INVERSE AGONISM OF SPHINGOSINE 1-PHOSPHATE RECEPTOR THREE MOBILIZES HEMATOPOIETIC STEM CELLS WITH LONG TERM ENGRAFTMENT CAPABILITY

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The thesis is submitted in partial fulfillment of the

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Abstract

The use of peripheral blood as an alternative to bone marrow for obtaining hematopoietic stem cells (HSC) has been successfully established. This noninvasive procedure presents several advantages such as elimination of pain and anesthesia during cell harvest. In order to enrich peripheral blood with stem cells they need to be pharmacologically mobilized with stem cell mobilizing agents. G-CSF is the gold standard but it is costly, time-consuming and does not always result in sufficient mobilization and engraftment. New strategies to mobilize sufficient numbers of HSC without impairing their functional capability would drastically improve the therapeutic use of peripheral blood stem cells. AMD3100, also known as plerixafor, is a CXCR4 antagonist that is being evaluated in conjunction with G-CSF to enhance stem cell mobilization. In this work we show that inverse agonism of sphingosine 1-phosphate receptor three ($S1P_3$) significantly mobilizes HSC from the bone marrow into peripheral blood without affecting their ability to engraft and repopulate blood cells.

HSC pre-treatment with VPC01211, an $S1P_1$ agonist and $S1P_3$ antagonist abolished chemotaxis towards SDF-1 in transwell migration assays and resulted in a 33% decrease in engraftment onto marrow-derived stromal cells and a 47% decrease in engraftment when combined with AMD3100. AMD3100 was used with or without VPC01091 to mobilize stem cells in GFP+ C57Bl/6 mice. Equal volumes of peripheral blood were used to reconstitute host C57Bl/6 mice after lethal irradiation. By 56 days post transplantation, animals receiving grafts mobilized with both AMD3100 and VPC01091 had a 162% increase in donor content in their blood and a 63% increase in donor content in their bone marrow over AMD3100 alone.

This work shows for the first time that $S1P_3$ receptor signaling can be regulated to mobilize stem and progenitor cells from their bone marrow niches into peripheral blood. The combination of AMD3100 and VPC01091 significantly enhanced HSC mobilization but did not impair the functional capability of these cells. $S1P_3$ receptor antagonism can be used alone or in conjunction with compounds like AMD3100 to significantly mobilize stem and progenitor cells for endogenous and exogenous stem cell therapies.

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Most importantly, God the father, who blessed me with the scholarly aptitude, inquisitive spirit and patience that was necessary to complete this thesis work. I am continually thanking and praising him for all he has blessed me with.

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List of Symbols/Abbreviations

A: AMD3100 (see below)

AMD3100: Stem cell mobilizing agent that reversibly blocks CXCR4 receptor

BMC: Bone marrow derived cell

CD11b: Cell surface receptor found on polymorphonuclear leukocytes, NK cells and mononuclear phagocytes

CD34: Human single pass transmembrane protein on early hematopoietic and vascular-associated cells

CD45: Leukocyte common antigen receptor

CFU: Colony forming unit, formed from one hematopoietic stem or progenitor cell

c-kit: Cytokine receptor expressed on the surface of hematopoietic stem cells

CXCR4: G-protein coupled receptor specific for the chemokine SDF-1α

FBMD-1: Flask Bone Marrow Dexter-1 bone marrow stromal cells with high SDF-1α expression

FTY720: Immunomodulating drug that is $S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$ agonist

G-CSF: Granulocyte colony-stimulating factor is used clinically as a stem cell mobilizing agent

GFP: Green fluorescent protein is a fluorescent tag used for tracking of cells

HSC: Hematopoietic Stem Cells

KO: Mice genetically void of $S1P_3$ globally

Lineage1: Standard cocktail of antibodies (CD2, CD3, CD4, CD6, CD11b, CD45, Gr-1, CD14, etc) used to exclude mature cell types from hematopoietic stem cells

LSK: Cells which are Lineage1 negative, Sca1 positive and c-kit positive and closely resemble human CD34 positive hematopoietic stem cells

MPK: Milligrams per kilogram weight

PB: Peripheral blood

RFP: Red fluorescent protein is a fluorescent tag used for tracking of cells

S1P: Sphingosine 1-phosphate

$S1P_{1-5}$: G-protein coupled sphingosine 1-phosphate receptors 1-5

Sca1: Stem cell antigen-1 marker for stem and progenitor cells

SDF-1α: Stromal derived factor 1-alpha, a potent chemoattractant for stem cells

SPHK1/2: Sphingosine kinase 1/2, phosphorylates sphingosine to create S1P

V: VPC01091 (see below)

VPC01091: FTY720 pro-drug analog that is an $S1P_1$, $S1P_4$ and $S1P_5$ agonist and $S1P_3$ antagonist

VPC01211: Phosphorylated analog of VPC01091

WT: Genetically and functionally wild type mice.

Introduction

The human body has the ability to regenerate cells, repair tissues and heal wounds through the differentiation and proliferation of multipotent stem cells. By applying the appropriate temporal and spatial molecular signals we can design strategies to recruit regenerative stem cells to sites of repair[1]. Over the last few decades, the use of peripheral blood as an alternative to bone marrow for obtaining hematopoietic stem cells (HSC) for transplantation has been successfully established[2-4], and this procedure presents several advantages such as elimination of pain and anesthesia during cell harvest. HSC from peripheral blood are used for various therapies involving autologous and allogeneic transplantation, and thus, improving their mobilization out of the bone marrow niche is important. However, it is imperative that this objective be achieved with minimal effect on the future engraftment ability of these cells and predictable mobilization kinetics.

The mechanisms of mobilization of stem cells from their bone marrow environment are not very well documented and are currently being widely researched. A key interaction that keeps the HSC tethered in their bone marrow niches is that of CXCR4 and SDF-1α[6]. CXCR4, a G protein-coupled receptor, is found on the cell surface of all HSC, and SDF-1α is produced by bone marrow stromal cells, particularly by immature osteoblasts to help retain these cells in the marrow. G-CSF, the most common mobilizing agent, secretes neutrophil associated extracellular proteases, such as elastase and matrix metalloproteinase-9, thus releasing HSC from their bone marrow niches[6]. However, as many as 30% of patients still fail to mobilize sufficient numbers of cells even after chemotherapy with G-CSF[7-9]. Furthermore, mobilization with G-CSF requires multiple repeated doses for several days, which is both costly and time consuming. G-

CSF's mechanism of mobilization also down-regulates CXCR4 expression which can impair signaling on the SDF-1α/CXCR4 axis and be detrimental to engraftment in both endogenous and exogenous stem cell therapies[10]. AMD3100, a recently approved drug by FDA, has been shown to improve HSC mobilization when used in conjunction with G-CSF[11,12]. It is a CXCR4 antagonist and causes the receptor to reversibly unbind to its ligand, SDF-1α[13]. Mobilization with a combination of AMD3100 and G-CSF results in both an increased number of cells and a higher percentage of primitive CD34+/CD38- cells but does not circumvent all of the drawbacks of G-CSF-induced mobilization. Many studies have looked at using AMD3100 alone and, though yielding mixed results, this does not seem sufficient to mobilize stem cells to the same capacity as G-CSF[14,15].

In this study we demonstrate that sphingosine-1-phosphate (S1P) receptor-selective agonists and antagonists are effective agents to induce and/or enhance HSC mobilization into the peripheral blood. S1P is a biologically active signaling lipid that is formed from the phosphorylation of sphingosine by one of two kinases (SPHK1/SPHK2) in a variety of cells. It is readily secreted from cells into blood where it binds to serum albumin or HDL and is a ligand for five high affinity G-coupled receptors ($S1P_1$-$S1P_5$) that direct a wide range of biological processes[16-20] (Figure 1). In recent years $S1P_1$ has become popular for its immunomodulatory effects. Egress of lymphocytes from lymphoid organs is dependent on $S1P_1$[21]. FTY720, a functional antagonist of $S1P_1$ that causes internalization and degradation of the receptor is currently being used to treat multiple sclerosis by sequestering lymphocytes in lymphoid organs[22]. Many groups have begun to look at S1P receptor signaling in the migration, egress, and homing of stem cells throughout the body[23]. Additionally, S1P gradients induce HSC mobilization into the

peripheral blood as previously reported, suggesting that the egress of these stem cells can occur independently of the SDF-1α/CXCR4 axis[24].

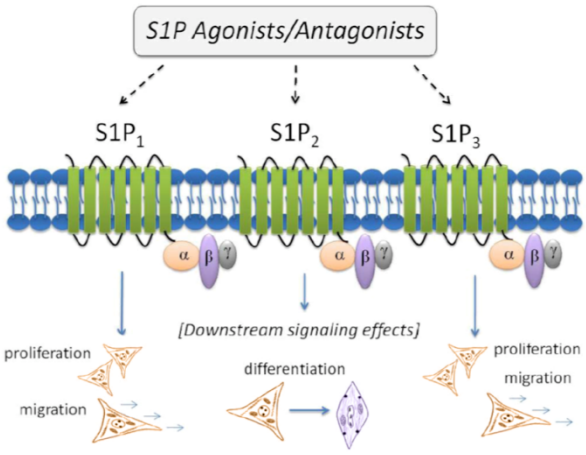

Figure 1. S1P receptor signaling. S1P receptors are prevalent on the vast majority of cell types at varying expression levels. S1P receptors have been implicated in proliferation, migration, differentiation, apoptosis, signal transduction and many other cellular functions. They are key signaling receptors in almost all organ systems and cell types and regulate a wide array of biological functions. $S1P_1$, $S1P_3$ and $S1P_2$ are the most prevalent receptors on hematopoietic stem cells, in that order.

The idea that S1P receptors interact with signaling on the SDF-1α/CXCR4 axis is not a new one. Kimura et. al showed that FTY720-pretreatment promoted the migration of human HSC towards SDF-1α and homing to the bone marrow[25]. Walter et. al showed that activation of $S1P_3$ on endothelial progenitor cells (EPCs) enhanced their migration towards SDF-1α and their functional capacity to recover blood flow after ischemia[26]. However, to date, the reverse has not been explored: attempting to antagonize S1P receptors to disrupt CXCR4/SDF-1α signaling and promote stem cell mobilization into peripheral blood. It has been shown that, along with CXCR4, HSC express $S1P_3$[27], and in these experiments we present that $S1P_3$ antagonism mobilizes a significant number of HSC from bone marrow that maintain their long-term ability to engraft and repopulate blood cells after transplantation. VPC01091, with a phenyl cyclopentyl linker is an

SPHK2 substrate, an $S1P_1$ agonist and an $S1P_3$ antagonist small molecule[28]. It demonstrates a mobilizing capacity equal to or higher than AMD3100 without affecting the re-populating capability of the mobilized HSC. We show that mice treated with VPC01091 alone and in combination with AMD3100 have more HSC mobilized into their peripheral blood and the long-term engraftment ability of these cells is not altered.

Methods

Mice. C57BL/6 mice (8-12 weeks old) were obtained from Jackson Laboratories. $S1P_3^{-/-}$ mice on the C57BL/6 background were a kind gift of Dr. Richard Proia at the National Institutes of Health. GFP+ (Tg(UBC-GFP)30Scha/J) and RFP+ (Tg(CAG-DsRed*MST)1Nagy/J) mice on a C57BL/6 background were obtained from Jackson Laboratories. All mice were used at 8-12 weeks old and were housed at the MR5 vivarium, clean room G320 at the University of Virginia. All mouse studies were approved by the Institutional Animal Care and Use Committee at the University of Virginia.

Mobilization. Mice were treated with 5 milligrams per kilogram weight (mpk) of VPC01091 or FTY720 in 5% DMSO in fatty acid free bovine serum albumin or saline intraperitoneally. Thirty minutes later, CXCR4 inhibition was induced by treating mice intraperitoneally with AMD3100 (Sigma) in saline at 5 mpk body weight. Peripheral blood was collected one hour after with a sterile cardiac stick into an EDTA coated blood tube.

Bone Marrow Cell Isolation. Wild type C57Bl/6 mice were sacrificed by CO_2 asphyxiation and their femurs and tibias were harvested and cleaned. Bones were washed in cold ethanol for 2 minutes followed by cold sterile PBS for 2 minutes. Bone cutters were used to cut the proximal end of the bone, which was then placed, cut side down, in a 1mL syringe and then placed into a 15mL centrifuge tube. The bones were spun at 400rcf for 5 minutes releasing a bone marrow pellet, containing bone marrow cells, which was re-suspended in sterile PBS and filtered.

Colony Forming Assay. 200µl of peripheral blood was collected from wild type C57Bl/6 mice via cardiac stick after bone marrow cell mobilization. Peripheral blood was resuspended in nine times the volume of red blood cell lysis solution-ammonium chloride (Stem Cell technologies) on ice for 10 minutes. After two washes with Iscove's medium (Invitrogen) the cell pellet was re-suspended in 400µl of Iscove's medium and added to 4ml of Methocult™ methylcellulose based medium with recombinant cytokines and erythropoietin (Stem Cell technologies). This was vortexed and allowed to rest for 20 minutes until all the bubbles disappeared. Using blunt tipped needles, samples were plated in triplicate in six well plates and, 6 days after plating, colonies were counted on a Carl Zeiss upright microscope at 10x magnification.

Cobblestone Assay. Flask Bone Marrow Dexter-1 (FBMD-1) cells (a kind gift from Dr. Van Zant, U. of Kentucky) were seeded in Iscove's media (Invitrogen) supplemented with 10% FBS, 5% horse serum, pen/strep/β-mercaptoethanol (Invitrogen) and 10-5M hydrocortisone (Sigma) at a density of 1,000 cells per well in a 96-well plate and allowed to grow to confluence at 33ºC for 2 weeks. Bone marrow cells were collected from the tibia of wild type C57Bl/6 mice, and the cell suspension was pre-treated with saline, 15nM AMD3100 (Sigma), 15nM VPC01211 (UVa) or both 15nM AMD3100 and 15nM VPC01211 for 30 minutes. The cells were then transferred to CAFC medium (100 ml horse serum, 5.5. ml pen/strep/β-mercaptoethanol and 5 ml hydrocortisone added to a 500 ml bottle of IMDM), and plated on the FBMD-1 stromal cells at 81,000, 27,000, 9,000 or 3000 cells per well (20 wells tested per density condition). To assess cell adhesion to the stromal layer, non-adherent cells were washed off the FBMD-1 after 2 hours and fresh CAFC medium was added to each well. The number of cobblestone positive wells was

determined 14 days after seeding. A well was scored positive if it had 6 or more cobblestone areas.

Transplantation. For competitive transplants, a constant volume of peripheral blood of GFP+ (donor) mice in combination with $2x10^6$ bone marrow-derived mononuclear cells collected from the tibiae and femurs of RFP+ (competitor) mice were transplanted into C57BL/6 recipient mice. Recipient mice were lethally irradiated with a total dosage of 10.5 Gy (5.5-Gy and 5-Gy doses, 3 h apart) before transplantation of donor cells, resuspended in 150µL PBS, via tail vein injection. Animals were kept on a water supplemented with sulfonamide for 8 weeks.

Flow cytometry. Chimerism of the peripheral blood and bone marrow was analyzed by flow cytometry with a panel of markers consisting of Sca1 for progenitor cells, CD45 for myeloid cells and CD11b for inflammatory cells. Flow cytometry was performed according to standard procedures and was analyzed on a 16 color Becton Dickinson LSRII Fortessa flow cytometer. FACS was performed according to standard procedures and was analyzed on a 12 color i-Cyt Reflection Cell Sorter. Monoclonal antibodies to Lineage 1, Sca1 and c-kit were used to label hematopoietic cells for FACS.

Transwell Migration Assay. LSK (hematopoietic stem) cells were sorted and serum starved for 3 hours. Cells were subsequently pre-treated with 15nM VPC01211 in 5% DMSO in 3% fatty acid free BSA or carrier at a concentration of 2 million cells/ml for 30 minutes and then were re-suspended in serum free DMEM (Invitrogen). 100µl of the pre-treated cell suspension was added to the top of 5µm transwell inserts (Costar) in a 24-well plate. The bottom of the wells contained 600µl of serum free DMEM or 12.5nM SDF-1 (Sigma) or S1P (Cayman Chemicals). LSK cells were allowed to migrate towards the

bottom well for 4 hours. After a 4 hour migration, the media in the top and bottom of the wells was collected and flow cytometry was used to determine cell count.

Statistical Analysis. Data are presented as means ± standard error. Multiple group comparisons were performed by one-way ANOVA followed by the Bonferroni or Tukeys posts tests to compare means and performed in GraphPad Prism statistical software. Comparisons between two groups were performed using an unpaired t test. For survival analysis, a log-rank (Mantel-Cox) test with a chi square distribution was used. Values of $P < 0.05$ were considered statistically significant.

Results

Basal level of circulating Sca1+ progenitors elevated in $S1P_3$-/- mice and further elevated after CXCR4 inhibition with AMD3100

Wild type and $S1P_3$-/- mice were given 200µl injections of saline or 5mpk AMD3100 dissolved in saline. One hour after injection, mice were euthanized with CO_2 and peripheral blood was collected via cardiac stick. The peripheral blood cells were stained with antibodies against Sca1, a progenitor cell marker, and counted with flow cytometry. In saline-treated wild type mice, 5.81% of the peripheral blood (PB) cells were Sca1+ (Figure 2). As expected, AMD3100 enhanced the peripheral blood pool of progenitor cells and 11.11% of the PB in these mice were Sca1+. Surprisingly, in the PB of $S1P_3$-/- mice given saline alone, there was a comparable percentage of Sca1+ cells to wild type mice treated with AMD3100 (11.46%) suggesting that $S1P_3$-/- alone may play a role in the mobilization of progenitor cells from their niches. There was a further and significant elevation of peripheral blood Sca1+ cells in $S1P_3$-/- mice given AMD3100 (20.83%) suggesting that antagonism of CXCR4 and $S1P_3$ is synergistic in the mobilization of stem and progenitor cells into peripheral blood.

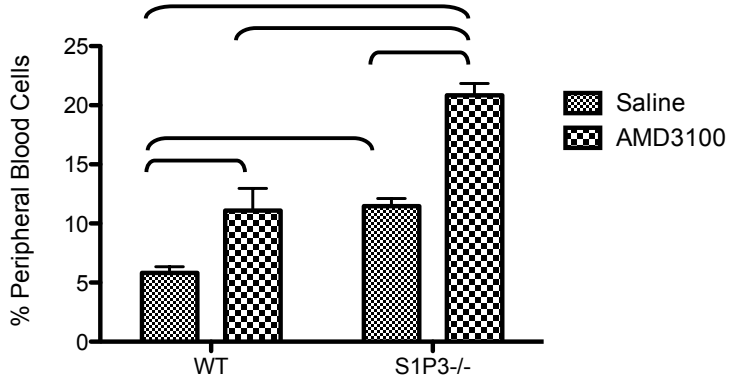

Figure 2. Flow cytometric analysis of peripheral blood Sca1+ cells. AMD3100 mobilizes Sca1+ progenitor cells into peripheral blood and significantly enhances their concentration over basal. Genetic knockdown of $S1P_3$ promotes a similar level of progenitor cell mobilization. $S1P_3$ knockdown in conjunction with AMD3100 substantially increases peripheral blood Sca1+ cell content.

Mobilization of Sca1+ progenitor cells from the bone marrow to the blood can be induced pharmacologically with VPC01091 and AMD3100

To assess whether pharmacological inhibition of $S1P_3$ could also mobilize progenitor cells into the peripheral blood, a novel $S1P_3$ antagonist, VPC01091, was used in conjunction with AMD3100. Wild type C57Bl/6 mice received 5mpk of VPC01091 followed, 30 minutes thereafter, by 5mpk AMD3100 intraperitoneally. One hour after AMD3100-induced mobilization, peripheral blood was drawn, incubated with Sca1 antibodies and counted with flow cytometry. AMD3100 in conjunction with VPC01091 significantly enhanced the percentage of Sca1+ progenitor cells (23.69%) in the peripheral blood of wild type mice compared to AMD3100 alone (9.54%) and was comparable to AMD3100-induced mobilization in the $S1P_3$-/- mice (Figure 3). To confirm that these cells were being mobilized from the bone marrow, the tibia and femurs of these mice injected with VPC01091 and/or AMD3100 were flushed and bone marrow cells were stained with antibodies against Sca1. There was a modest decrease in Sca1+ cell content in bone marrow of mice that had been treated with both VPC01091 and AMD3100 (52.92%) compared to AMD3100 alone (62.85%) suggesting that the Sca1+ progenitors that are elevated in circulation are indeed mobilized from bone marrow (Figure 3).

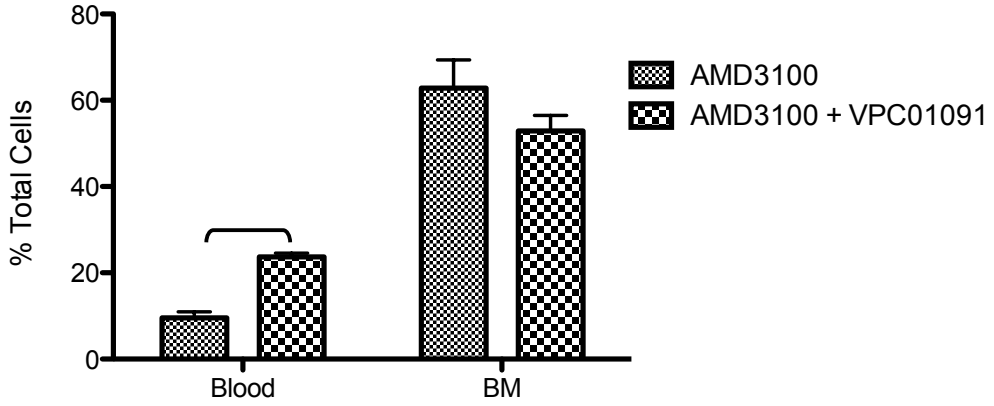

Figure 3. Flow cytometric analysis of peripheral blood and bone marrow progenitor cells. Pharmacological antagonism of $S1P_3$ with VPC01091 (checkered) significantly mobilizes Sca1+ progenitor cells in conjunction with AMD3100. The Sca1+ progenitor cell content in the bone marrow of mice treated with VPC01091 and AMD3100 was slightly decreased compared to AMD3100 alone.

VPC01211, a phosphorylated $S1P_3$ antagonist, attenuates the migration of Lineage1-/Sca1+/c-kit+ hematopoietic stem cells towards SDF-1α in transwell migration assays

To assess if $S1P_3$ inhibition affects HSC chemotaxis, whole bone marrow was flushed from the tibias of wild type mice and incubated with Lineage1, Sca1 and c-kit antibodies. FACS was used to isolate and sort Lineage1-/Sca1+/c-kit+ (LSK) cells. These LSK cells closely resemble human-derived CD34+ primitive hematopoietic stem cells. The cells were serum starved for 2 hours and pre-treated for 30 minutes with either plain media or VPC01211. VPC01211, a phosphorylated analog of VPC01091, was used because VPC01091 is a pro-drug that is only active when phosphorylated by sphingosine kinase 2 in vivo. Transwell migration assays were used to measure the chemotaxis of these cells towards SDF-1α and S1P, both known chemotactic agents for HSC. As expected, untreated LSK cells show a strong chemotaxis towards SDF-1α and S1P (Figure 4). Pre-treatment with VPC01211, however, abolished chemotaxis towards SDF-1α ($p=0.0502$) but did not affect chemotaxis towards S1P.

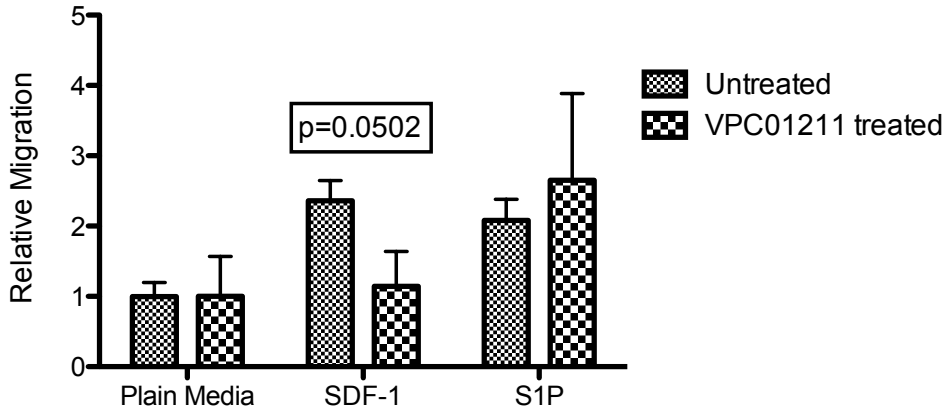

Figure 4. Transwell chemotaxis assay of murine HSC. Murine Lineage1-/Sca1+/c-kit+ hematopoietic stem cells were sorted, serum starved and treated with VPC01211 (checkered) or plain media (gray) for 30 minutes. They were seeded in the top of 5μm transwells and allowed to migrate towards plain media, SDF-1 or S1P for 4 hours. VPC01211 pre-treatment impaired chemotaxis towards SDF-1 but not S1P.

$S1P_3$ antagonism decreases engraftment of bone marrow-derived stem cells to bone marrow-derived stromal cells and is synergistic with CXCR4 antagonism.

We wanted to assess whether $S1P_3$ receptor antagonism affected the physical engraftment of HSC to stromal cells. Bone marrow niches contain stromal cells that secrete high concentrations of SDF-1α to keep stem and progenitor cells effectively tethered in these environments. We employed a cobblestone assay to measure the ability of marrow-derived cells to engraft to stromal feeder layers after pre-treatment with CXCR4 and/or $S1P_3$ antagonists. BMCs were flushed from the tibiae of wild type mice and pre-treated for 30 minutes with saline, AMD3100, VPC01211 or a combination of the two compounds. Cells were then seeded, at varying concentrations, onto a confluent layer of FBMD-1 stromal cells and allowed to attach for 2 hours. The non-adherent cells were washed off and the adherent cells were left in culture for 14 days to form cobblestones. Wells that had 6 or more cobblestones were considered positive. Untreated cells seeded at the highest density, 81,000 cells per well, were 75% positive for cobblestones at 14 days while wells that had cells pre-treated with AMD3100 were 70%

positive (Figure 5). VPC01211 treatment strikingly decreased the number of positive wells at all concentrations (50% at the highest concentration) and even further so in conjunction with AMD3100 (40% at the highest concentration). As Figure 5 shows, these trends were persistent at all four initial seeding concentrations of cells.

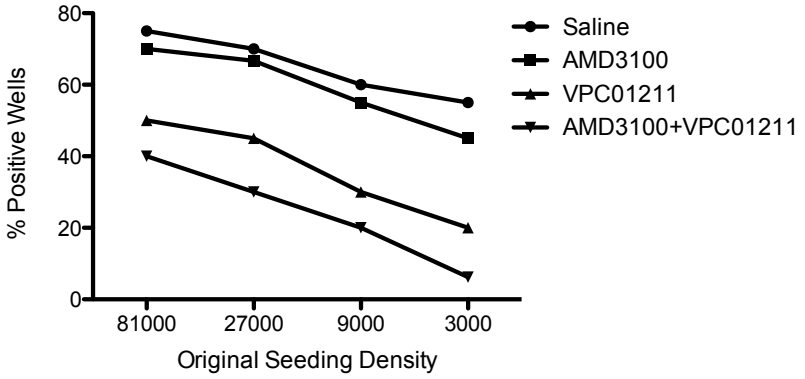

Figure 5. Cobblestone engraftment assay on FBMD-1 cells. Decreasing densities of bone marrow derived mononuclear cells were seeded on confluent layers of FBMD-1 stromal cells in wells after pretreatment with saline (circle), AMD3100 (square), VPC01211 (up-pointing triangle) or AMD3100+VPC01211 (down-pointing triangle), allowed to adhere for 2 hours and then washed off. After 14 days wells were assessed for cobblestone presence. AMD3100 and VPC01211 pre-treatment decreased engraftment of HSC. Combination of the two worked synergistically to drastically decrease engraftment of HSC to FBMD-1 layer.

$S1P_3$ inhibition mobilizes a significant number of stem cells without affecting their differentiative capabilities in vitro as measured with the colony forming unit assay

It became clear that $S1P_3$ antagonism alone or in combination with CXCR4 antagonism significantly mobilized cells from the bone marrow into peripheral blood by impairing signaling on the CXCR4/SDF-1α axis and attenuating the ability of bone marrow stromal cells to tether stem cells in their marrow niches. However, to this point, the functionality of the cells once mobilized had not been assessed. In order to measure the ability of stem cells mobilized into peripheral blood with $S1P_3$ antagonism to grow and differentiate we employed the colony forming unit (CFU) assay. Peripheral blood cells were washed and plated on Methocult stem cell media for 6 days and hematopoietic

stem cell-derived colonies were counted. As expected, the number of CFUs formed from 100μL of peripheral blood mobilized with AMD3100, 89, was significantly higher than the saline-treated cells, 31.42. Saline-treated $S1P_3$-/- mice and VPC01091-treated WT mice also showed an elevation in CFUs formed, 65 and 126.25 respectively, when compared to saline-treated wild type mice. Peripheral blood from wild type mice treated with VPC01091 + AMD3100 (185) or from $S1P_3^{-/-}$ mice treated with AMD3100 (219) both showed significant increases in CFUs formed after 6 days compared to all other groups (Figure 6). To confirm that this was a result of the antagonism at $S1P_3$ and not activation, or functional antagonism, at $S1P_1$, 5mpk FTY720 was used as a control. Wild type mice treated with FTY720 alone (33.75) or FTY720 + AMD3100 (104.75) failed to mobilize hematopoietic stem cells to the same extent as mice treated with VPC01091 alone or VPC01091 + AMD3100, respectively. This confirmed that it is $S1P_3$ antagonism and not $S1P_1$ activation that elicited hematopoietic stem cell mobilization.

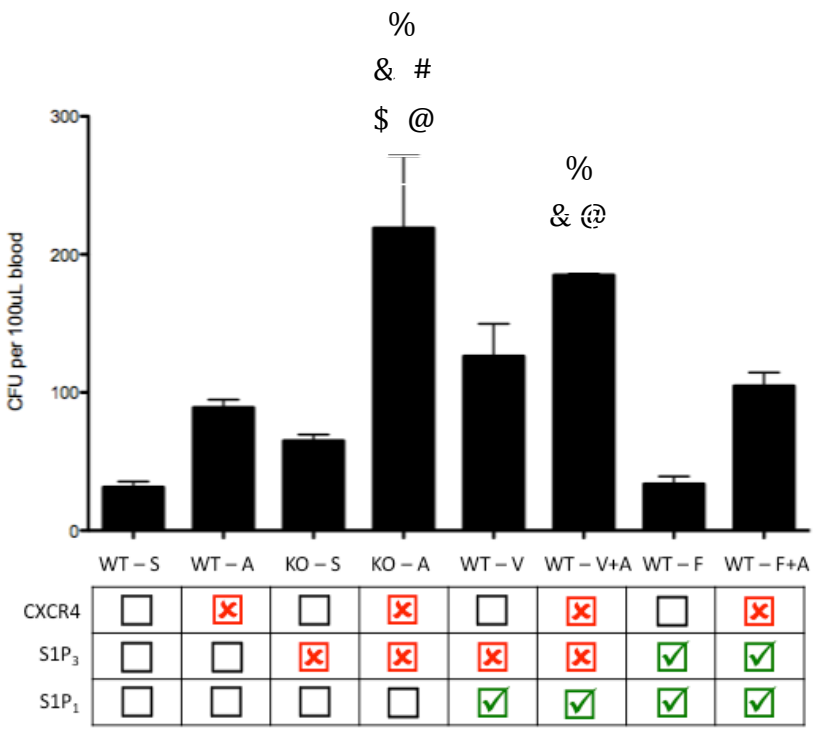

Figure 6. Colony forming unit assay for hematopoietic progenitor cell mobilization. 100µl blood was plated onto Methocult medium after pharmacologically/genetically induced stem cell mobilization. VPC01091 treatment with AMD3100 (WT-V+A) and genetic knockdown of $S1P_3$ with AMD3100 (KO-A) significantly enhanced mobilization efficiency. $S1P_1$ and $S1P_3$ activation with FTY720 (WT-F+A) did not reproduce these results. Black squares represent unaffected receptors, red x´s represent antagonized/knocked down receptors, green checks represent agonized/stimulated receptors. @: significant compared to WT-S. $: significant compared to WT-A. &: significant compared to KO-S. #: significant compared to WT-F. %: significant compared to WT-F+A.

Mice receiving peripheral blood grafts mobilized with AMD3100 and VPC01091 after lethal irradiation show improved survival over mice receiving grafts mobilized with AMD3100 alone

The final test on whether or not $S1P_3$ antagonism could mobilize functionally active HSC was an in vivo bone marrow transplant. GFP+ mice on a C57Bl/6 background were used as peripheral blood graft donors. Stem cells were mobilized with AMD3100 alone or AMD3100 + VPC01091 and equal volumes of peripheral blood were used to reconstitute wild type host mice after lethal irradiation. RFP+ bone marrow cells were used as a competitive graft to avoid graft failure (Figure 7).

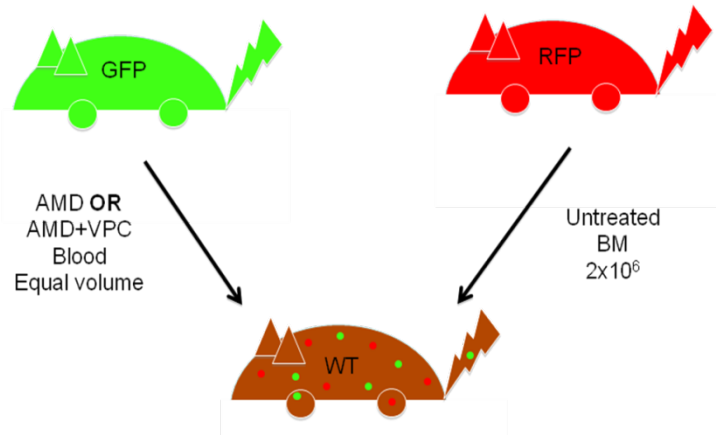

Figure 7. In vivo peripheral blood derived bone marrow transplant. Equal volumes of peripheral blood from GFP+ mice mobilized with AMD3100 alone or AMD3100 with VPC01091 were combined with 2 million bone marrow derived mononuclear cells from RFP+ mice and used to reconstitute wild type C57Bl/6 mice after lethal gamma irradiation. Chimerism (GFP/RFP) expression and repopulation efficiency (Sca1/CD45/CD11b) expression was measured over 56 days with flow cytometry.

An unexpected result was observed in mice that received AMD3100 alone mobilized grafts after lethal irradiation. By 14 days post transplantation there was 50% viability in the group treated with the AMD3100-mobilized grafts compared to 100% viability in the group treated with the AMD3100 + VPC01091-mobilized grafts (Figure 8). This persisted throughout 56 days of observation. The cause of death was not explored but it was most likely due to the lack of enough HSC to repopulate blood cells, infection, or a combination of the two.

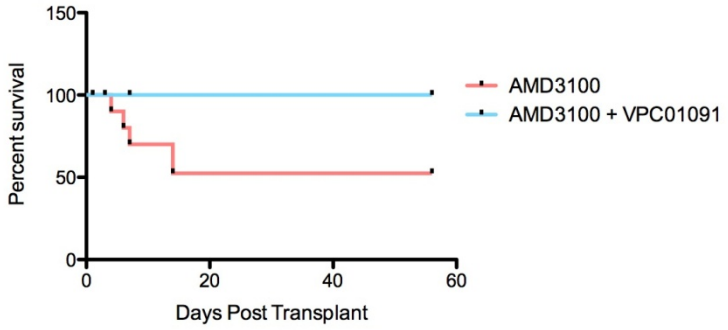

Figure 8. Survival of host mice after lethal irradiation and reconstitution with mobilized peripheral blood grafts. Survival significantly enhanced in mice that received peripheral blood graft that was mobilized with AMD3100 + VPC01091 (blue) relative to AMD3100-induced mobilization alone (red).

Donor chimerism increased in both blood and bone marrow of mice receiving peripheral blood stem cells mobilized with the combination therapy

For 56 days after donor reconstitution in wild type mice, blood and bone marrow were harvested for flow cytometry. Chimerism was assessed by measuring GFP+ (donor) cell content in the blood and bone marrow with flow cytometry. Figure 9 shows the kinetics of chimerism between the two groups in the blood. By the seventh day, there was a significant increase in peripheral blood donor cell content in the mice that received the AMD3100 + VPC01091 mobilized grafts (5.32%) compared to those which received the AMD3100 alone mobilized grafts (2.35%). This enhancement in chimerism persisted, and was amplified, throughout day 56 post transplantation when 11.4% of blood cells were peripheral blood donor derived with the AMD3100 graft compared to 29.92% with the AMD3100 + VPC01091 mobilized graft (Figure 9).

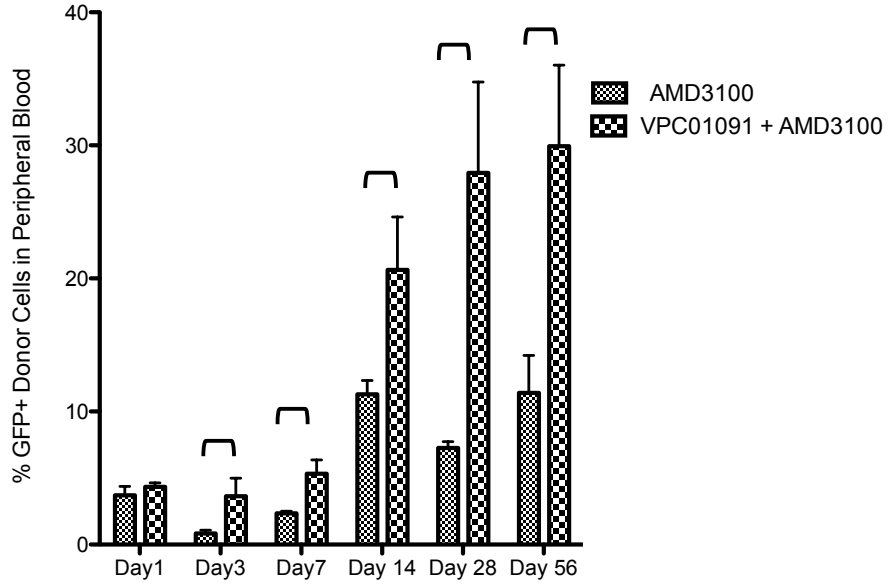

Figure 9. Peripheral blood donor cell chimerism for 56 days after transplant. Mice that received peripheral blood mobilized with VPC01091 and AMD3100 (checkered) show enhanced donor cell chimerism after 7 days post transplantation compared to mice that received graft mobilized with AMD3100 alone (gray)

The kinetics of chimerism in the bone marrow were very different than in the blood as it took a much longer time for donor cells to establish themselves in the marrow, suggesting that differentiated cells rapidly egress from the bone marrow into PB. Still, by 56 days post transplantation there was a significant increase in the donor content of mice which received the combination graft, 19.16%, compared to those which received the graft mobilized with AMD3100 alone 11.99% (Figure 10).

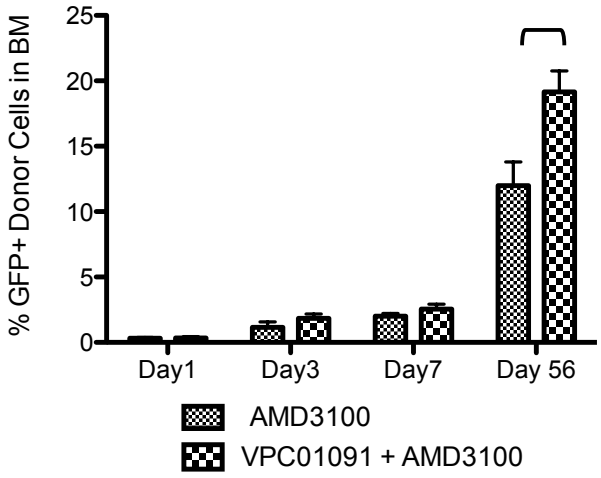

Figure 10. Bone marrow donor cell chimerism for 56 days after transplant. Mice that received peripheral blood mobilized with VPC01091 and AMD3100 (checkered) show enhanced donor chimerism in bone marrow 56 days post transplantation compared to mice that received graft mobilized with AMD3100 alone (gray)

Donor derived stem cells mobilized with VPC01091 and or AMD3100 maintain ability to repopulate mature blood cells but donor-derived repopulation efficiency is elevated in mice receiving graft mobilized with combination therapy.

To confirm that donor hematopoietic stem cells mobilized into peripheral blood and used in transplant grafts maintain the ability to differentiate and repopulate blood cells in vivo antibodies against Sca1, a progenitor cell marker, CD45, a myeloid cell marker, and CD11b, an inflammatory cell marker were used to assess repopulation efficiency in the blood and bone marrow for 56 days after transplantation. By 14 days post transplantation most of the host-derived cells in the peripheral blood had died and

were present at low levels in the circulation. The host-derived cell percentage dropped equally across both groups to less than 10% by 56 days. In the first week there were no significant differences in the percentage of Sca1+, CD45+ or CD11b+ peripheral-blood derived cells in the circulation of mice treated with AMD3100 or the combination therapy. By day 14, however, there were noticeable and significant increases in Sca1+, CD45+ and CD11b+ donor cells in mice treated with the combination graft compared to the AMD3100 alone graft (Figure 11D). The majority of the cells in peripheral blood were derived from the bone marrow competitive graft (RFP+) but significant increases in peripheral blood derived (GFP+) cell chimerism were observed in the combination group. Only in the combination group were peripheral blood-derived cells (CD11b+) observed to outnumber bone marrow-derived cells (Figure 11F). By 56 days the percentage of donor derived Sca1, CD45 and CD11b positive cells in the peripheral blood were 38.23%, 38.73% and 66.22% respectively, with the combination compared to 18.65%, 26.14% and 36.09% respectively with AMD3100 alone.

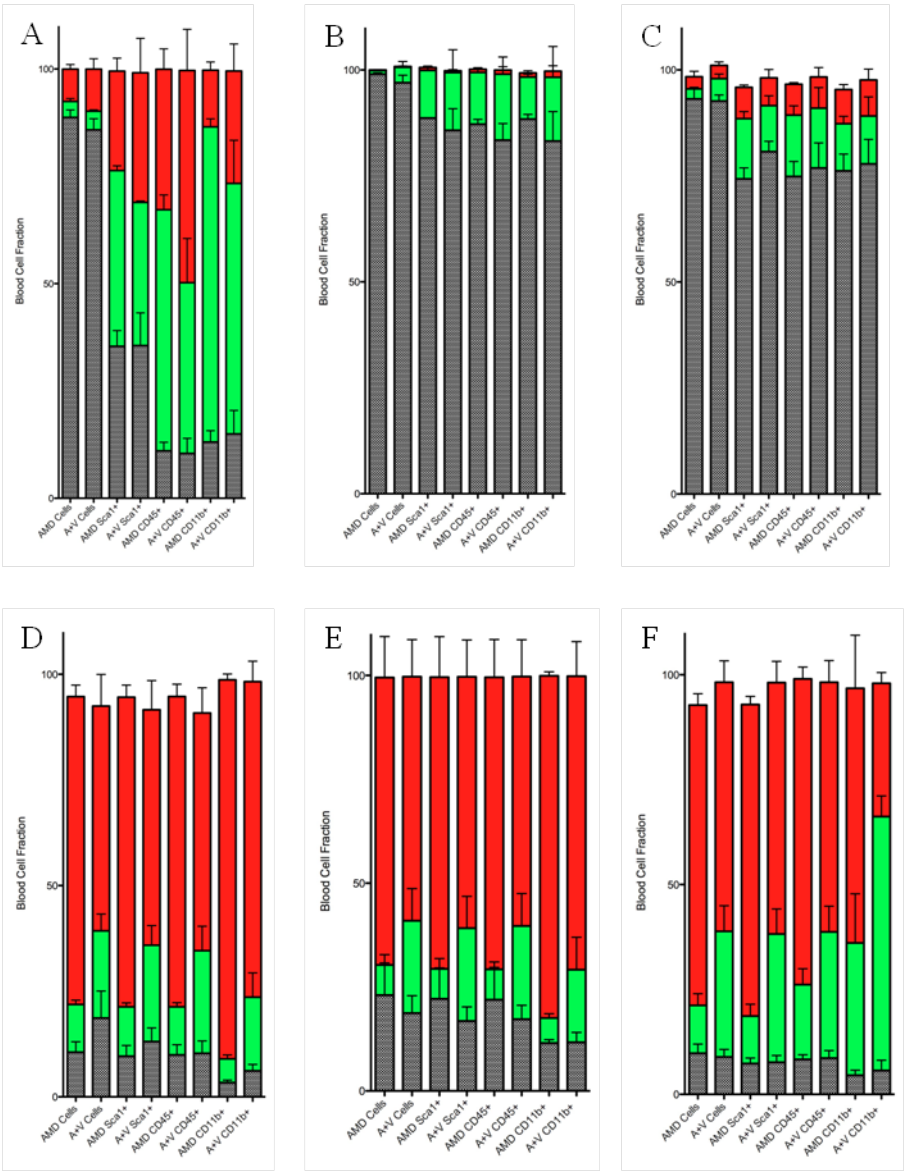

Figure 11. 56 day blood cell chimerism and repopulation. Peripheral blood was measured with flow cytometry for GFP, RFP, Sca1, CD45 and CD11b expression in host mice 1 (A), 3 (B), 7 (C), 14 (D), 28 (E) and 56 (F) days after mobilized peripheral blood graft transplantation. By 14 days post transplantation (D) the majority of host-derived cells had died and there are significant increases in peripheral blood derived (GFP+) cells that are Sca1, CD45 or CD11b positive in mice that received grafts mobilized with AMD3100 and VPC01091 (D-F). Bone marrow derived cells were the most prevalent in all of the cell types by, and after, 14 days post transplantation. A: Mice received graft mobilized with AMD3100. A+V: Mice received graft mobilized with AMD3100 + VPC01091. Red: Cells from competitive bone marrow derived graft. Green: Cells from peripheral blood mobilized graft. Gray: Host-derived cells.

Chimerism and blood cell repopulation in the bone marrow occurred on a much slower scale. Similar to in peripheral blood, the majority of bone marrow cells were host-derived at 1, 3 and 7 days post transplantation. There were no observable differences in

peripheral blood-derived (GFP+) cell chimerism in the bone marrow at 1, 3 and 7 days post transplantation. Surprisingly, there was a slight decrease in competitive graft bone marrow cell-derived bone marrow cells that were Sca1+, CD45+ and CD11b+ in mice that received grafts mobilized with AMD3100 and VPC01091. The overall percentage of PB-derived cells remained very small, between 0.32% and 1.82%, until 7 days post transplantation. By day 56 there was an elevation in peripheral blood derived Sca1+, CD45+ and CD11b+ cells in the bone marrow of mice that received the combination graft. Bone marrow Sca1+, CD45+ and CD11b+ PB-derived cells (32.84%, 29.47% and 32.55% respectively) were elevated in mice that received a graft mobilized with both VPC01091 and AMD3100 compared to those that received a graft mobilized with AMD3100 alone (24.89%, 20.41% and 29.39% respectively) (Figure 12 D).

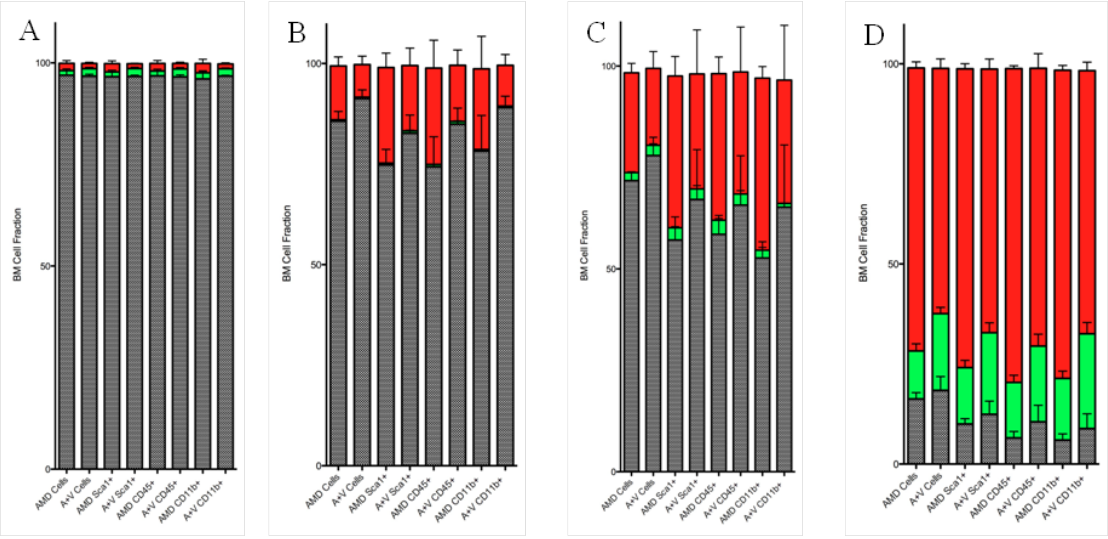

Figure 12. 56 day bone marrow cell chimerism and repopulation. Bone marrow was measured with flow cytometry for GFP, RFP, Sca1, CD45 and CD11b expression in host mice 1 (A), 3 (B), 7 (C) and 56 (D) days after mobilized peripheral blood graft transplantation. No observable differences in overall or Sca1+, CD45+ or CD11b+ peripheral blood derived (GFP+) cell chimerism was observed in the first 7 days of engraftment (A-C). By 56 days post transplantation (D) the majority of host-derived cells in the marrow had died and there were suggestive increases in peripheral blood derived (GFP+) cells that were Sca1, CD45 or CD11b positive in mice that received grafts mobilized with AMD3100 and VPC01091 (D). Bone marrow derived cells were the most prevalent in all of the cell types by 56 days post transplantation. A: Mice received graft mobilized with AMD3100. A+V: Mice received graft mobilized with AMD3100 + VPC01091. Red: Cells from competitive bone marrow derived graft. Green: Cells from peripheral blood mobilized graft. Gray: Host-derived cells.

Discussion

This thesis details work done to characterize the role of $S1P_3$ antagonism in hematopoietic stem cell mobilization from the bone marrow into peripheral blood and subsequent engraftment after peripheral blood-derived hematopoietic stem cell transplantation. Our main findings are the following: 1) $S1P_3$ antagonism disrupts the chemotaxis of HSC towards SDF-1α and decreases adhesion to BM stromal cells. 2) Pharmacological $S1P_3$ antagonism significantly mobilizes stem and progenitor cells from the bone marrow niches into peripheral blood. 3) Peripheral blood stem cells mobilized with $S1P_3$ and/or CXCR4 antagonism maintain their ability to repopulate blood cells in vitro. 4) Peripheral blood grafts mobilized with AMD3100 and VPC01091 enhanced survival, chimerism and repopulation efficiency in the host after hematopoietic reconstitution in vivo.

In these studies we aimed to compare mobilization induced by concurrent $S1P_3$ and CXCR4 antagonism to CXCR4 antagonism alone. The investigational drug AMD3100 is currently being used in conjunction with G-CSF to boost peripheral blood stem cell mobilization in poor mobilizers and we hoped to further enhance this elevation in stem cell mobilization. AMD3100 maximally mobilizes stem cells one hour after injection[29] therefore all of the time points correspond to this time frame of mobilization/treatment. Peripheral blood and bone marrow was collected one hour after mobilization and cells were pre-treated in vitro for one hour with AMD3100 unless otherwise stated. Other therapies, such as endogenous stem cell homing, may require different time scales of mobilization but we focused on this because it is relevant to hematopoietic stem cell transplantation.

The precise mechanism of how $S1P_3$ contributes to stem cell retention or mobilization has yet to be elucidated but this work provides sound and experimentally validated evidence that $S1P_3$ plays a critical role. Former studies have shown that $S1P_3$ activation induces the phosphorylation of CXCR4 by Src kinases and enhances migration towards SDF-1α[26]. Our data suggest that the reverse is also true and that antagonism of S1P3 may induce the dephosphorylation of CXCR4 or in other ways render the receptor inactive and impair migration towards SDF-1α. We showed that this is true with in vitro transwell migration assays of HSC pre-treated with VPC01211 and in cobblestone engraftment assays after pre-treatment. When HSC were pre-treated with VPC01211 they lost their ability to migrate towards SDF-1α but maintained their ability to migrate towards S1P. This supports that $S1P_3$-induced mobilization of stem cells from bone marrow niches is due to the inability of the cells to respond to SDF-1α gradients after S1P3 antagonism. Furthermore S1P is present at high levels in circulation, and often elevated during stress, so the preserved chemotaxis towards S1P may be beneficial in a therapy seeking to mobilize cells into circulation. Therefore, using a drug like VPC01091, which is an $S1P_1$ agonist and $S1P_3$ antagonist may be dually beneficial for therapies that seek to mobilize stem cells into the circulation by interrupting SDF-1α chemotaxis and enhancing, or at least maintaining, S1P chemotaxis.

An interesting result was observed in the cobblestone assay. It was expected that direct CXCR4 antagonism would impair HSC engraftment to stromal cells more than indirect $S1P_3$ antagonism. On the contrary, whole bone marrow cells pre-treated with VPC01211 engrafted, on the short term, less than cells treated with AMD3100 alone. $S1P_3$ activation has been shown to lie upstream of CXCR4 phosphorylation, or activation,

and our data supports that $S1P_3$ antagonism deactivates CXCR4. This finding speaks to the kinetics of receptor inactivation and suggests that $S1P_3$ antagonism may more rapidly deactivate CXCR4 than AMD3100. If the speed of de-phosphorylation induced by $S1P_3$ antagonism is quicker than the speed of competitive inhibition then the results observed would be expected. In other words, quickly de-phosphorylating CXCR4 may render the receptor inactive faster than direct inhibition and lead to more rapid mobilization when compared to AMD3100 inhibition. Both drugs used together caused the largest decrease in engraftment, as expected, suggesting that the two receptors work synergistically to tether stem cells in stromal cell-rich bone marrow niches.

Another potential benefit of mobilization with $S1P_3$ antagonism is that by solely targeting $S1P_3$ we are not directly blocking, inhibiting or down-regulating CXCR4 like many other drugs do. By avoiding any direct targeting of CXCR4 we can better preserve the long-term functionality of the cells and their ability to ultimately engraft in other tissues or a new host. It will be important, however, to look at the kinetics of VPC01091-induced $S1P_3$ antagonism and mobilization with VPC01091 alone. In designing endogenous therapies, for example, a transient dephosphorylation, inactivating CXCR4, followed by a reversal in this, allowing the mobilized cells to engraft in injured tissues, would be ideal. If $S1P_3$ antagonism by VPC01091 is long-lived and the cells do not recover from CXCR4 inactivation in enough time to home to other tissues the therapy may not be efficacious but preliminary studies do not support this.

The most important experimental question we answered in these studies was whether or not the cells mobilized with $S1P_3$ and CXCR4 antagonism would be fully functional in vivo. We showed that $S1P_3$ antagonism promotes stem cell mobilization

from the bone marrow into peripheral blood and that these cells do not lose their ability to differentiate in vitro, however it was important to address the efficacy of these cells in vivo. We chose to study chimerism and repopulation after lethal irradiation because of the recent advancements in using peripheral blood derived stem cells as donor grafts for hematopoietic stem cell transplantation. AMD3100 has already been explored as an agent to bolster G-CSF-induced mobilization but, as addressed earlier, it directly antagonizes CXCR4 and alone does not mobilize as many stem cells as with G-CSF therapy. We used AMD3100 alone and in conjunction with VPC01091 to see if the increase in hematopoietic stem cells in a donor graft caused by $S1P_3$ antagonism lead to an overall enhancement in therapy. Our first result, though surprising, answered this question directly. Host animals receiving grafts mobilized with AMD3100 and VPC01091 showed a 2-fold increase in survival by 14 days after reconstitution. In allogeneic transplants there is always some level of graft versus host disease as the immune systems between the graft and the host compete to dominate. In this study, however, the mice were syngeneic so the idea that VPC01091 + AMD3100-mobilized grafts attenuate GVHD is not valid. Gratwohl et. al reported that second to graft versus host disease, infection is the next leading cause of death after bone marrow transplant[30]. After lethal irradiation and reconstitution, the mice are susceptible to infection due to their weakened (or absent) immune systems. Over the course of days to weeks, the hematopoietic stem cells in the transplanted graft can overcome this by essentially creating a new immune system. In the AMD3100 alone-mobilized grafts it is possible that since fewer hematopoietic stem cells are mobilized there is not an adequate amount to replace the immune system before infection results in death while VPC01091+AMD3100 mobilized grafts mobilize enough

stem cells to overcome this deficit. It will be important to understand how these animals die after the transplant. Monitoring the weight, physical appearance and blood content of these animals will give key information to the cause of their death and inform this hypothesis. Another hypothesis regarding the increased survival in mice that received VPC01091+AMD3100 mobilized grafts is with respect to the profound neutropenia that results from the conditioning regimen and irradiation. As neutrophils are the first responders to infection, their absence would create a very vulnerable environment for infection of patients after irradiation. The levels of neutrophils in a graft can be a great predictor of the ability of that graft to rescue a patient from infection. It has been shown, and also corroborated by some of our work, that mice treated with VPC01091 show profound neutrophilia. It is thought that neutrophils are mobilized rapidly from the bone marrow as a form of compensation that results from the lymphopenia stimulated by $S1P_1$ functional antagonism. Therefore, it is possible that the AMD3100 + VPC01091 - mobilized grafts used to transplant mice are neutrophil-rich and thus able to protect these mice from infection and ultimately preventing death. Both theories are potentially valid and need to be explored in greater detail in the future.

We also looked at chimerism over a 56-day period as the transplanted cells began to replace the host's hematopoietic system. At early time points there were no significant differences in donor derived PB cell chimerism. It takes several days to weeks for donor cells to home to the bone marrow, engraft, and then begin to proliferate, differentiate and ultimately egress back into circulation. By 7 days post transplantation, however, there were significant increases in donor peripheral blood cell chimerism in the blood in the combination group, which was sustained throughout 56 days. By 56 days a significant

increase in bone marrow cell chimerism from donor PB derived cells was also observed in the combination group. Not only were peripheral blood donor-derived (GFP+) cells enhanced with the combination therapy, but differentiated peripheral donor-derived cells displaying Sca1, a progenitor cell marker, CD45, a myeloid cell marker, and CD11b, an inflammatory cell marker, were increased as well. Animals that received grafts mobilized with the combination therapy had higher fractions of mature, differentiated GFP+ cells which points to an overall enhancement in therapeutic efficacy. This data supports that the grafts mobilized with both AMD3100 and VPC01091 more rapidly and effectively engraft in the host bone marrow, differentiate into naïve and mature blood cells and egress from the marrow back into circulation in the host. This occurred in the peripheral blood and, on a slower scale, in the bone marrow. Enhancing stem cell mobilization in the PB grafts with $S1P_3$ antagonism creates a cell population that engrafts and repopulates significantly better in the host. The improvements in therapy are most likely multi-factorial, and another hypothesis is that the HSC mobilized are somehow primed by VPC01091 to engraft and repopulate more effectively. This could be due to anti-apoptotic signaling or an $S1P_1$/$S1P_3$-dependent signaling cascade leading to improved engraftment. Another contributing consideration, addressed above, is the idea that the strong survive. Creating a healthier graft that can more successfully fight infection but also more easily protect the donor cells as they engraft and differentiate could also be a factor in the enhancement in therapy we observed.

We now understand more clearly the role that S1P and its receptors play in stem cell retention, mobilization and engraftment and how modulation of S1P receptor signaling can be used to mobilize stem cells from their niches into circulation. There is no

debate that SDF-1α remains the primary signal to retain and attract stem cells but it is clear that other receptor/ligand families play important supporting roles in stem cell trafficking and can be regulated with, or independent of, CXCR4/SDF1-α signaling to mobilize stem cells for cell based therapies. Avoiding irreversibly impairing SDF-1α signaling is something that must always be considered when designing stem cell mobilizing regimens.

The present work is significant to the field of stem cell based therapies. Stem cells as forms of therapy are becoming more and more popular but there are many roadblocks to their use in vivo. Isolating and culturing stem cells while maintaining their functionality has proved very difficult. The idea of mobilizing stem cells in a donor and using these allogeneic cells in an impaired host has been widely explored but oftentimes these cells are not maximally mobilized and face rejection in the host and fail to engraft due to defective SDF-1α signaling and other factors. Even in endogenous stem cell therapies, where culture and rejection are not issues, the current gold standard to mobilizing stem cells can be proteolytic to CXCR4 and is not an ideal therapy to allow stem cells to home to target tissues and organs. The new knowledge that S1P receptors, specifically $S1P_3$, can be used to enhance the mobilization of stem cells without affecting their functionality is a step in the right direction towards creating efficacious therapies relying on patient stem cells.

Conclusion

Peripheral blood as a source of stem cells for therapy has become increasingly popular. In hematological therapies it has served as a replacement for harvesting stem cells directly from the bone marrow and is less painful and easier to obtain. However, due to the small fraction of stem cells in circulation, stem cell mobilizing agents must be used to enrich peripheral blood with stem cells mobilized from their niches. The current gold standard does not always achieve a sufficient level of mobilization, impairs signaling on the CXCR4 axis, oftentimes permanently, which is a detriment to subsequent engraftment or homing, is very expensive and takes several injections over several days to maximally mobilize cells. Strategies that rapidly mobilize sufficient numbers of cells without impairing engraftment or homing ability would greatly enhance the efficacy of therapies using peripheral blood stem cells. We have shown that inverse agonism of $S1P_3$ rapidly mobilizes a significant number of hematopoietic stem cells, with or without an exploratory CXCR4 antagonist, AMD3100. Furthermore, these cells are functionally active and capable of engrafting in vivo and contributing to host blood cells through differentiation and repopulation, after lethal irradiation, in both the bone marrow and the blood. Though $S1P_3$ and CXCR4 antagonism were synergistic and produced maximal mobilization levels together, $S1P_3$ antagonism alone seemed to mobilize at least as many stem cells as AMD3100, suggesting that it is just as efficacious. Using S1P receptor signaling, specifically VPC01091, to mobilize cells into an allogeneic graft also seemed to enhance survival after transplant which is most likely attributed to the enhancement of stem cell content as well as neutrophilia which would help combat infection and immune system rejection. Furthermore, by avoiding directly interacting with SDF-1/CXCR4

signaling, the mobilized cells may be quickly primed for re-introduction and engraftment in the host or target tissue in both exogenous and endogenous therapies, respectively. As we continue to look for pharmacological methods to regulate stem cell trafficking, S1P should consistently be regarded. Many regenerative stem and progenitor cells, outside of HSC, are chemotactically regulated by S1P and its receptors. With an increasing pool of S1P receptor specific agonists and antagonist, creating strategies to control where and when these cells traffic with S1P signaling may help develop effective strategies to use these stem cells in regenerative medicine while circumventing many of the issues that have become seemingly insurmountable obstacles in pushing stem cell based therapies to the clinic.

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