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Introduction

Hematopoietic stem cells (HSCs) are the source of cells that maintain hematopoiesis [1]. Hematopoietic stem cells maintain hematopoiesis in two ways, firstly through their ability to differentiate and develop into mature blood cells (leukocytes, erythrocytes and platelets), and secondly, through their ability to self-renew [2].

Hematopoietic stem transplantation is currently used to treat a variety of neoplastic and non-neoplastic conditions. The dose of HSCs infused, as quantified by the number of CD34+ cells infused per kilogram of body weight, influences marrow recovery [3]. As HSC dose increases, recovery of marrow function is accelerated [3]. At suboptimal HSC doses, hematopoietic recovery becomes delayed or incomplete [3]. Therefore, collecting a sufficient number of CD34+ hematopoietic stem cells is crucial for the success of transplant engraftment.

There are treatments that are used to try to collect/catch stem cells and there are treatments to drive stem cells into the marrow (integrins and other mediators on the cell surface) which are critical for hematopoietic stem cell transplantation.

Harvesting bone marrow from the iliac crests while the donor is under general anesthetic is one method of collecting hematopoietic stem cells [1]. Other methods include the use of chemotherapy or cytokines such as G-CSF to mobilize/release hematopoietic stem cells from the bone marrow into the peripheral blood and thereby allow for their collection by apheresis [4]. Allogeneic growth factor mobilized collections require that the healthy donors are given hematopoietic growth factors only, namely G-CSF [5]. For autologous collections, the use of chemotherapeutic agents in combination with G-CSF are used to mobilize stem cells into the peripheral blood [5]. AMD3100, also known as plerixafor, reversibly inhibits SDF-1α binding to CXCR4 and administration of this drug mobilizes CD34+ cells into the circulation [3]. See figure 1. The combination of AMD3100 and G-CSF has been shown to be safe, effective and superior to G-CSF alone for autologous HPC mobilization, particularly in poor mobilizers [3].

Once hematopoietic stem cells have been infused into a patient, the HSCs must migrate/home to the bone marrow's microenvironment [6] where they will settle into their niche [4]. Engraftment of transplanted HSCs occurs when the HSCs proliferate and differentiate into the different cell lines and recover hematopoiesis [4].Therefore, the outcome of hematopoietic stem cell transplantation is dependent on the HSCs homing and engrafting in the bone marrow niche [7].

Homing and lodging of HSCs into the bone marrow niche is a complex process. It is thought that hematopoietic stem cells roll and adhere to bone marrow endothelial cells and migrate though the extracellular matrix [7,8] .These events involve adhesion molecules, chemokines, proteolytic enzymes, and growth factors [7,8]. Therefore, cell adhesion molecules found on the surface of HSCs bind to ligands on bone marrow endothelial cells, which allows rolling and firm adhesion. Transmigration of HSCs through endothelial cells into the bone marrow occur where they reside within the bone marrow endosteal niche, beside osteoblast cells [8].

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The purpose of this study was two-fold. Firstly, we set out to determine the expression of specific adhesion molecules, chemokines and proteolytic enzymes found on the surface of CD34+ hematopoietic stem cells collected from steady-state bone marrow compared to those collected by mobilized peripheral blood in normal donors, patients who were poor mobilizers and subsequently treated with plerixafor vs. those who mobilized easily. Secondly, we set out to determine if there were any differences in biologic activity of the CD34 positive cells from the different clinical groups by measuring colony-forming cells and time to neutrophil and platelet recovery post transplant.

The specific adhesion molecules involved in hematopoietic stem cell migration, homing and engraftment that we examined in this study include the chemokine CXCR4, the cell adhesion molecules CD44/H-CAM and CD49d/VLA-4, and a proteolytic enzyme known as CD26/DPPIV. These were chosen due to research done by others showing their involvement in HSCs migration and homing.

CD184/CXCR4 is a chemokine receptor that is expressed on hematopoietic stem cells [6]. SDF-1a/CXCL12 is expressed in the bone marrow microenvironment[5] and is a chemoattractant for CXCR4[6]. CXCL12/CXCR4 interaction is important for trafficking CD34+ hematopoietic stem cells to and from the bone marrow [5,9]. Studies have shown that treating CD34+ hematopoietic stem cells with SDF-1α/CXCL12 or anti-CXCR4 antibody enhances migration of hematopoietic stem cells [9]. The use of neutralizing antibodies to block the function of CXCR4 has been shown in previous studies to impairs homing of transplanted human CD34+ hematopoietic stem cells to the bone marrow of transplanted immunodeficient mice and impairs engraftment [5]. AMD3100 is a mobilizing agent that is an antagonist to CXCR4 and disrupts the SDF-1α/CXCR4 interaction[5]. Studies have shown that the use of AMD3100 in mice, non-human primates and in humans causes rapid mobilization of hematopoietic stem cells [5]. Therefore, CXCR4 plays a crucial role in mobilization and homing of hematopoietic stem cells.

CD26/dipeptidylpeptidase IV is a membrane-bound extracellular peptidase whose enzymatic activity is cleaving the N-terminal dipeptides of polypeptide chains after a proline or an alanine [10, 11]. CD26/DPPIV has been shown to have selectivity toward stromal cell-derived factor 1α (SDF-1α)/CXCL12 [12]. CXCL12/SDF-1α is a chemoattractant for CD34+ HSCs and therefore is a key component of migration, homing and mobilization of human CD34+ HSCs [12]. Christopherson et. al. has shown that CD26/DPPIV is present on a subpopulation of CD34+ HSCs when looking at cord blood and that these cells possessed CD26 peptidase activity [12]. Studies have also shown that treatment of CD34+ cord blood cells with diprotin A, a CD26/DPPIV inhibitor, enhance the migratory response of these cells[12]. Previous studies have shown that the N-terminal truncated form of CXCL12/SDF-1α is unable to induce migration of CD34+ hematopoietic stem cells, however, it has the ability to bind the CXCR4 receptor and block migration of cells induced by normal CXCL12/SDF-1α [12]. Therefore, a potential novel therapeutic approach for increasing the effectiveness of homing and engraftment of HSCs to the bone marrow may be through suppressing the activity CD26/DPPIV [12].

Hematopoietic stem cells express a form of CD44, previously known as homing-associated cell adhesion molecule (H-CAM) [13]. CD44 molecule has been shown to be important in HSC homing to the bone marrow [8, 13]. CD44 is a receptor for hyaluronan/hyaluronic acid which is found in the bone marrow extracellular matrix [13]. In a study by T. Deguchi et. al., flow cytometry was used to characterize the expression of CD44 molecules on CD34+ HSCs from CB, PB and BM [13]. This study found that the frequency of CD44 expression was lower on BM CD34+ cells than on PB and CB CD34+ cells. They also found that there was a subset in BM of CD34+ CD44- HSCs. Avigdor et. al. examined the role of CD44 receptor and its ligand hyaluronic acid in the homing and engraftment of human HSCs in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice by blocking CD44 function with antihuman CD44 mAb, BU52, that inhibits cell adhesion to hyaluronic acid. Antihuman CD44 mAb was shown to block homing of HSCs into the bone marrow [7]. They also showed that HSC homing to the bone marrow is affected directly by interactions between CD44 on HSCs and hyaluronic acid in bone marrow with experiments where they masked the cell surface CD44 with its ligand HA prior to transplantation which reduced homing by 70-80%. They also performed studies where they injected mice intravenously with hyaluronidase, an enzyme that degrades hyaluronic acid. This also resulted in the impairment of HSC homing to the bone marrow [7]. These studies suggest that CD44 plays an important role in HSC migration to and from the bone marrow.

CD49d/VLA-4 (very late antigen-4) found on hematopoietic stem cells [14] binds to VCAM-1 (vascular adhesion molecule-1) expressed by bone marrow stromal cells [5]. Studies have shown in mice and primate models, that neutralizing antibodies for VLA-4 or VCAM-1 result in mobilization of hematopoietic stem cells into the peripheral blood [5]. Antibody treatment blocking VLA-4/VCAM-1 results in reduced homing and increased hematopoietic stem cell mobilization [14]. Studies have also shown that human CD34+ hematopoietic stem cells mobilized with G-CSF express less active VLA-4 when compared to steady-state bone marrow CD34+ hematopoietic stem cells [5]. Therefore, VLA-4/VCAM-1 may play a key role in migration of hematopoietic stem cells to and from the bone marrow [5, 14].

The importance of the number of CD34+ hematopoietic stem cells collected for successful transplantation cannot be overstated. Determining the expression of other chemokines, cell adhesion molecules and enzymes found on CD34+ HSCs that are involved in migration and homing of HSCs can be very valuable. New ways for increasing collection of HSCs and improving homing and engraftment can be further developed and utilized through the manipulation of other cell surface markers found on hematopoietic stem cells. Our study seeks to further our understanding of the expression of other surface markers found on hematopoietic stem cells when collected in a variety of ways and whether this affects engraftment outcomes.

Materials and Methods:

Study Sample Sources: Hematopoietic stem cells (HSC) collected from peripheral blood and bone marrow were used in these studies. Peripheral blood hematopoietic stem cells were collected from healthy allogeneic donors following G-CSF mobilization and cryopreserved. Patients undergoing autologous transplantations were part of a prospective phase II clinical trial evaluating the ability to convert patients who were poorly mobilizing to successfully collect by

administering plerixafor on the evening prior to collection. The first group, referred to as good mobilizers, consisted of patients successfully mobilized utilizing chemotherapy and G-CSF. The second group, referred to as poor mobilizers, were successfully mobilized with the addition of AMD3100/plerixafor to their mobilization regimen of chemotherapy and G-CSF. All peripheral blood hematopoietic stem cells collected were cryopreserved in DMSO and stored in liquid nitrogen until tested. Bone marrow hematopoietic stem cells were obtained from normal healthy donors for related/unrelated bone marrow transplantation through direct iliac aspiration. Cells left-over in the bags and in line filters from the bone marrow harvest were eluted and then cryopreserved as above. Our group sizes consisted of three samples from healthy bone marrow donors and a total of seven samples collected by mobilized peripheral blood. Three of the seven mobilized peripheral blood samples were from healthy allogeneic donors, two from patients who were good autologous mobilizers and the remaining two patients who were poor autologous mobilizers.

Product Thaw and Wash: Cryopreserved cells were thawed in a 37°C water bath in 2-3 minutes. Thawed cells were diluted 1:1 with Plasmalyte [15].

Ficoll Isolation of Mononuclear Cells: Isolation of mononuclear cells was performed. Lymphocyte density medium (Lymphoprep, density 1.077g/mL, MJS Biolynx Inc.) was used for the separation. The volume of sample processed was a 1:1 ratio of cell sample to lymphocyte density medium. The cell sample was carefully layered on top of the lymphocyte density medium without mixing the interface. The cell samples were centrifuged at 400g for 30 minutes, brake off. The interface containing the mononuclear cell layer was removed and placed into another sterile tube. The mononuclear cells were then washed 2x with PBS + 2% FBS for 10 minutes at 300g, brake on low [16]. 0.25mL of 1.0mg/mL deoxyribonuclease I solution was added to the mononuclear cell suspension to prevent clumping. The cells were then washed once with 10-15mL of PBS + 2%FBS and centrifuged for 10 minutes at 300g. The mononuclear cells were then resuspended to the desired cell concentration in preparation for the EasySep positive selection [17].

CD34+ cell selection: CD34 positive selection was performed using Easy Sep®. A mononuclear cell suspension was prepared in PBS + 2%FBS and 1mM EDTA. EasySep Positive Selection Cocktail (which includes anti-CD34) was added at 100uL/mL cells and was incubated at room temperature for 15 minutes. EasySep Magnetic Nanoparticles were then added at 50uL/mL cells and were incubated at room temperature for 10 minutes. The cell suspension was then brought to a total cell volume of 5.0mL (for $< 5.0 \times 10^8$ cells) or 10mL (for 5×10^8 to 2×10^9 cells) with PBS + 2%FBS and 1mM EDTA. The cells were mixed with gentle pipetting and then placed in the magnet for 5 minutes. After 5 minutes, the supernatant was poured off by inverting the magnet. This was considered 1 x 5-minute separation. The tube was then removed from the magnet and 5.0mL (for $< 5 \times 10^8$ cells) or 10mL (for 5×10^8 - 2×10^9 cells) of PBS + 2% FBS and 1mM EDTA was added. A total of 4 x 5-minute separations were done in the magnet. After the final separation, cells were re-suspended in 1mL of PBS supplemented with 2%FBS. A cell count was performed using the Coulter Counter [18].

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Flow Cytometry/Antibody labeling:
Single Platform Flow Assay - Cells were stained with the following antibodies, for each study subject, in individual tubes. All antibodies were purchased from BD Biosciences.

- CD45FITC/CD34PE, CD44PECy-7, and 7AAD for viability.
- CD45FITC/CD34PE, CD49dAPC, and 7AAD for viability.

Antibodies were placed in trucount tubes (BD Biosciences) with 75uL of cell sample at a concentration of WBC $\leq$ 10 x 10^6/mL. Cells were vortexed and incubated in the dark at room temperature for 15 minutes. 450uL of PBS + 2% FBS was added to each tube, vortexed, and incubated in the dark at room temperature for 10 minutes. Samples were then acquired using FACSCanto II Flow Cytometer [19].

Dual Platform Flow Assay - Appropriate amounts of antibodies were added to microcentrifuge tubes according to manufacturer's instructions, CD26FITC, CD44PECy-7, CD49dAPC, CD184PE. CD3PE antibody, which reacts with CD3antigen/T-cell antigen receptor (TCR) complex on T-lymphocytes was used as a negative control, as CD34+ HSCs do not express this antigen receptor. 75uL of cell sample at a concentration of WBC < 10 x 10^6/mL were added to each microcentrifuge tube, mixed gently, and incubated at room temperature in the dark for 20-30 minutes. 800uL of PBS + 2% FBS was added to each microcentrifuge tube, and samples were microcentrifuged at 3000rpm for 3 minutes. The supernatant was aspirated off using a sterile transfer pipette and cells were resuspended. 500uL of PBS + 2% FBS was added to each cell sample. Samples were then acquired using the FACSCanto II Flow Cytometer [19].

Colony-Forming Cell (CFC) Assays: CFC assays were used to detect the ability of hematopoietic stem cells to proliferate and differentiate in vitro. This assay was performed using aseptic technique in a biological safety cabinet. Fresh marrow required mononuclear light density separation prior to performing CFC assays. Three sterile 35mm culture dishes were placed into a 100mm Petri dish. 3mL tubes Methocult GF H4434 were thawed at room temperature. A cell count was performed on the cell therapy product. The desired plating concentration was based on the number of CD34 positive cells in the sample in order to yield the optimal number of colony forming units. Cell concentrations were adjusted using Iscoves Modified Dulbecoo's Medium (IDMD) + 2% Fetal Bovine Serum (FBS). 300uL of adjusted cell concentration was added to each Methocult tube (1:10 dilution). Methocult tubes were vortexed for 5 seconds and then left standing for 5 minutes or until all the bubbles had risen. A 3mL syringe with a blunt end needle was used to draw up the mixed MC media. 1.1mL was dispensed into each 35mm culture dish. The culture dishes were tilted and rotated gently to evenly spread the MC and cells on all sides of the dish. Sterile water was added to the third dish for hydration. The culture plates were incubated in a fully humidified incubator with 5% CO2 at 37 degrees Celsius for 14-16 days. Enumeration of the CFC cultures was performed using an inverted light microscope and a manual cell counter. Hematopoietic progenitor cell colonies were enumerated, including colony forming unit erythroid (CFU-E), burst forming unit erythroid (BFU-E), colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) and colony-forming unit granulocyte, macrophage (CFU-GM).

Results:

CD34 positive Human EasySep Separation and Enumeration - CD34+ selected hematopoietic stem cells were enumerated by flow cytometry, using the single platform flow cytometric assay based on the ISHAGE methodology for the staining, acquisition, gating and analysis of CD34 cells [19, 20]. % CD34+ cells in each sample as a fraction of leukocytes was calculated. The mean %CD34+ cells were calculated for each group.

$$X\%\ CD34+\ cells = \frac{100 \times number\ CD34+\ cells}{number\ CD45+\ events}$$

The mean %CD34+ HSCs was ≥90% for all peripheral mobilized HSCs and was 76% for HSCs collected by bone marrow harvest. This shows that the use of the EasySep Separation was successful in selecting for CD34+ HSCs and we can be confident, especially with the peripherally mobilized samples, that the majority of cells that we were examining for other surface markers were truly CD34+ hematopoietic stem cells. The CD34 enumeration by flow was used as a positive control for this study.

Table 1. Purity of isolated CD34 positive cells

Type of Hematopoietic Stem Cell Tranplant	Mean % CD34 positive HSCs
PB – Allogeneic	94%
PB – Autologous, good mobilizer	90%
PB – Autologous, poor mobilizer, given plerixafor	94%
Bone Marrow (BM)	76%

CD26/DPPIV: The mean % CD26 positive for peripheral mobilized HSC samples was calculated to be 61 +/- 16% and was 74 +/- 9.1% for bone marrow HSC samples. However, the expression of CD26 was very dim on all samples making it difficult to clearly distinguish which cells were truly CD26 positive. See Figure 2.

CD44/H-CAM: CD44/H-CAM was found to be highly expressed on CD34+ HSCs collected by bone marrow harvest and by peripheral mobilization. The mean % CD44 positive for PB HSCs was 100 +/- 0.1% and was 100 +/- 0.3% for bone marrow HSCs. See Figure 2 and 3. Our results show that different mobilization strategies did not affect the expression of CD44/H-CAM on CD34+ HSCs collected and that the adhesion molecule CD44 is highly expressed on CD34+ HSCs when collected from bone marrow and peripherally mobilized HSCs.

CD49d/VLA-4: CD49d was found to be highly expressed on CD34+ HSCs collected by bone marrow harvest and peripheral mobilization. The mean % CD49d positive for PB HSCs was 100% +/- 0.2% and for BM HSCs was 100 +/- 0.2%. Our results suggest that the adhesion molecule CD49d is highly expressed on CD34+ HSCs collected from bone marrow and peripherally mobilized blood. Our results also suggest that different mobilization regimens used for peripherally mobilized HSCs did not affect the expression of CD49d on the CD34+ HSCs collected. See Figures 2 and 4.

CD184/CXCR4: Our results suggest that CXCR4 is more highly expressed in bone marrow samples than in peripheral mobilized HSCs. The mean CD184 % positive for PB HSCs was 34 +/- 15% and was 62 +/- 12% for BM HSCs. A t-test to compare the difference in means was done with $p \leq 0.01$ being significant. A p-value of 0.001 indicates that the mean % CD184 positive for bone marrow and peripheral mobilized HSCs is significantly different. There was a different pattern of expression of CXCR4 observed when comparing the different peripheral mobilization regimens. There is a second population that is more highly expressing CD184/CXCR4 in the plerixafor treated patients. See Figures 2 and 5.

Colony-Forming Cell Assay: Enumeration of the different colonies was performed and it was found that all samples contained erythroid progenitors including CFU-E and BFU-E, granulocyte/macrophage progenitors including CFU-GM and multi-potential CFU-GEMM. The mean CFU/CD34 was 0.54 +/- 0.36 for peripheral blood compared to 0.72 +/- 0.42 for bone marrow. A t-test was done to compare the means, and showed non-significance. Therefore, we have shown that CD34+ HSCs from both bone marrow and peripherally mobilized blood have hematopoietic potential. See Figure 6 and 8. CFU/CD34 was also examined for the different mobilization strategies. See Figure 7.

Table 2. Mean CFU/CD34 for HSCs collected peripherally by different mobilization strategies

	Allogeneic	**Autologous – good mobilizers**	**Autologous – received plerixafor**
Mean CFU/CD34	0.56 +/- 0.23	0.74 +/- 0.56	0.30 +/- 0.03

When comparing the different clinical groups collected by peripheral mobilization, the differences in results were found to be non-significant. Therefore, the biologic potential of CD34+ HSCs collected by different mobilization regimens is not significantly different.

Engraftment: Time to ANC and platelet recovery was recorded for each patient and the mean was calculated for bone marrow transplant patients and for peripheral mobilized HSC transplant. For two of the bone marrow transplant recipients, time to neutrophil and platelet recovery was not yet available. Therefore, two bone marrow recipients were randomly selected, and their time to neutrophil and platelet recovery was utilized for this portion of the study. Mean time to ANC count $\geq 0.5 \times 10^9$/L for bone marrow HSC recipients was 22 +/- 7.9 days, compared to 16 +/- 7.5 days for peripheral mobilized HSC recipients. Mean time to ANC count $\geq 1.0 \times 10^9$/L was 32 +/- 10 days for bone marrow HSC recipients and 20 +/- 9.6 days for the peripherally mobilized HSC recipients. The mean time for platelet count $\geq 20 \times 10^9$/L was 23 +/- 3.6 days for bone marrow HSC recipients and 20 +/- 9.6 days for peripherally mobilized HSC recipients. The mean time for platelet count $\geq$ 50 x 10^9/L was 27 +/- 4.5 days for bone marrow HSC recipients and 22 +/- 9.7 days for patients receiving peripherally mobilized HSCs. Our data shows that ANC and platelet recovery occurred for both clinical groups. T-tests were performed and showed non-significance for every neutrophil and platelet recovery time. See Figure 8.

Discussion:

CD184/CXCR4 is a chemokine receptor expressed on hematopoietic stem cells [6], whose interaction with CXCL12/SDF-1α is important for trafficking CD34+ HSCs to and from the bone

marrow [5, 9]. Previous studies have shown that CD34+ HSCs in the bone marrow expressed a higher percentage of CXCR4+ than mobilized peripheral CD34+ HSCs [9]. Results from our studies are comparable. Mean % $CD184^{+}CD34^{+}$ HSCs for peripheral mobilized blood was 34.02 +/- 14.98% and 62.2 +/- 12.3% for bone marrow. When we took a closer look at expression of CD184/CXCR4 on $CD34^{+}$ HSCs from peripheral blood collected using different mobilization strategies. We found that there is a different pattern of expression of CXCR4 when comparing the three clinical groups of peripherally mobilized HSCs. Patients who received plerixafor as an additional mobilization agent showed a second population of CD34+ HSCs expressing a higher signal of CD184/CXCR4. Plerixafor/AMD3100 mobilizes CD34+ HSCs into the circulation by reversibly inhibiting SDF-1 alpha binding to CXCR4 on HSCs [3]. Therefore, our results may suggest that recruitment of this additional population of $CD34^{+}CD184^{high}$ HSCs may be due to the use of plerixafor as an additional mobilizing agent. Importantly we found that the increased numbers of CD34 positive cells obtained by this maneuver resulted in the same number of functional precursors (CFU) and engraftment time.

CD26/DPPIV is a peptidase that has been shown to have selectivity toward SDF-1a/CXCL12 [12], a chemoattractant for CD34+ HSCs. Studies done by Christopherson et. al. have shown using multivariant flow cytometry that CD26/DPPIV is enriched on a subpopulation of $Lin^{-}CD34^{+}$ HSCs when looking at cord blood[12]. Christopherson et. al. has also shown that treatment of CD34+ cord blood cells with diprotin A, a CD26/DPPIV inhibitor, enhances the migratory response of these cells [12]. Our study found dim expression of CD26 on CD34+ HSCs from bone marrow and peripherally mobilized HSCs. It was difficult to distinguish which CD34+ HSCs were truly positively expressing CD26/DPPIV, and as a result, it may not be prudent to draw conclusions from our study with regards to CD26+ expression on CD34+ HSCs from bone marrow and peripherally mobilized blood.

CD44/H-CAM is a cell adhesion molecule found on the surface of CD34+ HSCs and is the receptor for hyaluronic acid, found in the bone marrow extracellular matrix [13]. Previous studies by T. Deguchi et. al. used flow cytometry to characterize the expression of CD44 molecules on CD34+ HSCs from CB, PB and BM [13]. Their study found the frequency of CD44 expression was lower on BM CD34+ cells than on PB and CB CD34+ cells. They also found that there was a subset in BM of CD34+ CD44- HSCs. Our study results are not concordant with what has been previously shown by others. Our study found that CD44 was highly expressed on CD34+ HSCs from bone marrow and peripherally mobilization blood. The mean CD44 % positive for PB CD34+ HSCs was 99.94 +/- 0.05% and was 99.64 +/- 0.291% for bone marrow CD34+ HSCs. We found that the high expression of CD44 on CD34+ HSCs did not change based on differing mobilization regimens of the peripheral blood samples.

CD49d/VLA-4 is an adhesion molecule present on hematopoietic stem cells [14] which binds VCAM-1 expressed by bone marrow stromal cells [5]. Young-Ho Lee et. al. has studied the expression of CD49d on CD34+ cells in bone marrow, peripheral blood and cord blood and found that the proportions of $CD34^{+}CD49d^{+}$ cells were not significantly different between CB, BM or PB. They observed the same percentage of CD49d+ cells within the CD34+ cell populations in BM, PB and CB [6]. The results from our study show that CD49d was highly

expressed on CD34+ HSCs from bone marrow and peripheral mobilized blood. The mean % CD49d positive for PB HSCs was 99.69 +/-0.231% and for BM was 99.72 +/- 0.175%. Our study results show that expression of CD49d on CD34+ HSCs were not affected by varying methods of collection.

This set of studies has certain limitations. Firstly, the number of samples in each group was limited. It is difficult to draw conclusions from such small group sizes. Therefore, a larger group of patients would have yielded more significant results.

Monoclonal antibodies CD26FITC and CD184PE had the same fluorochromes as CD34PE and CD45FITC. Therefore, analysis of these monoclonal antibodies could not be performed simultaneously – thus we were unable to test for coexpression. We used CD34PE/CD45FITC flow assay as a guide for where the CD34+ cell population was located, and developed our own gating strategy for expression of CD26 and CD184 on a separate assay. See Figures 9 and 10. However, the use of samples that were CD34+ selected eliminated the majority of cellular debris, making this task simpler and more accurate.

Sample quality was another limitation to this study. All samples for this study were thawed from cryopreservation, and thus contained dead CD34+ cells, platelet aggregates, and other cellular debris which can pose as obstacles to reliable CD34+ cell enumeration [20]. For our study, CD34+ selection removed most cellular debris present, however, working with fresh samples ultimately results in more accurate CD34+ cell enumeration.

Selection of our negative control, CD3, was chosen for several reasons. Firstly, CD3 is a cell surface marker found on mature T lymphocytes. CD34+ HSCs do not express CD3 as a cell surface marker, and thus CD3 serves as a useful negative control. For our study, we did not use isotype- and fluorochrome-matched MAbs to control for non-specific staining of CD34+ HSCs as it is well known that CD34+ cells have low auto-fluorescence or non-specific binding. Sutherland et. al. have found that the Boolean gating strategy used for CD34+ cell enumeration in these experiments essentially eliminates the cell populations typically involved in nonspecific MAb binding, including platelets, dead cells and myelomonocytic cells [20]. However, the use of CD3 as an isotype control for CD44 and CD184 poses some limitations to our study. CD3 was composed of mouse IgG_1 heavy chains and kappa light chains, the same isotypes as CD26 and CD49d. However, CD44 was composed of mouse IgG_{2b} heavy chains and kappa light chains and CD184 was composed of mouse IgG_{2a} heavy chains and kappa light chains. Therefore, CD3 was not an appropriate isotype control for CD44 and CD184 monoclonal antibodies. As a result, it was difficult to ascertain percent positive populations using the monoclonal antibody CD44 and CD184.

Future Directions:

Future directions for this project include the use of flow cytometry to determine if CD26/DPPIV, CD44/H-CAM, CD49d/VLA-4 and CD184/CXCR4 are concurrently expressed on CD34+ HSCs. It would also be of interest to explore the differential expression pattern of the above surface markers on different CD34+ HSCs collected by bone marrow harvest and by peripheral

mobilization of HSCs. Selecting out for CD38+ and CD38- stem cell populations would identify more primitive stem cell subsets. Finally it would be of value to develop an in vitro migration assay using the transwell platform.

Conclusion:

In conclusion, research on migration and homing of hematopoietic stem cells is important clinically with regards to hematopoietic stem cell transplantation. Our study undertook the task of trying to better understand the expression of chemokines, cell adhesion molecules and enzymes present on CD34+ hematopoietic stem cells that are known to have a role in migration and homing of HSCs. Our data on CXCR4 show that there are two populations of HSCs present for patients mobilized with plerixafor: $CD184^{high}$ and $CD184^{dim}$. Patients mobilized without the use of plerixafor show only one population, $CD184^{dim}$. Our data on CD44/H-CAM and CD49d/VLA-4 shows that there are a lack of differences when observing expression of CD44/H-CAM and CD49d/VLA-4 in bone marrow and peripherally mobilized blood. CD44/H-CAM and CD49d/VLA-4 were both highly expressed on bone marrow and peripheral mobilized HSCs. Our data on CFU/CD34 also showed no difference when comparing bone marrow to peripherally mobilized HSCs, showing that the biologic activity of HSCs from bone marrow and peripherally mobilized blood are similar. Also, our data on CFU/CD34 showed no difference when comparing the clinical groups who underwent different peripheral mobilization strategies. Engraftment data also showed no difference in the time to neutrophil and platelet recovery when comparing bone marrow to peripherally mobilized HSCs.

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Miranda Charette

Figure 1: Stem cell mobilization by plerixafor/AMD3100.

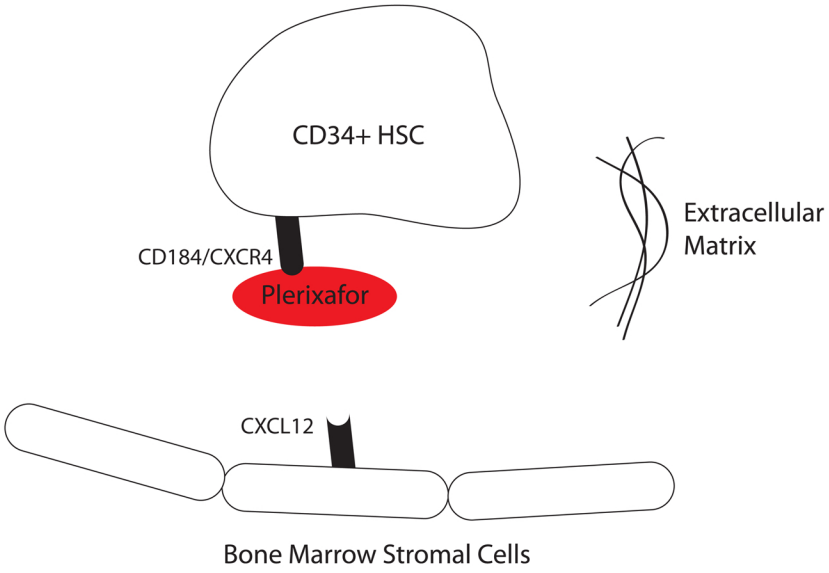


Figure 2. Cell surface markers present on CD34+HSCs when collected from steady state bone marrow compared to peripherally mobilized HSCs. (*) indicates statistical significance

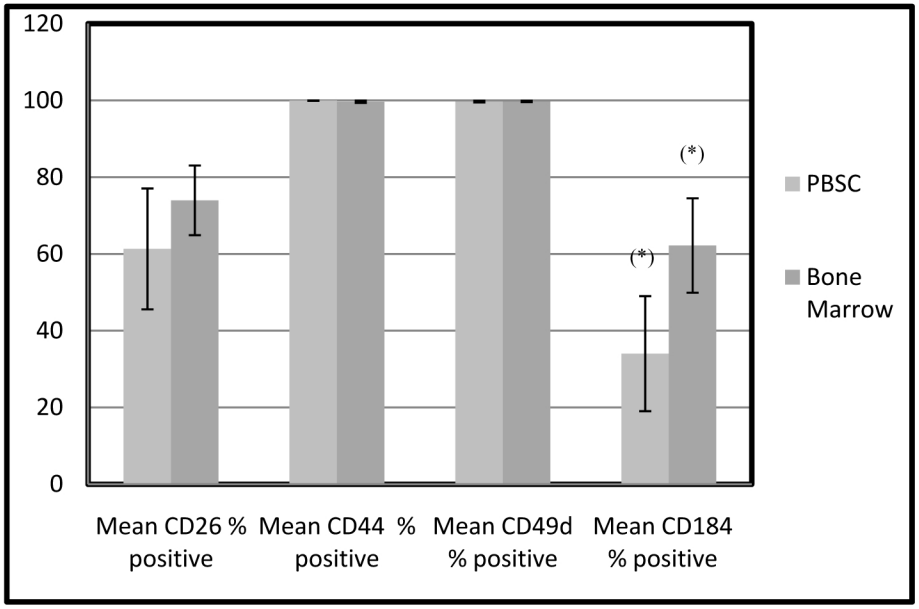


Figure 3. Expression of CD44 on bone marrow CD34+ HSCs compared to peripheral blood CD34+ HSCs. Left-bone marrow. Right – PBSC. Red histogram is CD44/H-CAM and black histogram is negative control.

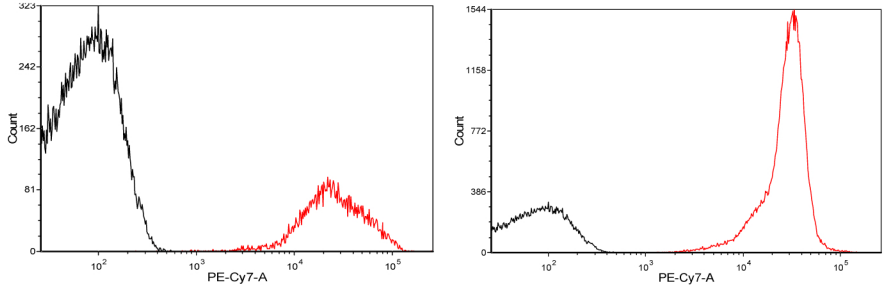


Figure 4. Expression of CD49d on bone marrow CD34+ HSCs compared to peripheral blood CD34+ HSCs. Left – Bone marrow. Right – PBSC. Red histogram is CD49d/VLA-4 and black histogram is negative control.

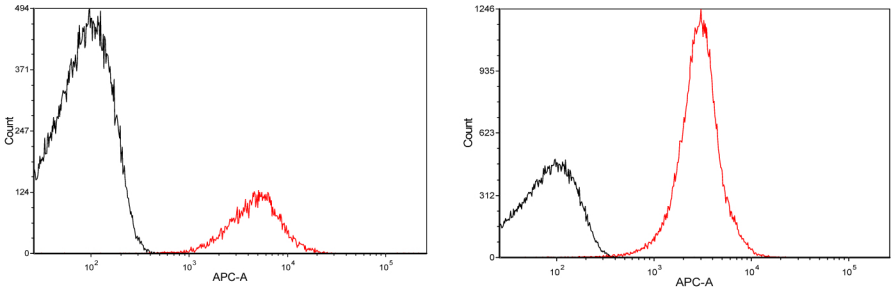


Figure 5. CXCR4 expression on CD34+ HSCs. Left – autologous given plerixafor. Middle – autologous good mobilizer, Right – allogeneic. Red histogram is CD184/CXCR4 and black histogram is negative control.

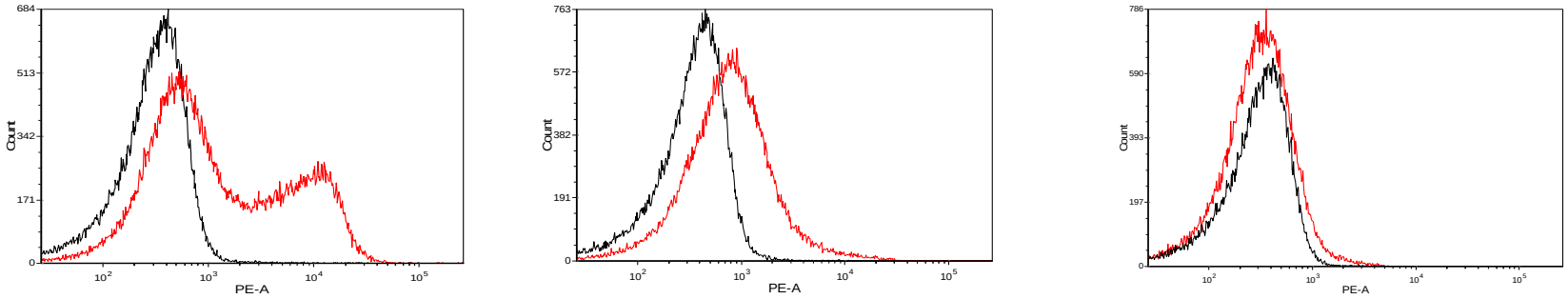


Figure 6. CFU/CD34 for peripheral blood and bone marrow

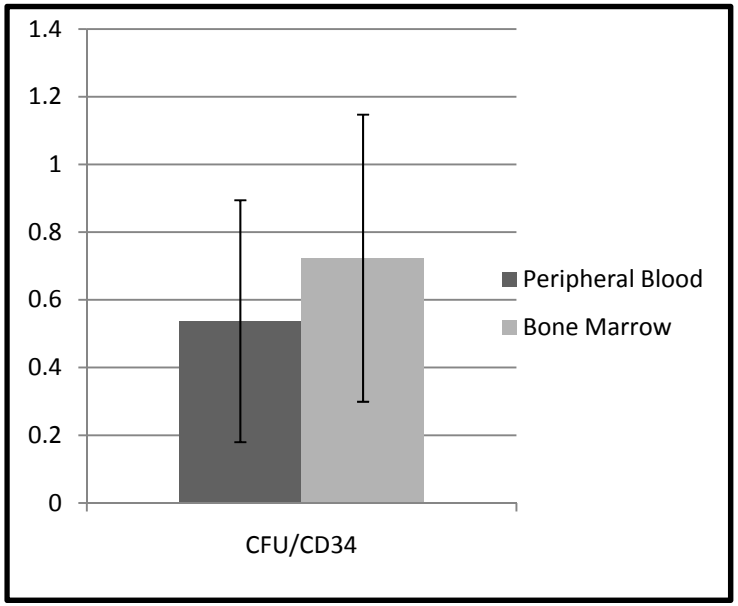


Figure 7. CFU/CD34 for peripherally mobilized blood.

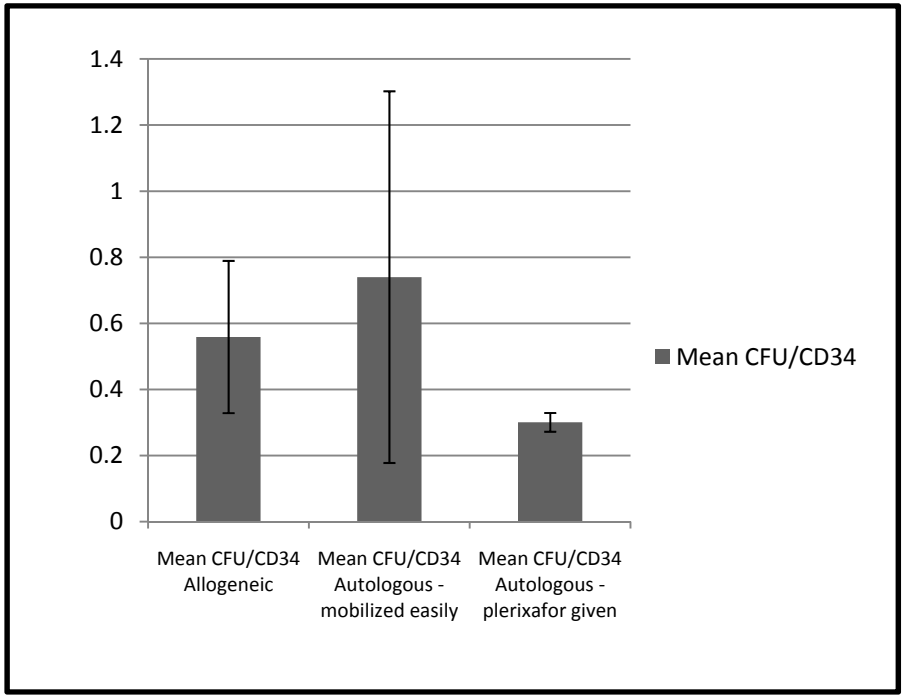
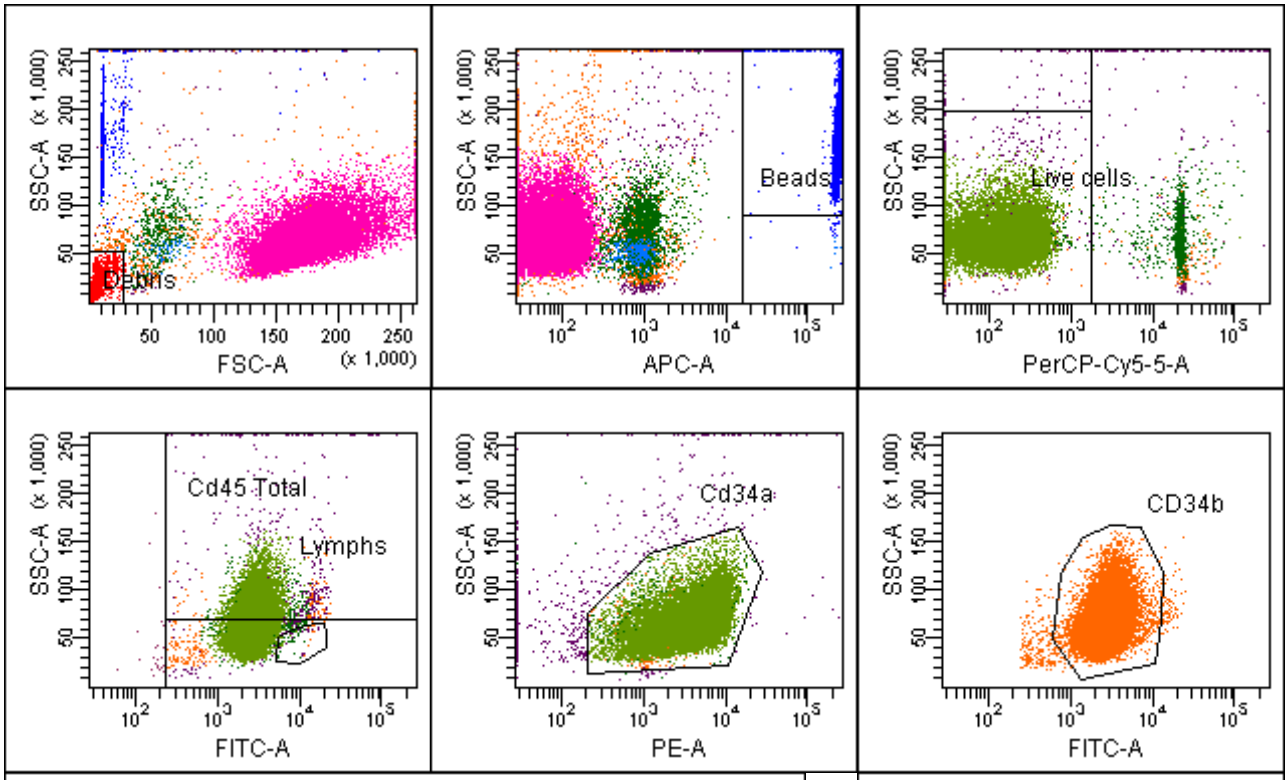


Figure 9. Enumeration of CD34+ Hematopoietic Stem Cells by Flow Cytometry.
Counterstaining CD34 with CD45 Mab (leukocytes) allows for the elimination of nonspecifically stained events and debris and discrimination of HSCs. HSCs express low levels of CD45 on their surface whereas lymphocytes and monocytes express high levels of CD45. Forward scatter(FS), side scatter(SS), CD34 expression and intensity of CD45 expression are the 4 parameters combined in a sequential Boolean gating strategy. The use of 7-AAD allows for the exclusion of dead cells. True CD34+ cells stain with CD45 and CD34, and the CD45dim, CD34+ events had to form a cluster with similar FS, SS characteristics as lymphocytes and blast cells. Absolute CD34+ cell count can be calculated with the use of fluorescent counting beads[20].



Tube: Tube_001

Population	#Events	%Parent	%Total
All Events	127,147		100.0
Debris	40,973	32.2	32.2
NOT(Debris)	86,174	67.8	67.8
Beads	11,053	12.8	8.7
NOT(Beads)	75,121	87.2	59.1
Cd45 Total	75,000	99.8	59.0
Cd34a	72,183	96.2	56.8
CD34b	71,317	98.8	56.1
CD34 total	68,013	95.4	53.5
Live cells	71,087	82.5	55.9
Lymphs	485	0.6	0.4
CD45 Live	71,042	82.4	55.9
CD34 Live	67,954	78.9	53.4

Figure 8: Neutrophil and platelet recovery post transplant.

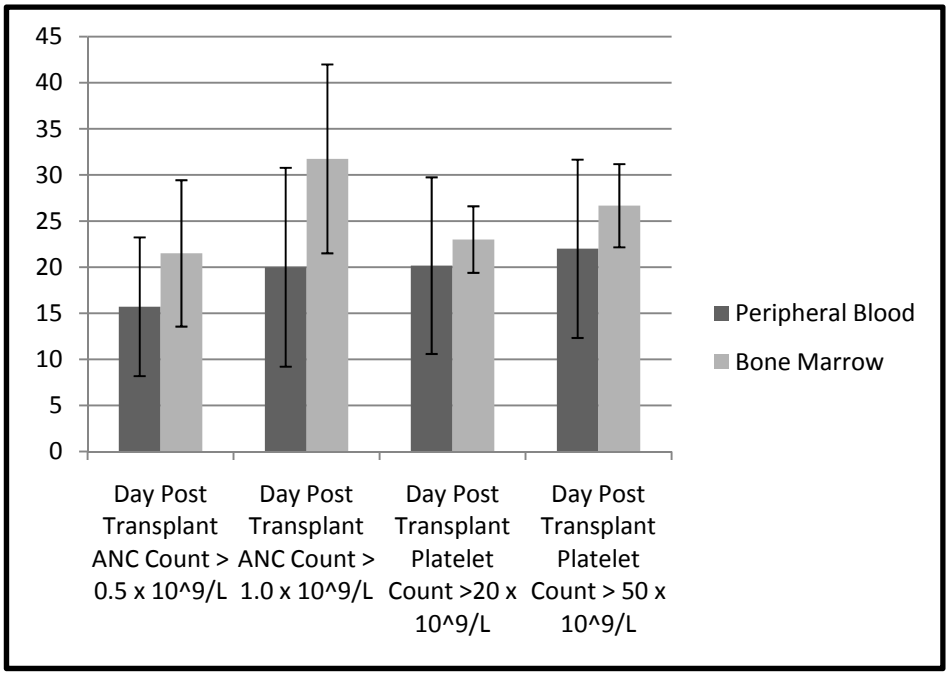


Figure 10. Expression of CD184/CXCR4 on CD34 positive HSCs. Use of the CD34 positive selection kit allowed for easier identification of CD34+ hematopoietic stem cells by flow cytometry. Using the FS and SC characteristics of CD34+ HSCs as determined in Figure 8, gating around the CD34+ HSCs is performed. Expression of CD184/CXCR4 is then analyzed on the selected CD34+ HSCs.