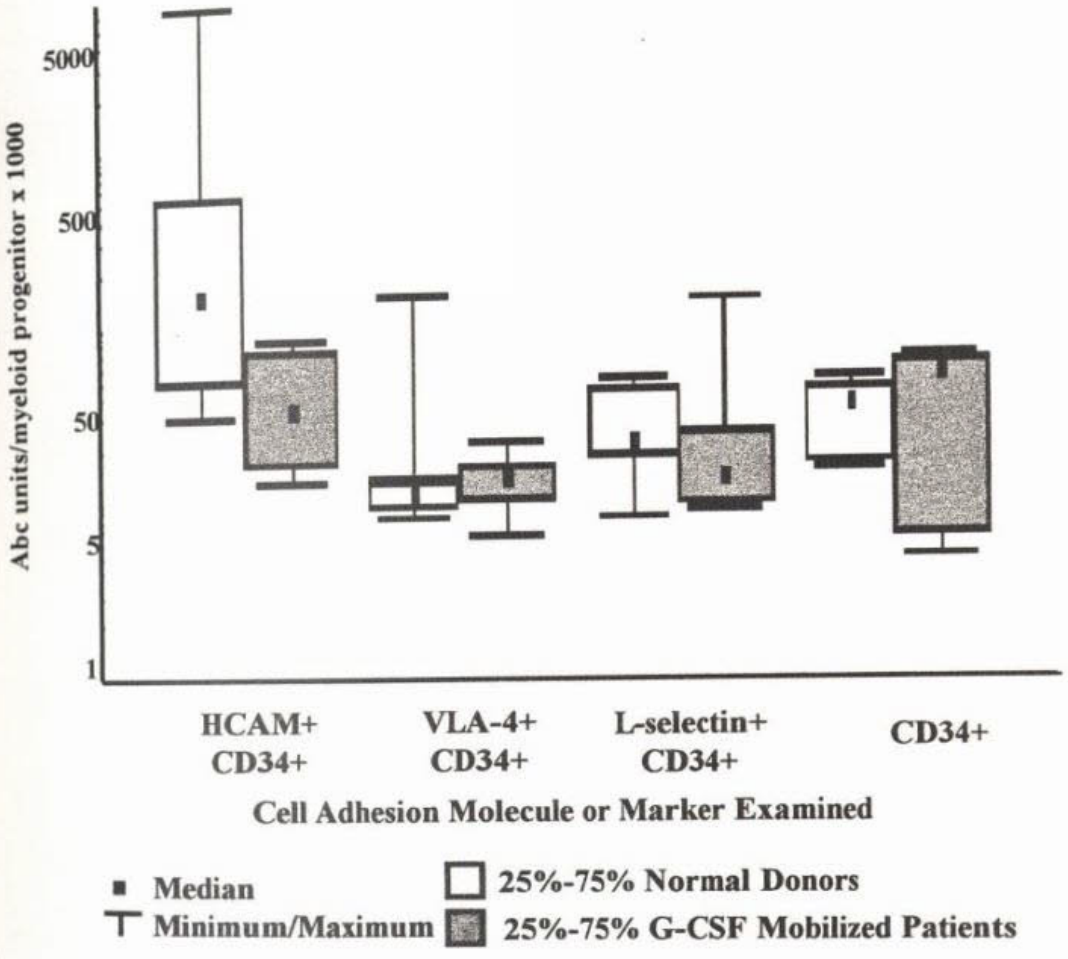


Figure 28. Comparison of marrow specimens derived from normal donors and G-CSF mobilized patients for quantitative differences in myeloid progenitor cell expression of HCAM, VLA-4, L-selectin and CD34. Marrow samples obtained from normal donors (n=7; white) or patients mobilized with G-CSF (n=7; gray) were enriched for CD34+ cells (same data set as presented in Figure 24). Myeloid progenitors were examined flow cytometrically for their coexpression of cell adhesion molecules: HCAM, VLA-4 or L-selectin with CD34; or their expression of CD34. No significant difference ($p>0.05$) was detected between marrow specimens derived from normal donors and those derived from G-CSF mobilized patients with regard to per myeloid progenitor cell expression of HCAM, VLA-4, L-selectin and CD34.

Table Three. Pre- and post-sort purities of sorted CAM^+CD34^+ myeloid progenitors based on post-sort FACS analysis. All percentages are presented as mean ± 2 x s.e.m.%.

Myeloid Progenitor Subset	Normal Marrow			Pooled Buffy Coats		
	N	Pre-sort	Post-sort	N	Pre-sort	Post-sort
$HCAM^+CD34^+$	10	32 ± 10%	98 ± 2%	6	34 ± 14%	97 ± 2%
$VLA\text{-}4^+CD34^+$	10	33 ± 16%	97 ± 2%	6	38 ± 24%	96 ± 4%
$VLA\text{-}4^-CD34^+$	9	14 ± 6%	98 ± 1%	3	14 ± 10%	97 ± 4%
$L\text{-selectin}^+CD34^+$	10	20 ± 10%	96 ± 2%	5	30 ± 16%	92 ± 2%
$L\text{-selectin}^-CD34^+$	9	22 ± 10%	97 ± 2%	3	16 ± 6%	97 ± 2%

Blood derived $CAM^{+/-}CD34^+$ myeloid progenitors produced a significantly greater ($p=0.00008$; ANOVA) total number of colonies than their respective marrow counterparts (Figure 29). Newman-Keuls analysis for specific differences are summarized in Table Four. $HCAM^+CD34^+$ myeloid progenitors sorted from normal blood produced a significantly greater ($p=0.013$) total number of colonies (391±128; mean±2xs.e.m.) than those derived from marrow (202±68; Figure 29 and Table Four). Blood derived $VLA\text{-}4^-$ and $L\text{-selectin}^-CD34^+$ myeloid progenitors produced a significantly greater total number of colonies (310±170, $p=0.031$; and 355±35, $p=0.0007$; respectively) than their marrow counterparts (139±44 and 99±43, respectively; Figure 29 and Table Four).

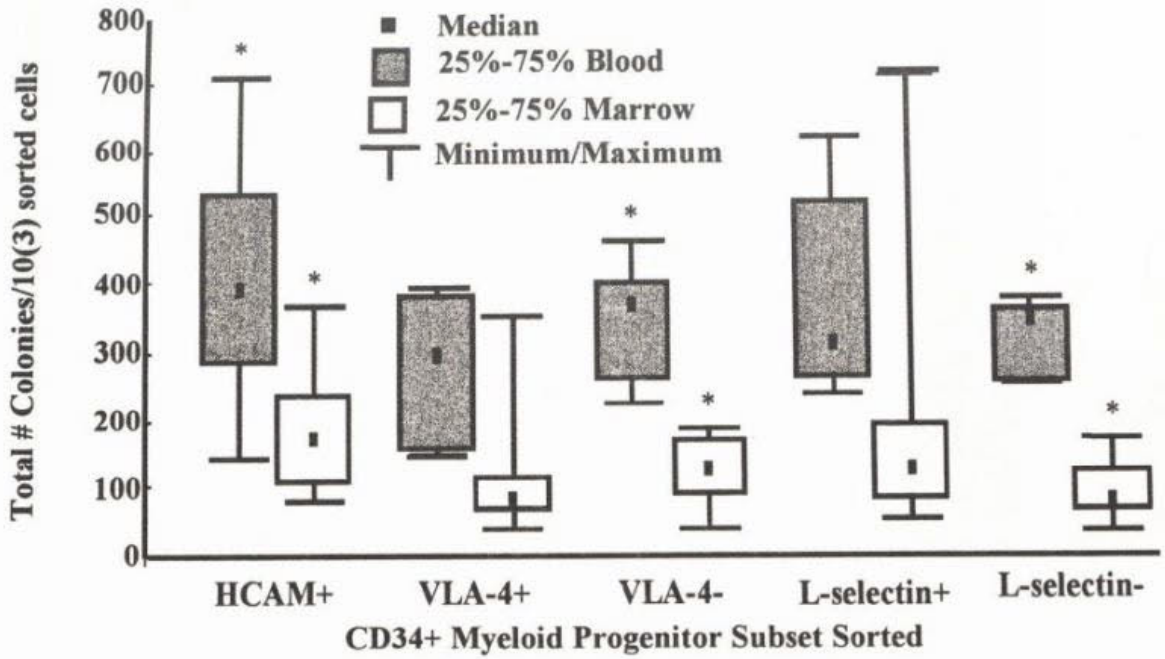


Figure 29. Total colonies formed by sorted CAM+/-CD34+ myeloid progenitors. Blood (gray; n=20) derived CD34+ myeloid progenitors formed significantly more (p=0.00008; ANOVA analysis) colonies *in vitro* than those derived from marrow (white; n=38). *Indicates significant difference was found between marrow and blood CD34+ myeloid progenitors also HCAM+ (p=0.013), VLA-4- (p=0.031), or L-selectin- (p=0.0007) using the Newman-Keuls Test for specific differences.

Table Four. Newman-Keuls Test of total colonies formed by blood versus marrow derived CAM$^{+/-}$ CD34$^+$ myeloid progenitors. N.S. indicates no significant difference ($p>0.05$) was found between the two variables; otherwise the specific p-value is listed.

Blood Derived CD34$^+$ Myeloid Progenitor Subset	Marrow Derived CD34$^+$ Myeloid Progenitors				
	HCAM$^+$	VLA-4$^+$	VLA-4$^-$	L-selectin$^+$	L-selectin$^-$
HCAM$^+$	0.013				
VLA-4$^+$		N.S.			
VLA-4$^-$			0.031		
L-selectin$^+$				N.S.	
L-selectin$^-$					0.0007

No significant difference ($p>0.05$) was detected in the total number of colonies formed between sorted CD34$^+$ myeloid progenitor subsets derived from blood ($n=20$) regardless of coexpression of HCAM, VLA-4 or L-selectin with CD34 (Figure 30). There was no significant difference ($p>0.05$) in the total number of colonies formed by marrow derived CD34$^+$ myeloid progenitors also HCAM$^+$, VLA-4$^{+/-}$ or L-selectin$^{+/-}$ (Figure 31).

No significant difference ($p>0.05$) in colony type distribution (i.e., %CFU-E versus %BFU-E, etc. of total colonies formed) was found between blood and marrow derived HCAM$^+$, VLA-4$^-$, L-selectin$^+$ or L-selectin$^-$ CD34$^+$ myeloid progenitors (Figures 32-35). Marrow derived VLA-4$^+$CD34$^+$ myeloid progenitors produced a significantly greater percentage ($p=0.013$; Student's t-test) of CFU-GM colonies ($24\pm11\%$; mean $\pm$ 2 x s.e.m.%) than their circulating counterparts ($4\pm2\%$; Figure 36).

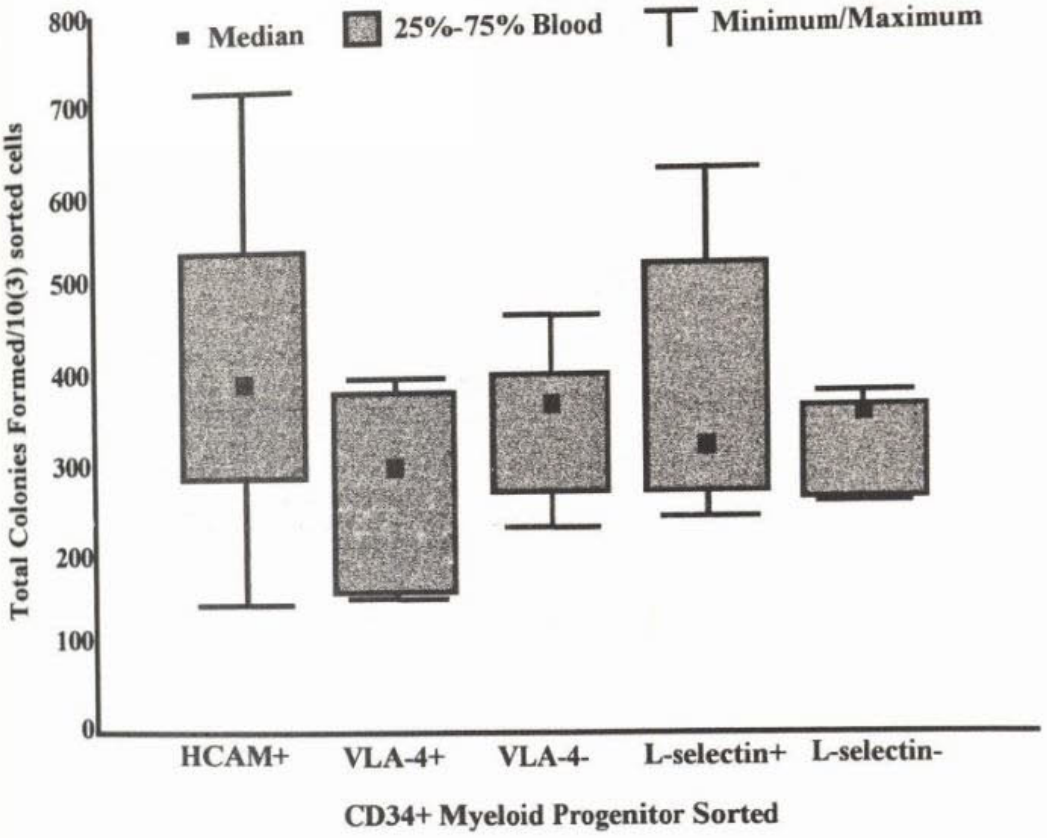


Figure 30. Total colonies formed from blood derived CAM+/-CD34+ myeloid progenitors. There was no significant difference ($p>0.05$) in the total number of colonies formed between sorted CD34+ myeloid progenitor subsets derived from blood (n=20) regardless of coexpression of HCAM, VLA-4 or L-selectin with CD34.

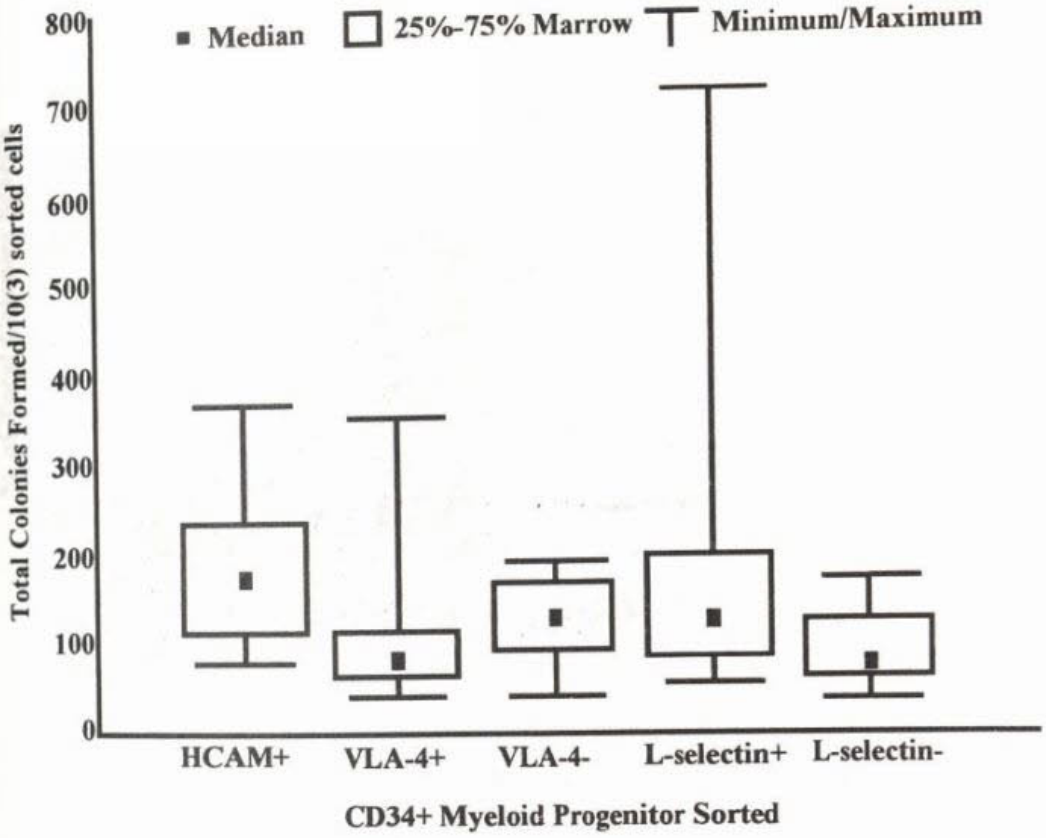


Figure 31. Total colonies formed from marrow derived CAM+/-CD34+ myeloid progenitors. There was no significant difference ($p>0.05$) in the total number of colonies formed between sorted CD34+ myeloid progenitor subsets derived from marrow (n=38) regardless of coexpression of HCAM, VLA-4 or L-selectin with CD34.

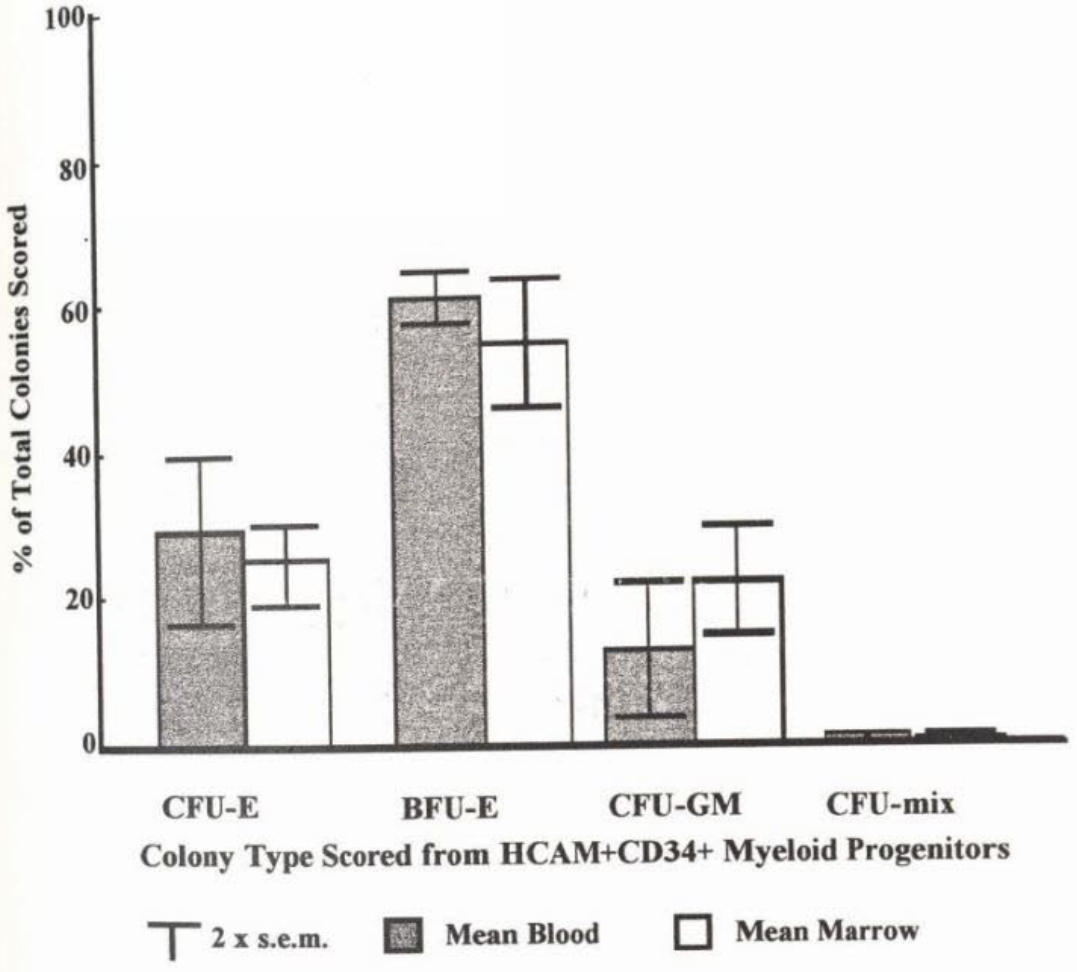


Figure 32. Comparison of CFU colony distribution between blood and marrow derived HCAM+CD34+ myeloid progenitors. CD34+ myeloid progenitors coexpressing HCAM were flow cytometrically sorted from blood and marrow specimens derived from normal donors and plated into CFU assays. No significant difference ($p>0.05$) in colony type distribution (represented as a percentage of total CFU colonies scored) was found between blood (gray; n=20) and marrow (white; n=38) derived HCAM+CD34+ myeloid progenitors.

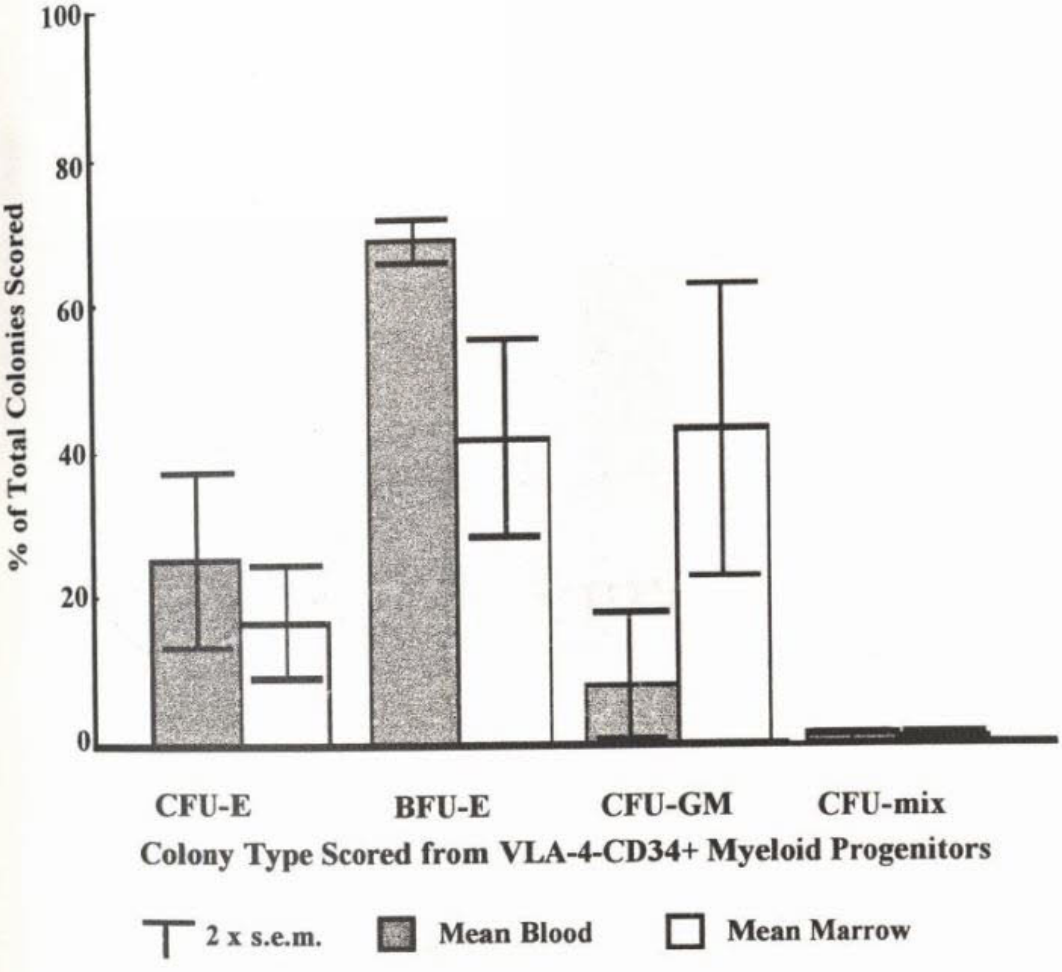


Figure 33. Comparison of CFU colony distribution between blood and marrow derived VLA-4-CD34+ myeloid progenitors. CD34+ myeloid progenitors not expressing VLA-4 (that is, VLA-4-) were flow cytometrically sorted from blood and marrow specimens derived from normal donors and plated into CFU assays. No significant difference ($p>0.05$) in colony type distribution (represented as a percentage of total CFU colonies scored) was found between blood (gray; n=20) and marrow (white; n=38) derived VLA-4-CD34+ myeloid progenitors.

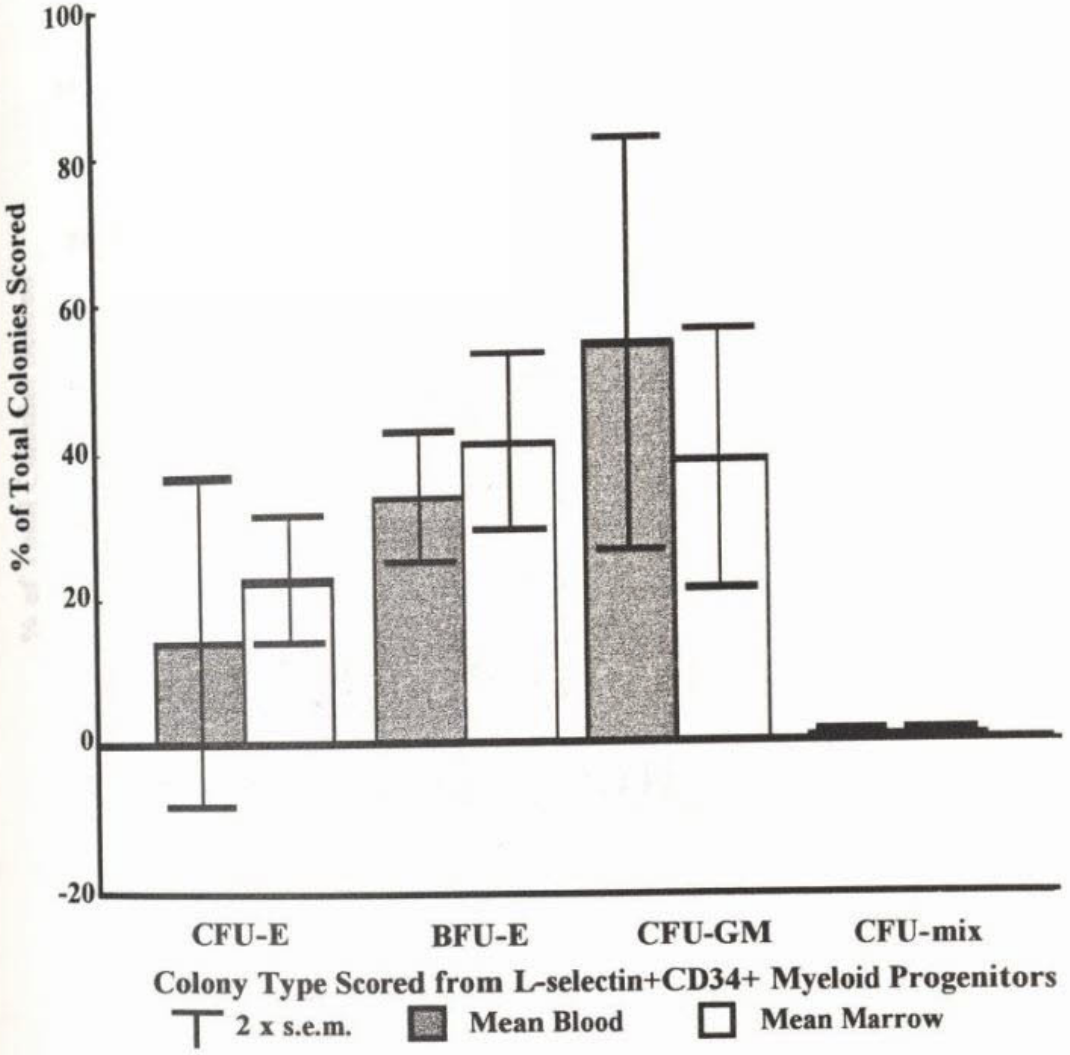


Figure 34. Comparison of CFU colony distribution between blood and marrow derived L-selectin+CD34+ myeloid progenitors. CD34+ myeloid progenitors coexpressing L-selectin were flow cytometrically sorted from blood and marrow specimens derived from normal donors and plated into CFU assays. No significant difference ($p>0.05$) in colony type distribution (represented as a percentage of total CFU colonies scored) was found between blood (gray; n=20) and marrow (white; n=38) derived L-selectin+CD34+ myeloid progenitors.

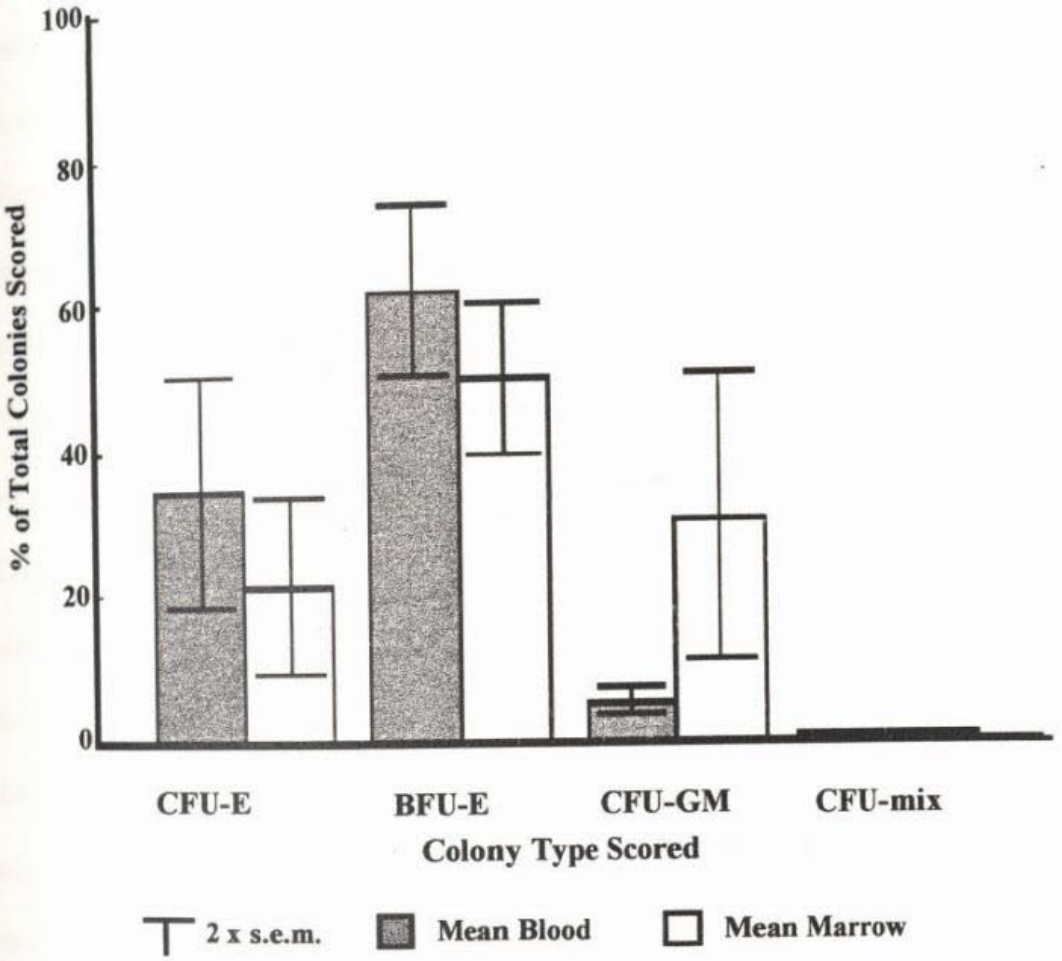


Figure 35. Comparison of CFU colony distribution between blood and marrow derived L-selectin-CD34+ myeloid progenitors. CD34+ myeloid progenitors not expressing L-selectin (that is, L-selectin-) were flow cytometrically sorted from blood and marrow specimens derived from normal donors and plated into CFU assays. No significant difference ($p>0.05$) in colony type distribution (represented as a percentage of total CFU colonies scored) was found between blood (gray; n=20) and marrow (white; n=38) derived L-selectin-CD34+ myeloid progenitors.

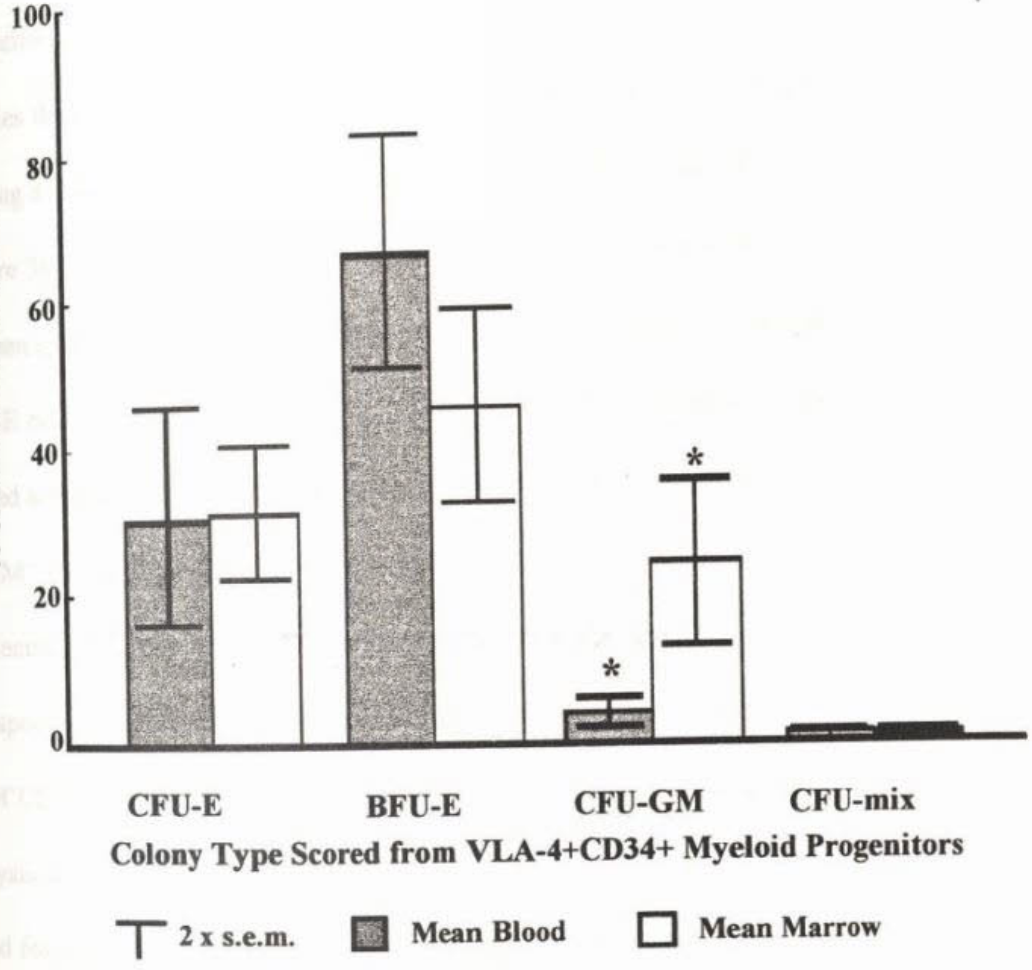
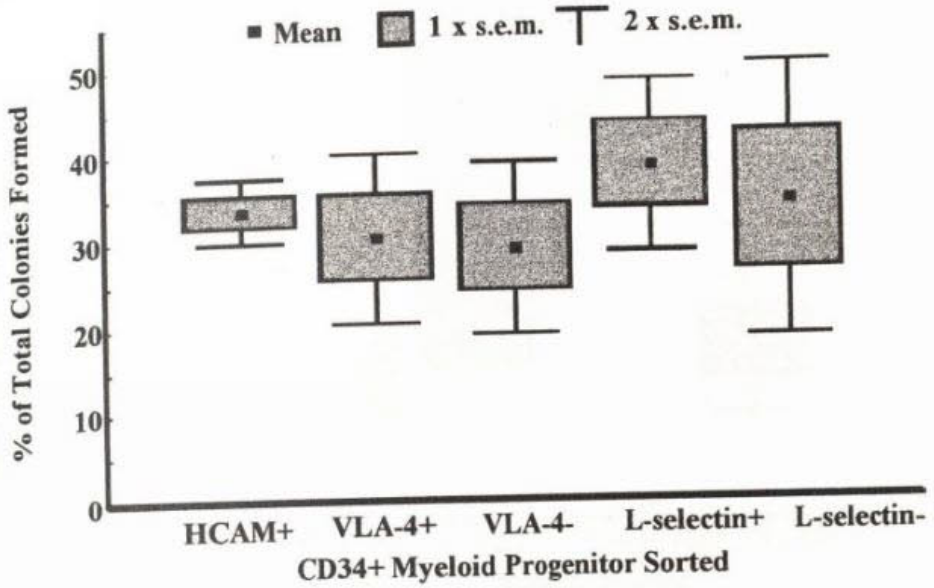


Figure 36. Comparison of CFU colony distribution between blood and marrow derived VLA-4+CD34+ myeloid progenitors. CD34+ myeloid progenitors coexpressing VLA-4 were flow cytometrically sorted from blood and marrow specimens derived from normal donors and plated into CFU assays. Marrow (white; n=38) derived CD34+ myeloid progenitors coexpressing VLA-4 produced a significantly greater ($p=0.013$) percentage of CFU-GM colonies than their circulating counterparts (gray; n=20). Significant differences indicated by asterisks.

In blood samples, the percentage of total colonies comprised of CFU-E, when comparing between sorted $CAM^{+/-}CD34^{+}$ myeloid progenitors, was not significantly different (Figure 37A). Blood derived $CD34^{+}$ myeloid progenitors coexpressing L-selectin formed a significantly lower ($p=0.002$; ANOVA) percentage of BFU-E colonies than all other sorted $CAM^{+/-}CD34^{+}$ myeloid progenitors (Figure 37B); while forming a significantly greater ($p=0.004$; ANOVA) percentage of CFU-GM colonies (Figure 38A). The Newman-Keuls test was used to determine the specific differences between sorted $CAM^{+}CD34^{+}$ myeloid progenitors with regard to the percentage of BFU-E colonies formed (Table Five). L-$selectin^{+}CD34^{+}$ myeloid progenitors from blood formed a significantly lower percentage of BFU-E colonies (33±9%;mean±2xs.e.m.) than $HCAM^{+}$ (60±4%; $p=0.007$), VLA-4^{+} (66±16%; $p=0.01$), VLA-4^{-} (68±3%; $p=0.005$), or L-$selectin^{+}$ (61±12%; $p=0.006$) $CD34^{+}$ myeloid progenitors (Figure 37B and Table Four). The specific differences between percentage of CFU-GM colonies formed from sorted $CAM^{+}CD34^{+}$ myeloid progenitors *in vitro* were determined using Newman-Keuls analysis and are summarized in Table Six. L-$selectin^{+}$ $CD34^{+}$ myeloid progenitors from blood formed a significantly greater percentage of CFU-GM colonies (53±28%; mean±2 x s.e.m.) than $CD34^{+}$ myeloid progenitors also $HCAM^{+}$ (12±9%; $p=0.009$), VLA-4^{+} (4±2%; $p=0.011$), VLA-4^{-} (8±9%; $p=0.007$), or L-$selectin^{-}$ (5±2%; $p=0.014$; Figure 38A and Table Six). No significant difference ($p>0.05$) between blood derived sorted $CAM^{+/-}CD34^{+}$ myeloid progenitors was found in the percentage of total colonies

A. CFU-E colonies



B. BFU-E colonies

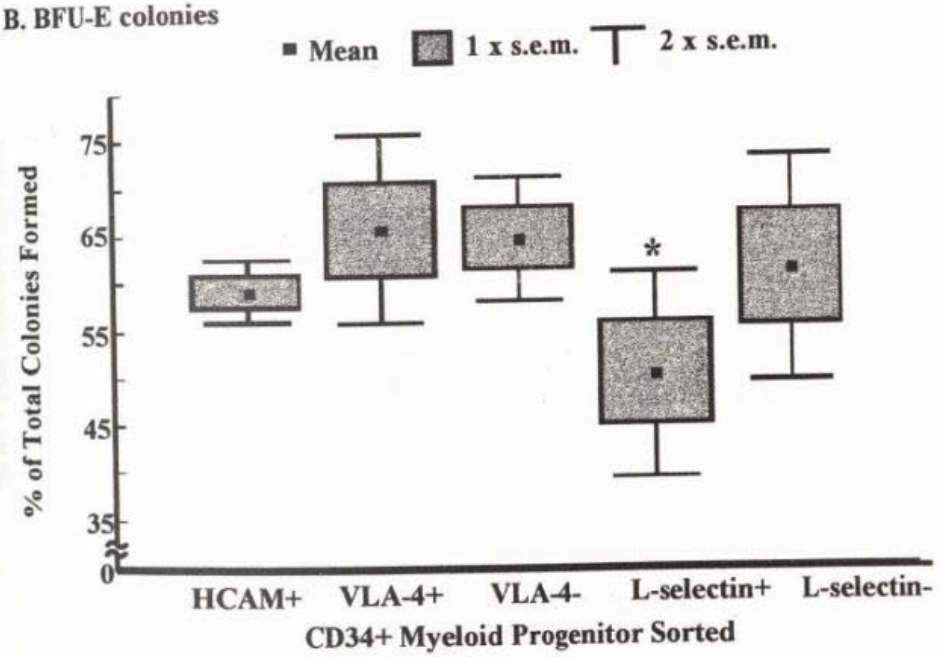


Figure 37. Percentage of CFU-E and BFU-E colonies formed from blood derived CAM+/-CD34+ myeloid progenitors. Flow cytometrically sorted CD34+ myeloid progenitors either expressing the cell adhesion molecule of interest (CAM+) or not (CAM-) were plated into CFU assays (n=20; pooled buffy coat samples). No significant difference between sorted CAM+/-CD34+ myeloid progenitors was found in the percentage of total colonies comprised of CFU-E colonies (A). CD34+ myeloid progenitors coexpressing L-selectin formed a significantly lower ($p=0.002$; indicated by asterisk) percentage of BFU-E colonies than all other sorted CAM+/-CD34+ myeloid progenitors (B).

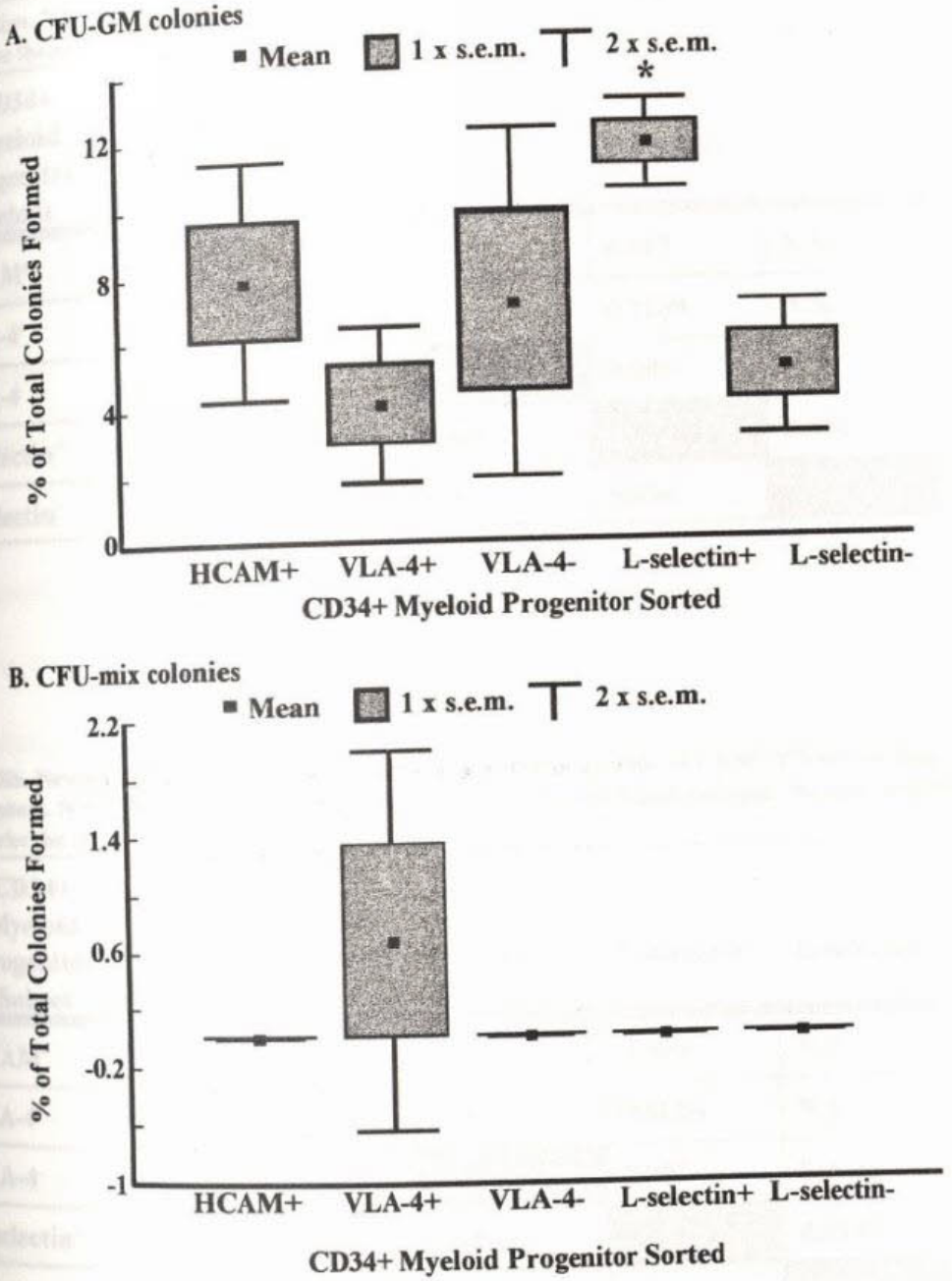


Figure 38. Percentage of CFU-GM and CFU-mix colonies formed from blood derived CAM+/-CD34+ myeloid progenitors. Flow cytometrically sorted CD34+ myeloid progenitors either expressing the cell adhesion molecule of interest (CAM+) or not (CAM-) were plated into CFU assays (n=20; pooled buffy coat samples). CD34+ myeloid progenitors coexpressing L-selectin formed a significantly greater ($p=0.004$; indicated by asterisk) percentage of CFU-GM colonies than all other CAM+/-CD34+ myeloid progenitors sorted (A). No significant difference ($p>0.05$) between sorted CAM+/-CD34+ myeloid progenitors was found in the percentage of total colonies comprised of CFU-mix colonies (B).

Table Five. Newman-Keuls Test of %BFU-E colonies formed by blood CAM$^{+/-}$CD34^{+} myeloid progenitors. N.S. indicates no significant difference ($p>0.05$) was found between the two variables; otherwise the specific p-value is listed.

CD34+ Myeloid Progenitor Subset	HCAM^{+}	VLA-4^{+}	VLA-4-	L-selectin^{+}	L-selectin-
HCAM^{+}		N.S.	N.S.	0.007	N.S.
VLA-4^{+}	N.S.		N.S.	0.0109	N.S.
VLA-4^{-}	N.S.	N.S.		0.005	N.S.
L-selectin^{+}	0.007	0.0109	0.005		0.006
L-selectin^{-}	N.S.	N.S.	N.S.	0.006	

Table Six. Newman-Keuls Test of %CFU-GM colonies formed by blood CAM$^{+/-}$CD34^{+} myeloid progenitors. N.S. indicates no significant difference ($p>0.05$) was found between the two variables; otherwise the specific p-value is listed.

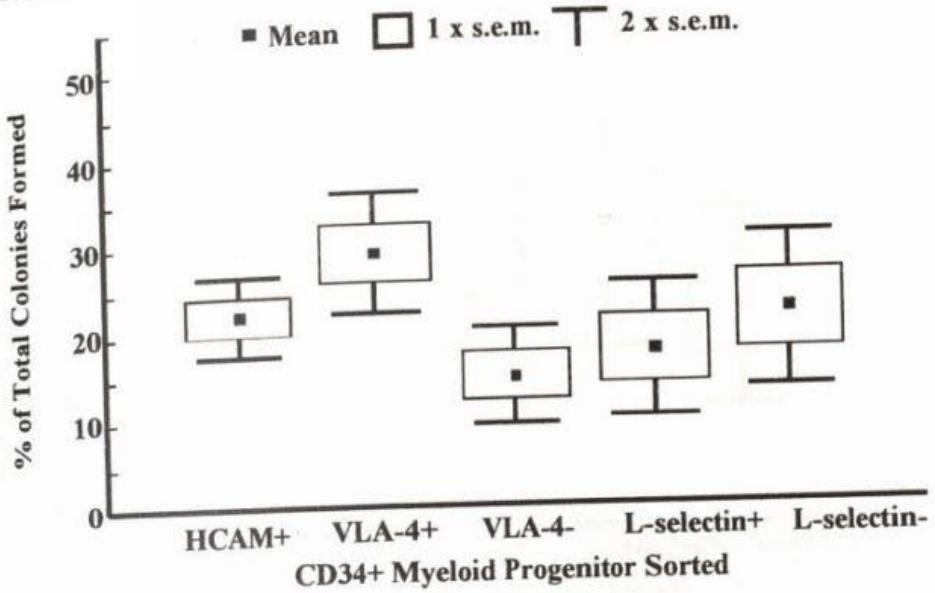
CD34+ Myeloid Progenitor Subset	HCAM^{+}	VLA-4^{+}	VLA-4-	L-selectin^{+}	L-selectin-
HCAM^{+}		N.S.	N.S.	0.009	N.S.
VLA-4^{+}	N.S.		N.S.	0.0118	N.S.
VLA-4^{-}	N.S.	N.S.		0.007	N.S.
L-selectin^{+}	0.009	0.0118	0.007		0.0144
L-selectin^{-}	N.S.	N.S.	N.S.	0.0144	

comprised of CFU-mix colonies (Figure 38B). Analysis of marrow samples revealed no significant differences ($p>0.05$) in the percentage of total colonies comprised of CFU-E, BFU-E, CFU-GM or CFU-mix colonies (Figures 39 and 40).

LTCIC assays of $CAM^{+/-}CD34^{+}$ myeloid progenitor populations from marrow and blood. Nucleated cell fractions of marrow or pooled buffy coat specimens from normal donors were enriched for $CD34^{+}$ cells, labelled with mAbs, and sorted flow cytometrically (Figures 4 and 15). Sorted cells were assayed using the LTCIC assay plated at dilutions of 3, 10, 30, 100, 300 and 1000 cells per well in replicates of 24 wells onto irradiated mouse bone marrow stroma capable of maintaining human hematopoiesis *in vitro* (Figure 20). After 5 weeks of culture, each well was individually harvested and subcultured into CFU assays. After 14 days, those wells containing at minimum of one colony per well were scored as positive. A standard curve of the number of cells plated per well versus percent positive wells was plotted on semi-log paper for each subset and the number of LTCIC per 10^6 sorted $CD34^{+}$ myeloid progenitor cell was determined (Figure 21).

No significant difference ($p>0.05$) in LTCIC enrichment was found between $CAM^{+}CD34^{+}$ myeloid progenitors sorted from blood (n=9; Figure 41A). All subtypes of $CD34^{+}$ myeloid progenitors sorted from normal marrow and plated onto M2-10B4 stroma contained significantly different ($p=0.0027$; ANOVA) enrichments of LTCICs per 10^6 sorted cells (Figure 41B). The Newman-Keuls test was used to determine the specific differences between LTCIC enrichment of sorted marrow derived $CAM^{+}CD34^{+}$ myeloid progenitors (Table Seven).

A. CFU-E colonies



B. BFU-E Colonies

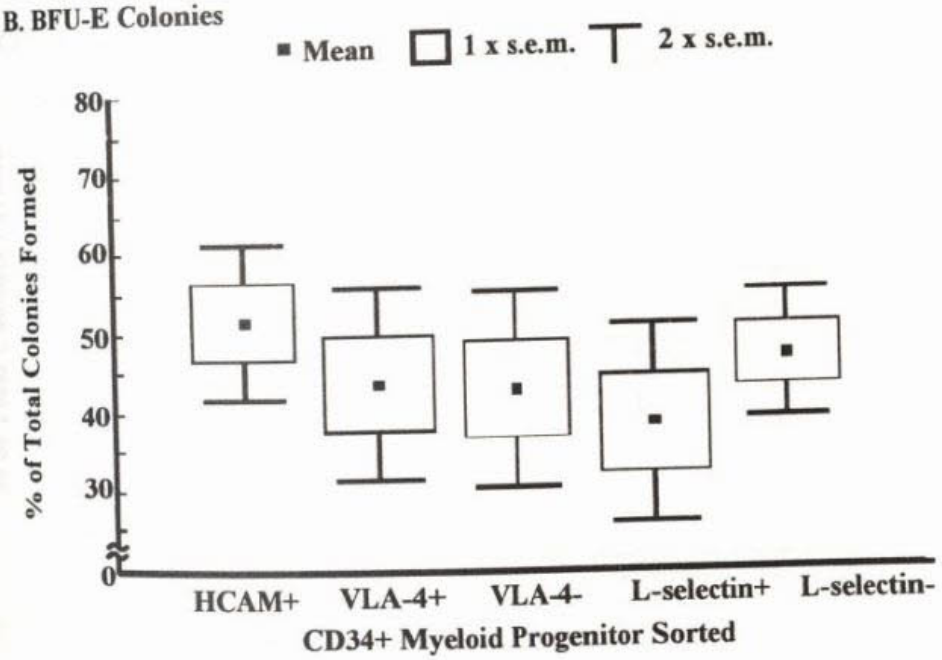


Figure 39. Percentage of CFU-E and BFU-E colonies formed from marrow derived CAM+/-CD34+ myeloid progenitors. Flow cytometrically sorted CD34+ myeloid progenitors either expressing the cell adhesion molecule of interest (CAM+) or not (CAM-) were plated into CFU assays (n=38; normal marrow specimens). No significant difference ($p>0.05$) between sorted CAM+/-CD34+ myeloid progenitors was found in the percentage of total colonies comprised of CFU-E (A) or BFU-E colonies (B).

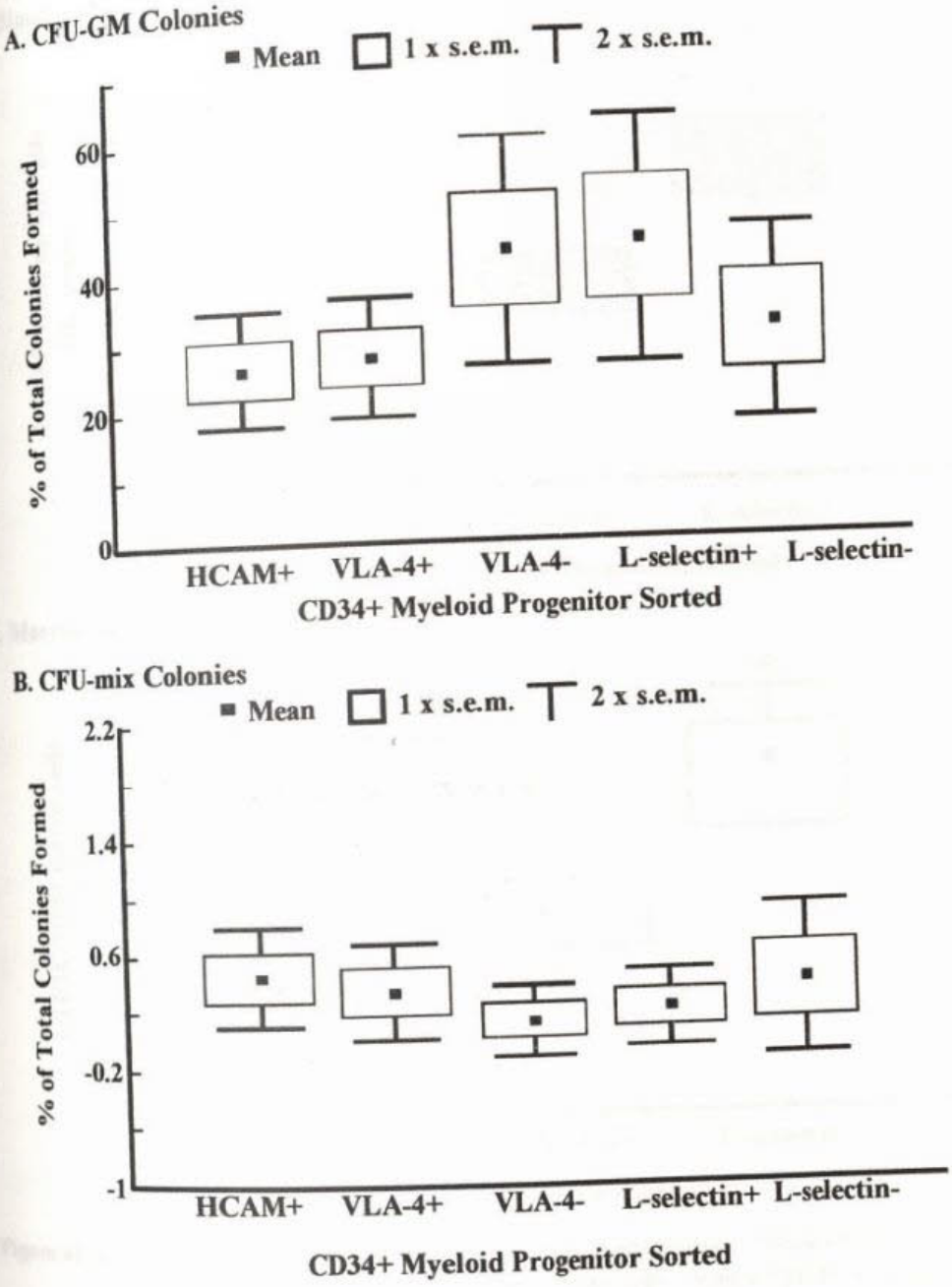


Figure 40. Percentage of CFU-GM and CFU-mix colonies formed from marrow derived CAM+/-CD34+ myeloid progenitors. Flow cytometrically sorted CD34+ myeloid progenitors either expressing the cell adhesion molecule of interest (CAM+) or not (CAM-) were plated into CFU assays (n=38; normal marrow specimens). No significant difference ($p>0.05$) between sorted CAM+/-CD34+ myeloid progenitors was found in the percentage of total colonies comprised of CFU-GM (A) or CFU-mix colonies (B).

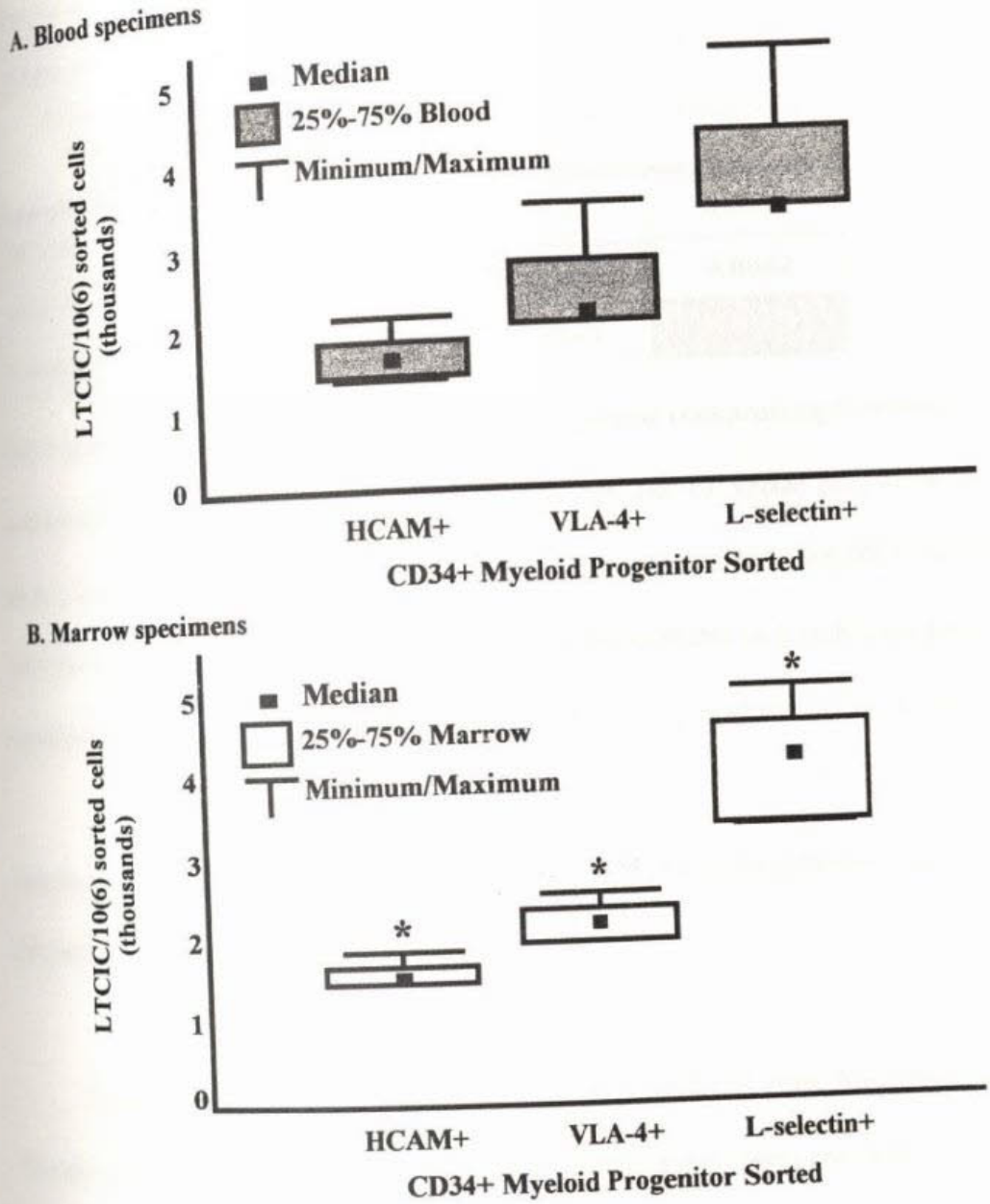


Figure 41. LTCIC content of sorted CAM+CD34+ myeloid progenitors. Blood (A) or marrow (B) from normal donors was enriched for CD34+ cells. Viable CD34+ myeloid progenitors coexpressing HCAM, VLA-4 or L-selectin with CD34 were flow cytometrically sorted at purities greater than or equal to 90% purity and plated into LTCIC assays. After seven weeks of culture on M2-10B4 stroma, the number of LTCIC per 10(6) sorted CAM+CD34+ myeloid progenitor cells plated was determined. No significant difference ($p>0.05$; Kruskall-Wallis ANOVA) in LTCIC content was found between CAM+CD34+ myeloid progenitors sorted from blood (n=9). CAM+CD34+ myeloid progenitors sorted from normal marrow (n=9) were significantly different ($p=0.0027$; ANOVA; indicated by asterisks) from one another.

Table Seven. Newman-Keuls Test of LTCIC enrichment of sorted marrow derived CAM$^+$CD34$^+$ myeloid progenitors. N.S. implies no significant difference ($p>0.05$) was found between the two variables; otherwise the specific p-value is listed.

CD34+ Myeloid Progenitor Subset	HCAM$^+$	VLA-4$^+$	L-selectin$^+$
HCAM$^+$		N.S.	0.0029
VLA-4$^+$	N.S.		0.0044
L-selectin$^+$	0.0029	0.0044	

CD34$^+$ myeloid progenitors derived from normal marrow coexpressing L-selectin contained a significantly greater enrichment of LTCIC per 10^6 sorted cell (41,667±9,642; mean ± 2 x s.e.m.) than those coexpressing HCAM (16,467±2,467; $p=0.0029$) or VLA-4 (22,333±3,697; $p=0.0044$). No significant difference ($p>0.05$) in LTCIC enrichment was found between blood and marrow CAM$^+$ CD34$^+$ myeloid progenitors (Figure 42).

Specific aim 3: demonstration that VLA-4 binds CD34$^+$ myeloid progenitors to fibronectin.

Binding of CD34$^+$ myeloid progenitors to fibronectin in vitro. Nucleated cell fractions of pooled buffy coat specimens from normal donors were enriched for CD34$^+$ cells (Figure 4). Cells were plated (50 x 10^3 cells/well) in replicates of four into four treatment groups. One group was plated onto blank, non-coated wells to serve as nonspecific binding controls. The remaining three treatment groups consisted of cells plated onto FN coated wells which received either buffer alone, mAb against the α4 chain of VLA-4, or an isotype identical mAb not specific for the α4 chain of the VLA-4

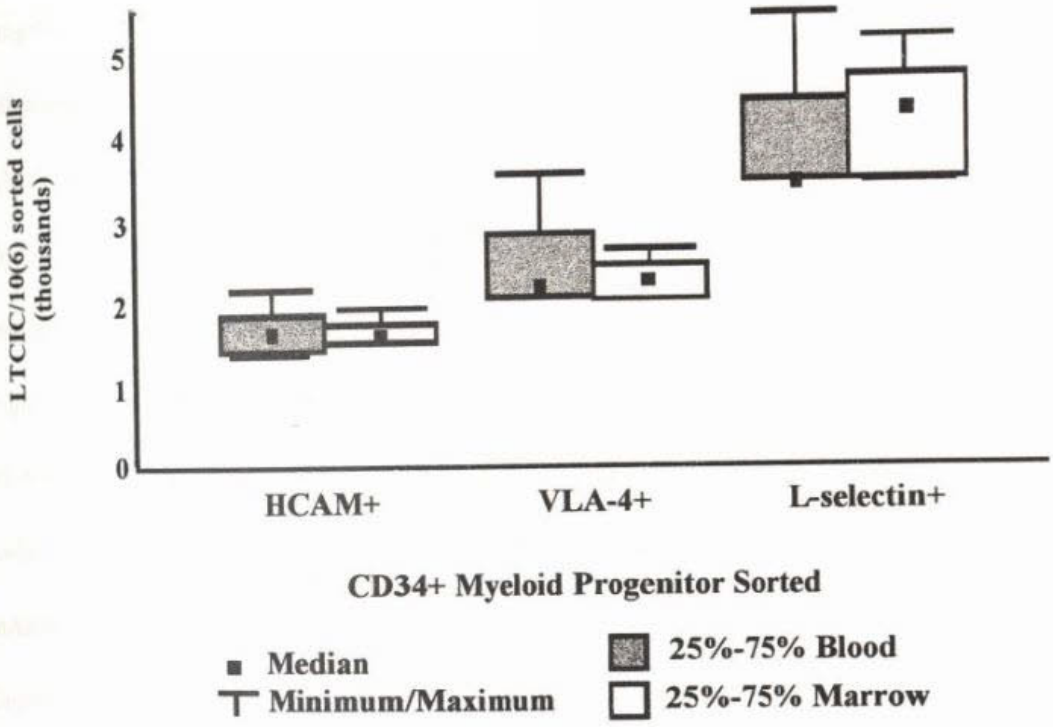
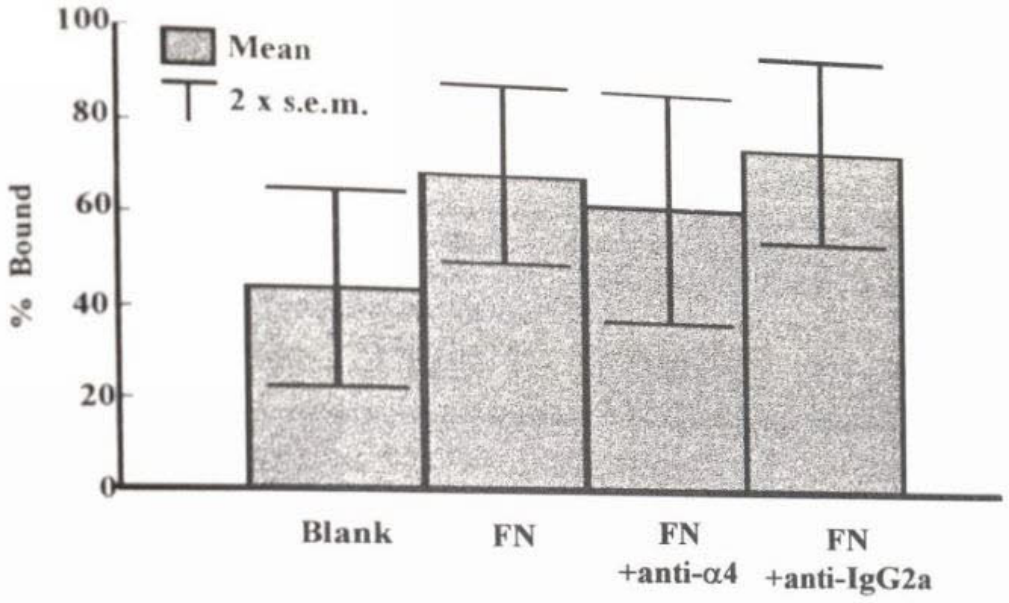


Figure 42. Comparison of LTCIC content between blood and marrow derived CAM+CD34+ myeloid progenitors. Blood (gray) and marrow (white) specimens obtained from normal donors were enriched for CD34+ cells. Viable CD34+ myeloid progenitors coexpressing HCAM, VLA-4 or L-selectin were flow cytometrically sorted at purities greater than or equal to 90% and plated into LTCIC assays. After seven weeks, the number of LTCIC per 10(6) sorted CAM+CD34+ myeloid progenitor cells plated was determined. No significant difference ($p>0.05$) in LTCIC enrichment was found between blood (gray; n=9) and marrow (white; n=9) CAM+CD34+ myeloid progenitors.

heterodimer. After stimulating adhesion to FN at 37°C, non-adherent and adherent cells were harvested, quantitated and labelled for flow cytometric analysis of CD34, CD45 and VLA-4 expression. The percentage of $CD34^+$ or VLA-4^+CD34^+ myeloid progenitors bound in each replicate was determined.

No significant difference ($p>0.05$) was found in the percentage of $CD34^+$ myeloid progenitors bound to FN in each of the four treatment groups (Figure 43A). The adhesion of VLA-4^+CD34^+ myeloid progenitors was significantly different ($p=0.0002$, ANOVA; Figure 43B). Post hoc comparisons to determine specific differences between the binding of VLA-4^+CD34^+ myeloid progenitors in each treatment group (and their respective p-values; Newman-Keuls Test) were performed and are summarized in Table Eight. A significantly greater ($p=0.0005$) percentage (mean ± 2 x s.e.m.) of VLA-4^+CD34^+ myeloid progenitors bound to FN coated wells than to blank, noncoated wells (41±8%) regardless of the addition of buffer alone, (71±12%, $p=0.0005$), anti-α4 mAb (55±8%, $p=0.0403$), or anti-IgG2a (71±8%, $p=0.0008$; Figure 43B and Table Eight). There was no significant difference ($p>0.05$) between the percentage of VLA-4^+CD34^+ myeloid progenitors bound to FN coated wells treated with buffer alone (71±12%) and those treated with anti-IgG2a (71±8%). The difference between the percentage of VLA-4^+CD34^+ myeloid progenitors bound to FN coated wells treated with anti-IgG2a (71±8%) and those treated with anti-α4 mAb (55±8%) approached significance ($p=0.0528$). The addition of mAb against the α4 chain of VLA-4 significantly ($p=0.0249$) reduced the binding of VLA-4^+CD34^+ myeloid progenitors to FN by an average of 16% (Figure 43B and Table Eight). Analysis of

A. Viable CD34+ Myeloid Progenitors



B. Viable VLA-4+CD34+ Myeloid Progenitors

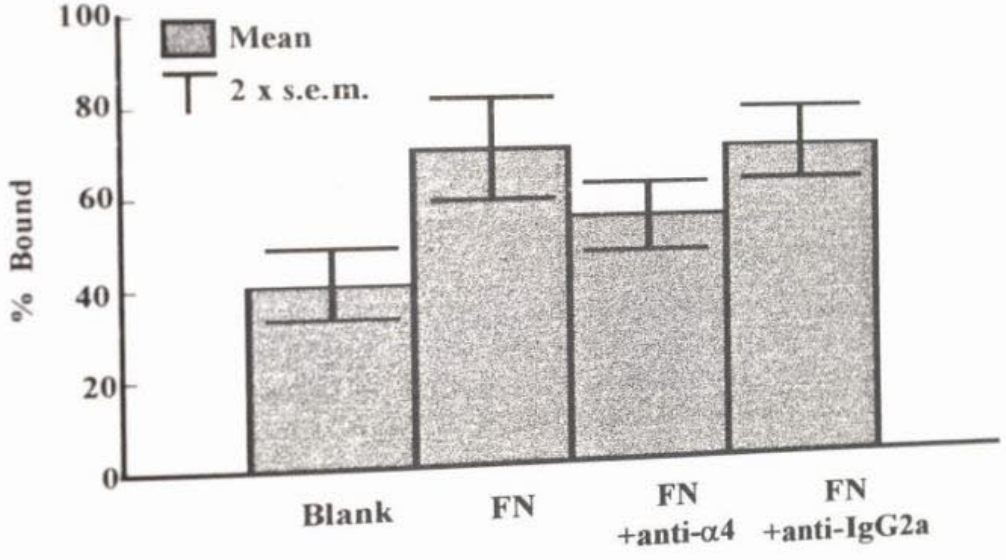


Figure 43. Results of adhesion assay. CD34+ cell enriched fractions from pooled buffy coat samples were plated into wells of microtiter plates (replicates of four; n=6) into one of the following well treatment groups: uncoated (Blank), coated with fibronectin alone (FN), FN and mAbs against the α4 chain of the VLA-4 heterodimer (FN+anti-α4), or FN and an isotype identical mAb (FN+anti-IgG2a). (A) Adhesion of viable CD34+ myeloid progenitors did not significantly differ ($p>0.05$) between treatment groups. (B) Adhesion of viable VLA-4+CD34+ myeloid progenitors was significantly different ($p=0.0002$) as revealed by ANOVA. Intergroup differences shown in Table Eight.

VLA-4$^-$CD34$^+$ myeloid progenitor adhesion was not possible because this cell type was a minority of the total CD34$^+$ cells (see Figure 23) and the number of VLA-4$^-$CD34$^+$ myeloid progenitor cells recoverable from the adhesion assay was highly variable.

Table Eight. Newman-Keuls Test of the percentage of VLA-4$^+$CD34$^+$ myeloid progenitors bound. CD34$^+$ cell enriched fractions of pooled buffy coats were plated into adhesion assays. The adhesion of VLA-4$^+$CD34$^+$ myeloid progenitors was assessed in uncoated wells (Blank); and FN coated wells with buffer, anti-α4, or anti-IgG2a. The specific p-values are listed.

Well Treatment	Blank	FN coated	FN coated + anti-α4	FN coated + anti-IgG2a
Blank		0.0005	0.0403	0.0008
FN coated	0.0005		0.0249	0.9391
FN coated + anti-α4	0.0403	0.0249		0.0528
FN coated + anti-IgG2a	0.0008	0.9391	0.0528	

DISCUSSION

Hematopoietic stem cell transplantation first became a feasible clinical therapy for the treatment of hematopathologies in the late 1960's with the advent of several successful allogeneic transplants in pediatric patients with immunodeficiencies.[18-20] Since that time, hematopoietic stem cell transplantation has been applied to a variety of hematologic as well as nonhematologic malignancies, benign pathologies and genetically determined diseases.[16]

In spite of the many advances in the field of stem cell transplant therapy, many problems remain. Two specific problems are efficiency and quantity of hematopoietic stem cells mobilized into the peripheral circulation for collection prior to transplant and the role of stem cell homing to the marrow during post-transplant hematopoietic recovery. Understanding the role that cell adhesion molecules play in stem cell peripheralization and homing could lead to the development of enhanced stem cell collection protocols and post-transplant engraftment rates, respectively.

This study was designed to address the hypothesis that modulation of the cell adhesion molecules HCAM, VLA-4 and L-selectin play a role in regulating hematopoietic progenitor cell trafficking between the marrow and the peripheral circulation. This hypothesized role for HCAM, VLA-4 and L-selectin in HPC trafficking

predicts that 1) differences in the surface expression of CAMs (HCAM, VLA-4, or L-selectin) are detectable between marrow and blood specimens, 2) both high and low CAM expressing $CD34^+$ cells can be functionally demonstrated to be hematopoietic progenitors and 3) VLA-4 mediates the adhesion of $CD34^+$ cells to fibronectin, a component of the hematopoietic microenvironment.

Specific aim 1: differential expression of CAMs between marrow and blood

In order to test the prediction that hematopoietic progenitors will differentially express cell adhesion molecules as a function of their anatomic derivation, samples of marrow and blood $CD34^+$ myeloid progenitors from the same individual were examined for differences in expression of CAMs. The CAMs examined, homing-associated cell adhesion molecule, very late antigen-4 and L-selectin, were selected because of established or potential importance on hematopoietic stem cells.[34-38,41-44]

Half of the paired samples were obtained from patients treated with mobilizing doses of granulocyte-colony stimulating factor (G-CSF) from whom bone marrow was being collected for autologous transplant. All of these patients had breast carcinoma and no current or previous histological evidence of disease in their marrow. The remainder of the paired samples were obtained from hematologically normal individuals donating bone marrow for allogeneic transplant. The nucleated cell fraction of each specimen was

isolated and enriched for $CD34^+$ cells. $CD34^+$ enriched specimens were analyzed via flow cytometry in conjunction with standards of antibody binding in order to determine the amount of antibody bound per cell.

In normal individuals, marrow $CD34^+$ myeloid progenitor cells bound more anti-CD34 per cell than those in the blood, suggesting a greater expression of CD34 on HPCs residing in the marrow than those in circulation. Although the function of CD34 is not known, studies have shown that as myeloid progenitors mature their expression of CD34 decreases suggesting an inverse relationship between CD34 expression and HPC maturation.[65,66] Since marrow derived HPCs expressed more CD34 per cell than those which have entered the circulation, one is tempted to speculate that marrow myeloid progenitors are less mature, that is have greater hematopoietic potential, than circulating myeloid progenitors. Assessment of *in vitro* hematopoietic potential (investigated in execution of specific aim two) demonstrated that blood derived HPCs rather than those derived from marrow had the greatest short-term hematopoietic potential. No significant difference in long-term hematopoietic potential was found between blood and marrow. These findings illustrate that physiologic compartment may play a more critical role in characterization of HPC maturation state than CD34 expression.

In normal individuals, no significant quantitative difference was found between blood and marrow in terms of HCAM, VLA-4 or L-selectin expression per $CD34^+$ myeloid progenitor cell. Lack of a significant quantitative differences in CAM expression suggests that modulation of expression during marrow ingress and egress during steady-state hematopoiesis involves alterations in CAM ligand affinity/avidity

rather than quantitative decreases in surface expression. Analysis of CAM ligand affinity/avidity interactions on marrow versus blood derived HPCs would provide insight into this issue.

In G-CSF mobilized patients, marrow derived $CD34^+$ myeloid progenitors expressed more VLA-4 antigens per cell than those in circulation, a finding which is consistent with the hypothesis that a quantitative difference in the expression of VLA-4 exists between HPCs residing in the marrow and those in circulation. These data are consistent with the hypothesis that down-regulation or masking of VLA-4 expression is involved in the release of HPCs from the marrow microenvironment into the peripheral circulation.

No significant quantitative differences in HCAM or L-selectin expression between the blood and marrow $CD34^+$ myeloid progenitors from G-CSF mobilized individuals were detected. These findings suggest that during G-CSF mobilization down-regulation of surface expression of HCAM or L-selectin does not play a direct role in egress from the marrow into the peripheral circulation. Thus, as in the case of steady-state hematopoiesis, the lack of significant quantitative differences in HCAM or L-selectin expression between G-CSF mobilized blood and marrow suggests that alterations in HCAM or L-selectin avidity and/or affinity might play a more critical role in HPC trafficking than quantitative changes in expression. Previously reported studies of HCAM expression on circulating mature lymphocytes and L-selectin expression on HPCs have suggested that these CAMs might play a key role in HPC homing.[30,38]

Analysis of HCAM and L-selectin mediated adhesion of HPCs to marrow endothelium under nonstatic conditions would provide insight into the role of these CAMs in HPC homing.

The above conclusions were based on experiments in which the precise molecular characteristics of the epitopes recognized by the mAbs, the number of epitopes per antigen and the mechanism of mAb binding to antigen were not known. The binding of mAbs to structurally repeated motifs, such as the α-helices and β-pleated sheets of L-selectin, could have resulted in more than one mAb binding to each antigen.[41,63,64] If this had occurred then the antigen binding observed flow cytometrically would have reflected a greater number of antigens present than actually occurring in nature. Although the amount of mAb bound to each cell would be linearly proportional to the number of antigens present, care must be taken in the interpretation of the number of CAMs present per cell. The QSC bead assay was utilized to demonstrate differences between blood and marrow per cell expression in terms of which cell type expressed more or less of the CAM under examination. Thus, this assay allows for relevant comparisons of antigenic expression rather than determination of exact number of antigens present per cell.

In addition to the limitations of the QSC bead assay, redistribution of antigens (patching, capping and pinocytosis of antibody-antigen complexes on the cell surface) could have adversely affected detection of antibody during flow cytometric analysis. In order to prohibit the redistribution of antigens on the cell surface and to minimize conformational changes of antigen, all experiments were carried out at 4°C. In addition, all mAbs utilized in these experiments contained sodium azide, a metabolic inhibitor.

Had these precautions not been employed, flow cytometric analysis of CAM and CD34 expression per myeloid progenitor cell might have estimated cell surface expression to be much lower than that present in nature.

To summarize the above data and conclusions regarding specific aim one, G-CSF mobilized marrow $CD34^+$ myeloid progenitor cells expressed more VLA-4 antigen than their circulating counterparts. These findings support the hypothesis that modulation of VLA-4 plays an important role in HPC peripheralization, namely during G-CSF induced HPCs egress from the marrow microenvironment. The lack of detectable quantitative differences in HCAM and L-selectin expression in G-CSF mobilized or normal individuals does not rule out the possibility that alterations in affinity and/or avidity of these CAMs for their ligands as a mechanism for HPC peripheralization and homing. The finding that normal marrow $CD34^+$ myeloid progenitor cells expressed more CD34 than those derived from blood suggested that marrow HPCs are less mature than those in the blood; however, subsequent experiments (specific aim two) refuted this possibility. These data are consistent with the hypothesis stated in specific aim one that hematopoietic progenitor cells differentially express the cell adhesion molecules only with regard to quantitative changes in VLA-4 expression. Quantitative changes in HCAM and L-selectin expression were not observed between the marrow and blood of either normal or G-CSF mobilized individuals suggesting that other mechanisms of modulating CAM function might be playing a role in HPC trafficking.

Specific aim 2: clonogenic potential and long term culture initiating cell content of marrow and blood

To test the prediction that both high and low CAM expressing $CD34^+$ cells can be functionally demonstrated to be hematopoietic progenitors, nucleated cell fractions of marrow or pooled buffy coat specimens from normal donors were enriched for $CD34^+$ cells. The specimens were labeled with mAb against CD34 and one of the following cell adhesion molecules: HCAM, VLA-4 or L-selectin. The labeled samples were analyzed and sorted flow cytometrically for $CAM^{+/-}CD34^+$ myeloid progenitors. Post-sort purities of ≥90% $CAM^{+/-}CD34^+$ myeloid progenitors were used to plate colony forming unit assays to assess short-term hematopoietic potential.

$CAM^{+/-}CD34^+$ myeloid progenitors sorted from either blood or marrow produced hematopoietic colonies *in vitro*. These findings are consistent with the prediction that each of the $CAM^{+/-}CD34^+$ myeloid progenitor populations examined can be functionally demonstrated to be hematopoietic progenitors with regard to establishment of short-term hematopoiesis *in vitro*.

Circulating $CAM^{+/-}CD34^+$ (i.e., $HCAM^+$, VLA-$4^{+/-}$ or L-$selectin^{+/-}$) myeloid progenitors produced a greater total number of colonies than those derived from marrow. The greatest total number of colonies were formed from blood derived $CD34^+$ myeloid progenitors also $HCAM^+$; followed by L-$selectin^-$, VLA-4^-, VLA-4^+ or L-$selectin^+$ subtypes. Since CAM^-CD34^+ myeloid progenitors formed more colonies than those expressing VLA-4 or L-selectin, this data suggests that VLA-4 and L-selectin expression

might not play an important role in terms of short-term hemopoietic reconstitution but does not rule out the possibility that VLA-4 or L-selectin function in a secondary fashion to enable HPCs to migrate to the appropriate physiologic compartment. The CFU assay data opposes the earlier supposition (specific aim one) that circulating myeloid progenitors are more mature than their marrow counterparts and suggests that CD34 expression does not solely predict hematopoietic potential. Parameters such as physiologic compartment (blood versus marrow) and the coexpression of other markers (e.g., cell adhesion molecules and/or lineage specific markers) may play a key role in defining which specific HPC subtype is a true stem cell.

Analysis of blood derived myeloid progenitors revealed those expressing VLA-4 and CD34 produced a smaller percentage of CFU-GM colonies than their marrow counterparts. These findings support the hypothesis that as myeloid progenitors mature, as evidenced by a decrease in their ability to form CFU-GM colonies, egress into the peripheral circulation might, in part, occur through alterations in ability to bind fibronectin.

The distribution of colonies types formed from blood derived $CD34^+$ myeloid progenitors was significantly affected by L-selectin expression, while the distribution of colony types formed by all subtypes sorted from marrow were not significantly affected. $CD34^+$ myeloid progenitors expressing L-selectin produced a significantly smaller percentage of BFU-E colonies and a concomitantly higher percentage of CFU-GM colonies. These findings suggest that L-selectin plays a role in establishment of

granulocytic and monocytic lineages. Whether L-selectin expression on HPCs results in enhanced establishment of granulocytic and monocytic lineages post-transplantation requires further investigation.

To test whether high and low CAM expressing $CD34^+$ cells were functionally demonstrative of long term hematopoiesis, sorted CAM^+CD34^+ myeloid progenitor populations from marrow and blood of ≥90% purities were plated into long-term culture initiating cell assays. All sorted CAM^+CD34^+ myeloid progenitor populations formed CFU colonies after 5 weeks of long term bone marrow culture. These findings are consistent with the prediction that each of the $CAM^{+/-}CD34^+$ myeloid progenitor populations examined can be functionally demonstrated to be hematopoietic progenitors with regard to establishment of long-term hematopoiesis *in vitro*.

Marrow derived L-$selectin^+CD34^+$ myeloid progenitor cells contained the greatest enrichment of LTCIC per sorted $CD34^+$ myeloid progenitor cell, suggesting that L-$selectin^+CD34^+$ cells have the greatest long term hematopoietic potential of the $CAM^{+/-}$ $CD34^+$ myeloid progenitor cells examined. This finding suggests that L-selectin might play an important role in establishment of long term hematopoiesis.

To summarize the above findings concerning the short-term and long-term hematopoietic potential of HPCs expressing HCAM, VLA-4 or L-selectin, circulating $CAM^{+/-}CD34^+$ myeloid progenitors have greater short-term hematopoietic potential than their counterparts residing in the marrow microenvironment. Marrow derived L-$selectin^+CD34^+$ myeloid progenitors contained the greatest enrichment of LTCICs, indicative of a functional stem cell, followed by those expressing HCAM and VLA-4.

Blood derived $CD34^+$ myeloid progenitors were not significantly enriched for stem cell content on the basis of CAM coexpression. These findings suggest that L-selectin$^+$ $CD34^+$ cells derived from marrow correspond with a less mature cell type. HCAM or VLA-4 expression provides no insight into the long-term hematopoietic potential of $CD34^+$ myeloid progenitors regardless of stem cell source. These findings expand the prediction that both high and low CAM expressing $CD34^+$ cells can be functionally demonstrated to be hematopoietic progenitors to suggest a role for L-selectin as a marker of a less mature hematopoietic cell type than HPCs expressing CD34 alone.

Specific aim 3: demonstration that VLA-4 binds $CD34^+$ myeloid progenitors to fibronectin.

Mature hematopoietic cells, such as monocytes, T cells and B cells, bind to the CS-1 domain of FN via the $\alpha 4$ chain of VLA-4.[67] To test the prediction that VLA-4 expressed on the surface of hematopoietic progenitors mediates binding to fibronectin, nucleated cell fractions of pooled buffy coat specimens from normal donors were enriched for $CD34^+$ cells. Cells were plated into adhesion assays in which $\alpha 4$ mediated binding of VLA-4 to FN was blocked with monoclonal antibodies. The percentage of bound $CD34^+$ or VLA-4$^+$$CD34^+$ myeloid progenitors was determined flow cytometrically.

In vitro adhesion assays revealed that mAb blockage of the $\alpha 4$ chain of VLA-4 significantly reduced the binding of VLA-4$^+$$CD34^+$ myeloid progenitors to FN by 16%

on average when compared to treatment with buffer alone. These findings are consistent with the prediction that VLA-4 mediates the binding of myeloid progenitors to FN.

It is unclear why on average 29% of the VLA-4$^+$CD34$^+$ myeloid progenitors failed to bind FN in these experiments (Figure 43B). It is known that marrow derived CD34$^+$ hematopoietic progenitors and hematopoietic cell lines express ß1 integrins (VLA-4 and VLA-5) in a low affinity state; that is, in a weakly adhesive phenotype to FN.[68] Upon stimulation with cytokines, interleukin-3 or GM-CSF, the binding of these cell types to FN increases in a dose dependent manner suggesting that cytokines specifically stimulate ß1 integrin function.[68] Perhaps, just as their marrow counterparts, blood derived CD34$^+$ myeloid progenitors require cytokine activation of VLA-4 in order to fully bind FN. Elucidation of the role of cytokine activation of VLA-4 and other ß1 integrins on HPCs warrants further study.

The adhesion assay contained intrinsic limitations. Adhesion of VLA-4$^+$CD34$^+$ myeloid progenitors to FN when treated with anti-α4 mAb when compared to those treated with isotype identical control approached but did not reach statistical significance ($p=0.0528$). Since the data presented herein were based on six experiments, a larger sample size might have resolved this issue. The nonspecific binding of 40% of VLA-4$^+$CD34$^+$ myeloid progenitors to the Nunc microtiter plates was not affected by blocking with human and bovine serum albumin. Considering these limitations, the adhesion assay requires further development in order to draw more conclusive inferences concerning VLA-4 mediated adhesion of HPCs to FN.

Based on current literature concerning CAMs and HPCs, a model for HPC trafficking between the marrow microenvironment and the blood was developed. This model suggested that HPCs utilize the cell adhesion molecules HCAM, VLA-4 and L-selectin to peripheralize from and home to the marrow microenvironment (Figure 1). According to this model, HPCs could utilize HCAM to adhere to components of the marrow microenvironment such as collagens and hyaluronic acid. HPCs could utilize VLA-4 to adhere to fibronectin, a component of the marrow microenvironment, and VCAM-1 expressing stromal cells. Peripheralization of HPCs could occur through a quantitative reduction in HCAM or VLA-4 surface expression and/or reduced affinity of these receptors for their respective ligands. Homing of circulating HPCs to the marrow microenvironment could occur via L-selectin mediated binding to marrow endothelium expressing vascular addressin or HCAM mediated binding to marrow endothelium. Thus HPC modulation of HCAM, VLA-4 and L-selectin could facilitate marrow ingress (via L-selectin or HCAM mediated homing) and marrow egress (via down regulation/masking of HCAM and VLA-4).

If this model of HPC trafficking accurately reflects the mechanics of HPC migration between the marrow microenvironment and the peripheral circulation, then HCAM, VLA-4 and L-selectin should have been differentially expressed between these anatomic compartments. The experiments presented herein demonstrated that VLA-4 but not L-selectin or HCAM are differentially expressed between circulating HPCs and those

residing in the marrow. VLA-4 expression was significantly decreased on circulating HPCs relative to those in the marrow in G-CSF mobilized individuals. These findings suggest that HPC peripheralization, at least in the context of G-CSF mobilization, involves the down regulation or masking of VLA-4 expression. The mechanism by which G-CSF mobilization affects VLA-4 mediated adhesion to fibronectin and/or VCAM-1 is not known. The experiments presented herein demonstrated no quantitative difference in per cell expression of HCAM or L-selectin suggesting that quantitative changes in expression of these CAMs might play a lesser role in HPC trafficking than changes in ligand binding affinity and/or avidity.

VLA- This model also predicts that HPCs expressing either HCAM, VLA-4 or L-selectin are functionally capable of establishing hematopoiesis. The results of the CFU and LTCIC assays confirmed this prediction of the model. L-selectin expression on HPCs was associated with an increased ability to restore granulocytic and monocytic lineages (CFU assays) and the greatest enrichment of stem cells (LTCIC assays) when compared to all other examined subtypes of $CD34^+$ myeloid progenitors. These findings suggest that in addition to mediating homing to the marrow post-transplantation, as suggested by the literature, L-selectin may be regarded as a marker of a less mature $CD34^+$ HPC cell type possessing substantial long-term hematopoietic reconstituting capabilities.

L-selc- If the model of HPC trafficking based on current literature accurately reflects the mechanics of HPC migration between the marrow microenvironment and the peripheral circulation, then blockage of VLA-4 mediated binding to FN should have resulted in

decreased adhesion of HPCs to fibronectin. This model was supported by results from the *in vitro* adhesion assays performed on HPCs from normal individuals which demonstrated that blood derived HPCs bound to fibronectin. Whether VLA-4 alone or in combination with other CAMs present on the surface of HPCs mediated this interaction is not clear. The observation that binding could be significantly decreased by blocking the FN binding site of VLA-4 with mAbs is consistent with the model of HPC trafficking suggested by the literature. However, the discovery that almost a third of the HPCs expressing VLA-4 did not bind to fibronectin requires modification of this model. Perhaps in addition to quantitative modulations in expression per cell, alterations in VLA-4 binding affinity for FN plays a significant role in HPC peripheralization.

The findings presented herein have resulted in modification of the model suggested by the current literature concerning the role of HCAM, VLA-4 and L-selectin in HPC trafficking (Figure 44). The proposed model of HPC trafficking suggests that quantitative changes in VLA-4 expression in addition to alterations in ligand affinity/avidity interactions plays a role in HPC egress from the marrow microenvironment. In addition this model proposes that affinity/avidity interactions rather than quantitative changes in surface expression govern L-selectin mediated homing and HCAM mediated homing/peripheralization. This finding opposes the model suggested by the literature which indicated quantitative changes in surface expression of L-selectin and HCAM play a role in HPC trafficking.

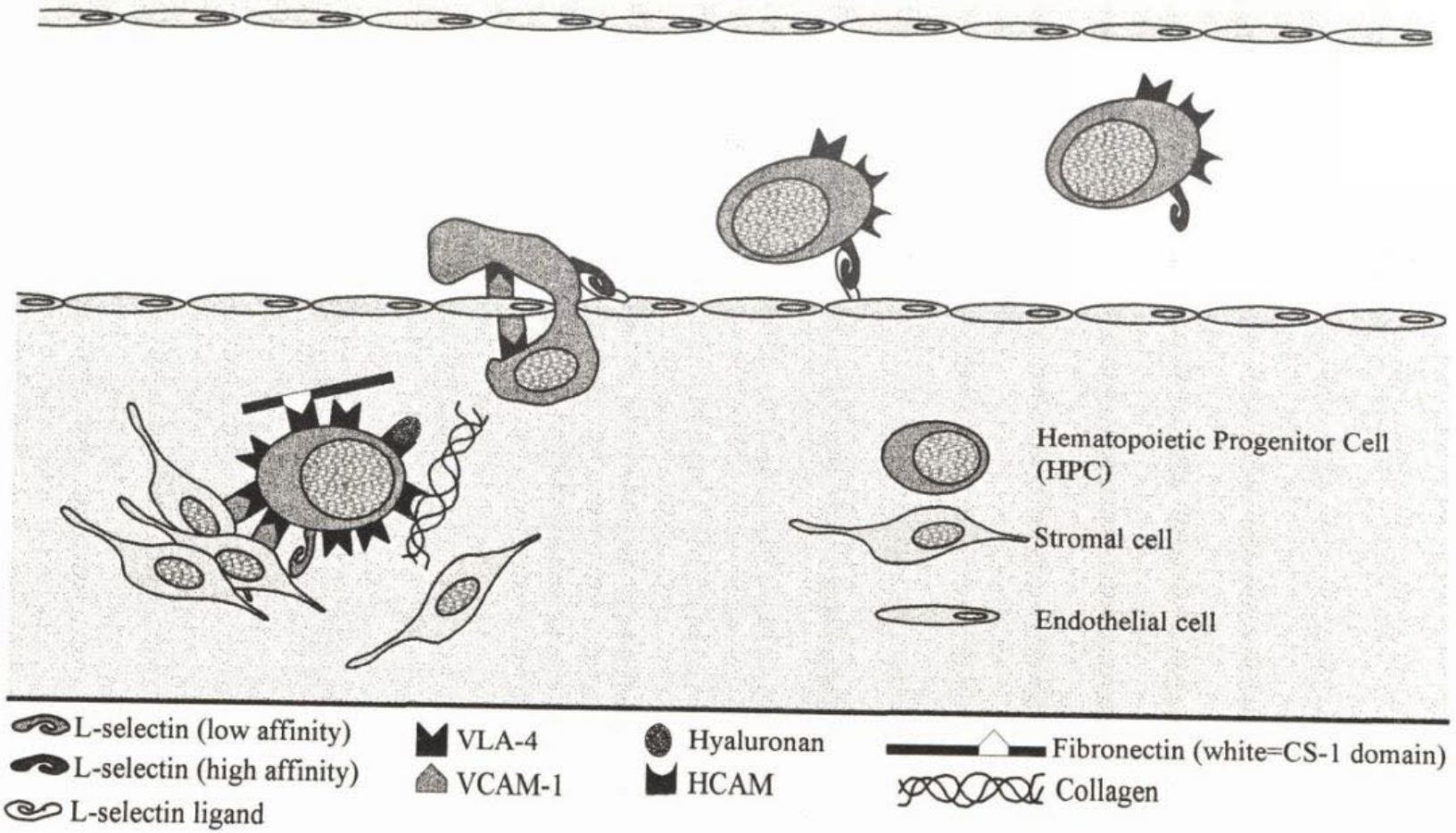


Figure 44. Proposed model of cell adhesion molecules in hematopoietic progenitor cell trafficking.

To summarize the proposed model of the role of HCAM, VLA-4 and L-selectin in HPC trafficking, peripheralization of HPCs can be promoted by quantitative decreases in surface expression of VLA-4. Thus HPCs may enter the vasculature by virtue of their decreased ability to bind components of the marrow microenvironment, namely fibronectin and VCAM-1 expressing stromal cells. Peripheralization does not appear to involve quantitative decreases in HCAM expression per HPC but instead involves alterations in HCAM affinity for hyaluronan and collagen. With regard to HPC homing to the marrow microenvironment, modulation of L-selectin and/or HCAM affinity rather than quantitative changes in surface expression most likely plays an important role in HPC homing to marrow endothelial cells. Once bound to endothelium by L-selectin, VLA-4 mediated adhesion to endothelial cells expressing VCAM-1 may enable HPC transmigration into the marrow microenvironment. Thus, these studies implicate the cell adhesion molecule VLA-4 and L-selectin as possessing an important role in HPC trafficking, while the role of HCAM in this complex process remains speculative

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APPENDICES

APPENDIX 1. PRINCIPLES OF DENSITY GRADIENT CENTRIFUGATION

The following principles of density gradient centrifugation were outlined by Boyum in the late 1960's apply for both Ficoll-Paque® Plus and Polymorphprep.[45] Anticoagulant treated blood is layered on the density gradient solution and centrifuged for a short period of time. Differential migration during centrifugation results in the formation of layers containing different cell types (Figure 4). The bottom layer contains erythrocytes which have been aggregated by the gradient and, therefore, sediment completely through the gradient. The layer immediately above the erythrocyte layer contains mostly granulocytes which at the osmotic pressure of the gradient solution attain a density great enough to migrate through the gradient layer.

Because of their lower density, the lymphocytes are found at the interface between the plasma and the gradient with other slowly sedimenting particles (platelets and monocytes). The lymphocytes are then recovered from the interface and subjected to a shorter washing step with a balanced salt solution to remove any platelets, gradient and plasma.

APPENDIX 2. MORPHOLOGIC CHARACTERIZATION OF HEMATOLOGIC CELL TYPES

The following morphologic characteristics were utilized in the differentiation of specific hematologic cell types within all cytocentrifuged slide preparations stained with Leukostat:

Myeloblast - (diameter=15-20 μm) moderate, bluish, unevenly stained nongranular cytoplasm which is lighter next to the nucleus than at the periphery. Nucleus is round and stains predominantly red. Two or more nucleoli are usually demonstrable.

Promyelocyte - (diameter=15-20 μm) cytoplasm same coloration as myeloblast except distinct dark-blue or reddish-blue granules of variable shapes are present. Nucleus is round and large relative to cytoplasm. Nucleoli may be visible but are usually indistinct.

Myelocyte, neutrophilic - Small islands of reddish granules present in cytoplasm adjacent to nucleus. Nuclei are round, oval or flattened on one side. Nucleoli are indistinct.

Metamyelocyte, neutrophilic - Slightly smaller than myelocytes. Small, pinkish-blue granules present in cytoplasm. Nucleus slightly indented.

Band neutrophil - Slightly smaller than metamyelocytes. Cytoplasmic granules are small, evenly distributed and stain various shades of pink and blue. The opposite edges of the nucleus become approximately parallel for an appreciable distance giving a horseshoe appearance.

Segmented neutrophil - Similar size to neutrophilic band. Cytoplasmic granules present as described for band neutrophil. Nucleus is separated into definite lobes with a very-narrow filament or strand connecting the lobes.

Eosinophil - Size comparable to segmented neutrophil. Cytoplasm contains large, spherical granules which stain red.

Basophil - Size comparable to segmented neutrophil. Cytoplasm contains large, spherical granules which stain deep purplish-blue to dark purple-red.

Monocyte - Slightly larger than neutrophils with their diameters 3-4 times those of erythrocytes in the same microscopic field. Dull gray-blue variably shaped cytoplasm (most are round or oval shaped) with blunt pseudopods. Nucleus has brain like convolutions.

Lymphocyte - (diameter=7-10 μm) Blue, nongranular cytoplasm with large nucleus in relation to cytoplasm. Nucleus size is comparable to diameter of normal erythrocytes. No visible nucleoli.

APPENDIX 2. MORPHOLOGIC CHARACTERIZATION OF HEMATOLOGIC CELL TYPES (CONTINUED)

Plasmocyte - (diameter=15-25 μm) Nongranular, translucent, dark-blue cytoplasm usually has round or oval shape with smooth or slightly irregular margins with distinct perinuclear clear zone. Nuclei are small, oval, round and eccentric. Nuclear chromatin is coarse and lumpy.

Pronormoblast - Visible nucleoli. Chromatin strand are linear and distinct. Cytoplasm stains light blue to dark, royal-blue color (similar to that seen in plasmocytes).

Basophilic normoblast - Smaller than pronormoblast. Cytoplasm has reddish tinge but predominant color is blue. Distinguishable from pronormoblast by the coarsening of the chromatin pattern and ill-defined or absent nucleoli.

Polychromatic normoblast - smaller than normoblast, have relatively more cytoplasm. Nuclear chromatin is thickened and irregularly condensed. Nucleoli are not visible.

Orthochromatic normoblast - Cytoplasm is predominantly red with minimal amount of residual blue. Nucleus is small and has a nonlinear clumped chromatin structure or a solid blue-black degenerated nucleus.

Erythrocyte - (diameter=6-8 μm) biconcave discs which appear in stained smears as red, circular objects with distinct, smooth margins. The intensity of the stain in the central portion where the cell is thinnest is less than at the thicker marginal area.

Megakaryocyte - Large cells with relatively large amount of cytoplasm, round shapes, even margins and multiple nuclei. Chromatin pattern of nucleus is linear, coarse with distinct spaces between the chromatin strands. Cytoplasm contains numerous small, uniformly distributed granules of reddish-blue hue. Numerous cytoplasmic granules, two or more nuclei.

Thrombocyte - (diameter=1-4 μm) Cytoplasmic fragment of a megakaryocyte which has multiple pointed filaments or tentacle like protrusions. Round, oval, spindle, and discoid shapes with smooth margins are also observable. Cytoplasm stains light blue and contains variable numbers of small, blue granules which tend to aggregate in the center.

The morphological characteristics described above were derived from an altas of hematologic cells by Diggs et al.[69]

APPENDIX 3. CITRATE PHOSPHATE DEXTROSE SOLUTION

1) Combined the following in volumetric flask and increased volume with distilled water (dH_2O) to ~50 mL:
   1.66 g sodium citrate (dihydrate) USP
   1.61 g dextrose (monohydrate) USP
   188 mg citric acid (anhydrous) USP
   140 mg monobasic sodium phosphate (monohydrate) USP
2) Adjusted pH to 7.2 to 7.4 using 2 M NaOH
3) Increased volume to 63 mL with dH_2O.
4) Stored in sterile reservoir at 4 °C until use.

APPENDIX 4. SUMMARY OF FLOW CYTOMETRIC FLUOROCHROME CONJUGATED MONOCLONAL ANTIBODY LABELLING

Tube Number	Sample Description	Monoclonal Antibody Conjugate			Purpose
		FITC	PE	PerCP	
1	Cells*	---	---	---	Compensation for autofluorescence
2	Cells*	CD14	IgG1	---	Compensation for emmission spectra overlap of each fluorochrome
3	Cells*	IgG1	CD14	---	
4	Cells*	IgG1	IgG1	CD45	
5	Cells*	IgG1	IgG2a	CD45	
6	Cells*	CD44	CD34	CD45	Analysis of CAM expression on $CD34^+$ myeloid progenitors
7	Cells*	CD49d	CD34	CD45	
8	Cells*	CD62L	CD34	CD45	
9	QSC Beads	CD44	---	---	Standardization of mAb binding
10	QSC Beads	CD49d	---	---	
11	QSC Beads	CD62L	---	---	
12	QSC Beads	---	CD34	---	

*Refers to mononuclear cells derived from marrow, blood or buffy coat specimens enriched for $CD34^+$ cells

--- implies no mAbs were added

APPENDIX 5. PHOSPHATE BUFFER FOR STERILE SORTING

[6.8×10^{-2} M NaCl, 1.3×10^{-3} M KCl, 4.1×10^{-3} M Na_2HPO_4, 7.1×10^{-4} M KH_2PO_4 in dH_2O]

1) Combined the following in volumetric flask and increased volume with distilled water (dH_2O) to ~470 mL:
   4.003 g NaCl
   0.097 g KCl
   0.575 g Na_2HPO_4
   0.096 g KH_2PO4
2) Adjusted pH to 7.2 to 7.4 using 2 M NaOH
3) Increased volume to 1 L with dH_2O.
4) Sterile filtered with 0.2 μm Nalgene filtration system (Nalge Company, Rochester, NY)
5) Stored in sterile reservoir at 4 °C until use.