P-selectin glycoprotein ligand regulates the interaction of multiple myeloma cells with the bone marrow microenvironment

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ABSTRACT

Interactions of multiple Myeloma (MM) cells with the bone marrow (BM) microenvironment play a critical role in the pathogenesis of MM and in the development of drug resistance. Selectins are involved in extravasation and homing of leukocytes to target organs. In this study, we focus on adhesion dynamics that involve P-Selectin-Glycoprotein-Ligand-1 (PSGL-1) on MM cells and its interaction with selectins in the BM microenvironment. We show that PSGL-1 is highly expressed on MM cells, and regulates the adhesion and homing of MM cells to cells in the BM microenvironment in vitro and in vivo. This interaction involves both endothelial cells and bone marrow stromal cells. Using loss of function studies and a small molecule pan-selectin inhibitor GMI-1070, we show that PSGL-1 regulates activation of integrins and downstream signaling. We also document that this interaction regulates MM cell proliferation in co-culture with BM microenvironmental cells, and their development of drug resistance. Furthermore, inhibiting this interaction with GMI-1070 enhances the sensitization of MM cells to bortezomib in vitro and in vivo. These data highlight the critical contribution of PSGL-1 to the regulation of growth, dissemination, and drug resistance in MM, in the context of the BM microenvironment.
INTRODUCTION

The progression and dissemination of multiple myeloma (MM) involves continuous spread of MM cells in and out of the bone marrow (BM)\textsuperscript{1,2}. Interactions of MM cells with the BM microenvironment play a critical role in the pathogenesis of MM and in the development of drug resistance\textsuperscript{3-5}. We have shown that blocking the CXCR4/SDF1 axis disrupts the interaction of MM cells with the BM microenvironment, which in turn enhances the efficacy of therapeutic agents against the MM cells\textsuperscript{6}.

Selectins are cell surface adhesion molecules that contain a lectin-like domain with selectivity for binding to specific saccharide chains \textsuperscript{7}. Each of the three types of selectins (E, L and P), has a unique tissue distribution (in endothelium, Leukocytes and platelets, respectively)\textsuperscript{7}, and distinct classes of leukocytes use specific combinations of selectins to interact with endothelium\textsuperscript{8,9}. The binding affinity of selectins to their ligands is relatively low, but is nonetheless strong enough to serve as a biologic brake that rapidly decelerates leukocytes as they roll on endothelial cells (ECs). While rolling, leukocytes are activated by binding to selectin, and by chemo-attractants such as CXCR4/SDF-1; activation increases the affinity of the integrins on leukocytes for ligands found in the endothelium. The presence of a chemotactic signal outside a venule induces leukocytes to squeeze between the ECs of the venule, and migrate into the target organ (extravasation)\textsuperscript{10,11}; inhibition of rolling, by blocking selectins, decreases this extravasation of leukocytes \textsuperscript{12-14}. In fact, small molecule inhibitors of selectins have clinical activity\textsuperscript{12,15-18}. Synthetic inhibitors of selectin also have a demonstrated ability to improve the manifestation of psoriasis, and of allergen-induced asthma in humans\textsuperscript{19}, and in mouse models of skin
inflammation\textsuperscript{20}; such agents are currently being tested in clinical trials for treatment of inflammatory diseases, and also preclinical studies of solid tumor metastasis\textsuperscript{21-24}.

P-selectin glycoprotein ligand-1 (PSGL-1) is a dimeric, mucin-type glycoprotein ligand expressed by all leukocytes and involved in homing of leukocytes to target tissues\textsuperscript{25}. PSGL-1 plays an important role in organ targeting during inflammation, and inhibition of PSGL-1 represents an attractive basis for anti-inflammatory strategies\textsuperscript{25,26}. Earlier studies show that PSGL-1 is highly expressed in MM\textsuperscript{22,27}. PSGL-1 was shown as a novel therapeutic target for monoclonal-antibody-mediated MM immunotherapy, in which PSGL-1 was shown to play a humoral immunotherapy of MM, and combination of treatment with monoclonal-antibody of PSGL-1 with chemotherapy improves tumor cytotoxicity\textsuperscript{23,28}.

In this study we targeted PSGL-1 by inhibiting its interaction with selectins in the microenvironment as a therapeutic prospect; however, we focus on adhesion dynamics that involve PSGL-1 on MM cells and its interaction with selectins in the BM microenvironment. We show that PSGL-1 regulates the adhesion and homing of MM cells to cells in the BM microenvironment, including ECs and bone marrow stromal cells (BMSCs); we also document that this interaction regulates MM cell proliferation and their development of drug resistance, both \textit{in vitro} and \textit{in vivo}. Furthermore, inhibiting this interaction with GMI-1070, a selective novel therapeutic agent that targets selectins, enhances the sensitization of MM cells to bortezomib \textit{in vitro} and \textit{in vivo}. These data highlight the critical contribution of PSGL-1 to the regulation of growth, dissemination and drug resistance in MM, in the context of the bone marrow microenvironment.
METHODS

Reagents

The pan-selectin inhibitor GMI-1070 was obtained from GlycoMimetics, Inc. (Gaithersburg, MD). Recombinant SDF-1, recombinant selectins, and antibodies against E, L and P selectins were purchased from R&D (Minneapolis, MN). Monoclonal antibodies for western blotting were procured from Cell Signaling Technologies (Danvers, MA), the anti-PSGL-1 antibody was from Millipore (Billerica, MA), and the one against β1-integrin (clone 12G10) was from Ab-Cam. Calcein-AM cell labeling dye was obtained from Invitrogen (Carlsbad, CA). Scramble-siRNA and SmartPool siRNAs for PSGL-1, E-selectin, L-selectin and P-selectin were bought from Dharmacon (Lafayette, CO, USA). Lipofectamin-2000 was purchased from Invitrogen (Carlsbad, CA).

Cells

The MM1s cell line was purchased from ATCC (Manassas, VA) whereas the OPM1, OPM2, H929, RPMI8226, U266, and U266LR7 lines were the kind gift of Prof. Jesús F. San Miguel (Salamanca, Spain). Human umbilical vein ECs (HUVECs) were purchased from Lonza (Walkersville, MD). CD138+ cell were isolated from MM patients by bead-selection, and BM stromal cells (BMSCs) cultures were established as previously described6.

Informed consent was obtained from all patients in accordance with the Declaration of Helsinki for primary MM patient samples. Approval of the animal study protocol was obtained from the Institutional Review Board of the Dana-Farber Cancer Institute.
Immunohistochemistry

To detect PSGL-1, BM aspirates from 17 MM patient and 3 normal subjects were rinsed with PBS, fixed with 4% formaldehyde in PBS, dehydrated with ethanol, embedded in paraffin and sectioned; tissue was immuno-stained with mouse anti-human PSGL-1.

Gene expression analysis

To determine the gene expression of PSGL-1 in MM, we used datasets from the Gene Expression Omnibus (Mayo Clinic, series number GSE 6477). Expression levels of PSGL-1 (probe ID 209879_at) were compared in plasma cells from normal subjects (n=15), MGUS patients (n=20), smoldering MM patients (n=23) and newly diagnosed MM patients (n=68).

Expression of PSGL-1 and selectins by Flow Cytometry

MM cell lines (MM1s, OPM1, OPM2, H929, RPMI8226, U266, and U266LR7) or HUVECs were treated with mouse anti-human antibodies for PSGL-1; or E-, L-, or P-selectin (5μg/ml), or with an isotype control, for 1 hr on ice; cells were then immersed in FITC-secondary antibody; expression was determined with flow cytometry analysis, and was quantified as the ratio of the mean-fluorescence-intensity (MFI) of detected target/MFI of isotype control.

To detect selectin on primary ECs, mononuclear cells from BM aspirates of MM patients were treated with primary antibodies against each selectin, followed by a FITC-anti-mouse secondary antibody, washed, and treated with APC-conjugated anti-CD31.
antibody. ECs were gated as APC-CD31+ cells, and the expression of each selectin was quantified as the ratio of the FITC-MFI of each selectin/ FITC-MFI of isotype control.

**Knock down of PSGL-1 in MM1s cells and of E, L and P selectins in HUVECs**

MM1s cells were cultured overnight in 6-well-plates in OPTI-MEM media, washed, and cells in each well were immersed overnight in a mixture of lipofectamin-2000 (7 μl) with scramble-siRNA or SmartPool PSGL-1 siRNA (100 pmole) in a final volume of 2ml of OPTI-MEM. Similarly, HUVECs were transfected with scramble siRNA or with SmartPool siRNA for E-selectin, L-selectin or P-selectin. Twenty four hours later, the transfection solution was replaced with complete media and cells were used after an additional 24–48hrs.

**Interaction of recombinant selectins with MM cells**

MM1s cells transfected with scramble siRNA or PSGL-1 siRNA, or treated with increasing concentrations (0, 250 and 500 μM) of GMI-1070, were incubated with free human Fc-chain (isotype control) or with chimeras of human-Fc chain and recombinant human E, L or P-selectin (10 μg/ml), followed by FITC-conjugated mouse-anti human Fc. The interaction of the selectins with MM cells was analyzed by flow cytometry and quantified as the ratio of the MFI of each selectin/ MFI of isotype control.

**Adhesion of MM cells to HUVECs and BMSCs**

HUVECs or BMSCs were cultured overnight to confluence in 96-well plates (5x10³ cells/well) before initiating the adhesion assay. Selectins on HUVECs were inhibited by
knockdown with siRNA for E-, L-, or P-selectin, by treatment with blocking antibodies against E-, L- or P-selectin (10 μg/ml, for 1 hr), or with increasing concentrations of GMI-1070 (0, 250 and 500 mM for 1 hr) before performing the adhesion assay. MM1s, OPM1 and H929 cells were serum-starved for 3 hours, pre-labeled with Calcein-AM, added to the HUVECs or BMSCs (1x10^5 cells/well), and allowed to adhere for 2 hours at 37°C. Non-adherent cells were aspirated away, the HUVECS or BMSCs were washed, and fluorescence intensity was measured using a fluorescent-plate reader (Ex/Em=485/520 nm). In some experiments MM cells were treated with scramble-siRNA or siRNA for PSGL-1; and in other experiments with isotype control antibody or with a blocking antibody of PSGL1 (clone KPL-1, Millipore, Billerica, MA).

**Adhesion of MM1s cells to HUVECs under shear flow**

HUVECs were grown to confluence in 96-well plates (5x10^3 cells/well) prior to performing the adhesion assay. HUVECs were then activated with vehicle or TNF-α (30U/ml) for 3 hours or with IL-4 (3ng/ml) for 24 hours, followed by histamine (2.25 mM) for 4 hours. The cells were then treated with mouse anti-human antibody against each of the selectins for 1 hour, followed by a secondary FITC antibody for 1 hour. Non-bound antibodies were washed away and the expression of selectins was assessed by measuring fluorescence intensity (Ex/Em=485/520 nm).

Adhesion of MM cells to HUVECs under conditions of shear flow was measured with use of a parallel plate flow chamber. HUVEC monolayers were grown in 35 mm tissue culture plates, and activated as above. A MM cell suspension (2x10^6cells/ml) was perfused through the chamber, over the HUVEC monolayers, at a shear rate corresponding
to a wall shear stress of 0.9 dynes/cm². For each experiment, the cell suspension was allowed to flow through the chamber for 3 minutes, after which digital images were collected to quantify the results. Inovision’s IC300 digital image system, driven by a Silicon Graphics Indigo2 workstation, was used to acquire and analyze the images.

**Trans-endothelial migration and Chemotaxis of MM cells**

HUVECs (5x10³ cells/basket) were incubated overnight in the upper chamber of an eight-micron pore filters (Costar, NY) before performing the adhesion assay. Selectins on HUVECs were inhibited by knockdown with siRNA or by 1hr treatment with blocking antibodies (10 μg/ml), or by 1hr treatment with GMI-1070 (0, 250 and 500 μM) prior to carrying out the trans-endothelial migration assay. MM1s cells were serum-starved for 3 hours, then added to the upper chamber of the basket (2x10⁵ cells/well), and left to migrate (for 4 hrs at 37°C) towards the lower chamber, which contained 0 or 30 nM of SDF1α. In some cases, PSGL-1 in the MM cells was knocked down by siRNA, and in other experiments MM cells were treated with isotype control antibody or with a blocking antibody of PSGL1. In other cases, migration was assayed without pre-coating the filter: in these instances, MM1s were treated with scramble siRNA, PSGL-1 siRNA, or with increasing concentrations of GMI-1070. Those cells that made it to the lower chambers were counted via flow cytometry.

**In vivo extravasation of MM cells**

*In vivo* flow cytometry was used to examine extravasation. SCID mice were pre-treated for 1 hour with vehicle, anti-P-selectin antibody (250 μg/kg, iv) or GMI-1070 (25
mg/kg, ip). Calcein-labeled MM1s cells (1 x 10^6) were injected into anesthetized mice and immediately thereafter, a 488-nm laser was focused on an artery in the mouse’s ear. Signals were detected through a 680/25-nm bandpass filter, and analyzed with Matlab software. Cell counts were obtained every 5 minutes for 40 minutes after the cell injection.

**In vivo homing to the BM**

We used *in vivo* confocal microscopy to test the homing of MM1s cells to the BM *in vivo*. Calcein-labeled MM1s cells (1 x 10^6) were injected into anesthetized SCID mice that had been pre-treated with vehicle or GMI-1070 (25 mg/kg, ip) for 1 hour prior to the cell injection. For some cases, PSGL-1 in the MM cells was knocked down by siRNA.

We performed in vivo confocal microscopy as described previously 6,29. Briefly, A skin flap was made in the scalp of the mice to expose the underlying skull surface and Evans Blue was injected intravenously immediately before imaging to visualize blood vessels. High-resolution images with cellular detail were captured 30 minutes after cell injection through the intact mouse skull at depths of up to 250μm from the surface of the skull using a 30x 0.9NA water immersion objective lens (Lomo, St. Petersburg, Russia). Multiple imaging depths were acquired and a maximum intensity z-projection was performed in Image J to merge the images. Calcein-labeled MM cells and Evans-Blue were excited with 491-nm and 638-nm single-photon lasers, and were detected via 528/19-nm and 680/25-nm bandpass filters, respectively.
Adhesion-related signaling

MM1s cells (5x10^6) were serum-starved for 3 hours, and stimulated for different amounts of time with recombinant P-selectin (10 μg/ml). Moreover, MM1s cell were treated with recombinant P-selectin (10 μg/ml, 30 minutes) in the presence or absence of GMI-1070 (500 μM). We also tested the effect of GMI-1070 on signaling induced in MM cells by adhesion to HUVECs. HUVECs were treated with 0, 250 and 500 μM of GMI-1070 for 1 hour, was followed by washing with PBS. Non-treated MM cells were co-cultured with the HUVECs for 1 hour, and then harvested by placing the dishes on ice for 10 min and dislodging the MM cells from the HUVECs by gentle pipetting. The MM cells were lysed, and immunoblotted for the detection of p-FAK, p-Src, coflin, p-AKT, p-GSK3 and α-tubulin.

Immuno-fluorescence for the detection of up-regulation of integrins

MM1s cells were treated for 30 minutes with recombinant P-selectin (10 μg/ml) in the presence or absence of GMI-1070 (500 μM); non-treated MM1s cells served as a control. Cells were fixed in formaldehyde for 15 minutes at room temperature, spun onto slides, washed, blocked, stained with (5 μg/ml) anti-β1-integrin antibody at 4°C overnight, followed by FITC-labeled secondary antibody for 1 hour, washed, mounted, and analyzed with use of a Nikon inverted TE2000 microscope equipped with a 20x Plan-Fluor DIC NA0.5 objective. The percent of highly labeled cells was calculated for each condition and normalized to the percent in the non-treated control.
**Proliferation assay**

HUVECs or BMSCs (5x10³ cells/well) were plated overnight in 96-well plates. MM1s cells (2x10⁵ cells/ml), transfected with scrambled siRNA, or with siRNA for PSGL-1, were cultured for 24hrs, with or without BMSCs or HUVECs. In some cases, HUVECs were pretreated with GMI-1070 (0 or 500μM) prior to the addition of the MM1s to the culture. In some cases, MM1s cells were treated with vehicle, bortezomib (5nM), or dexamethasone (25nM). Proliferation was quantified with use of the BrdU assay, 24hrs post co-culture used according to manufacturer’s instructions (BD, Franklin Lakes, NJ).

**Tumor initiation in vivo.**

MM1s cells, transfected with scramble siRNA or PSGL-1 siRNA, were injected intravenously into the tail vein (3x10⁶ cells in a 100μL of PBS per mouse) or into the BM of the tibia (2x10⁵ cells in a 20μL of PBS per mouse) of SCID/bg mice. Mice were sacrificed after 1 week; BM was extracted from two femurs of mice injected intravenously and the tibia of injected mice. Red blood cells were lysed, and mononuclear cells were incubated for 1 hr with mouse-anti-human-CD138 antibody conjugated to PerCP-Cy5.5 (5 μg/ml) and analyzed using a flow cytometer.

**Sensitivity of MM cell to chemotherapy in vivo**

MM1s cells that express Luciferase were injected iv into 40 SCID mice; treatment was started after tumors were first detected by bioluminescence imaging. Mice were divided to four groups of 10 each: (1) control group, received weekly ip injection of vehicle and implanted with Alzet®-Pump-2002 (flow rate 0.5μl/hr) loaded with vehicle
every two weeks (for 4 weeks); (2) GMI-1070 treated group, which received weekly ip injection of vehicle and was implanted with a pump loaded with 200 μl of 150mg/ml GMI-1070 which was changed every two weeks (for 4 weeks); (3) bortezomib treated group, which received weekly ip injection of bortezomib 1.5 mg/Kg and implanted with a pump loaded with vehicle which was changed every two weeks (for 4 weeks), and (4) combination group, which received weekly ip injection of bortezomib 1.5 mg/Kg and was implanted with a pump loaded with 200 μl of 150mg/ml GMI-1070 which was changed every two weeks (for 4 weeks). Tumor progression was monitored once a week by bioluminescence imaging, while the mice were on treatment (4 weeks) and after the treatment was stopped (4 weeks).

**Statistical analysis**

Results were reported as the mean +/- standard deviation for three experiments. Samples were compared by the Student T-test, Two Factor ANOVA or Log Rank test. Results were considered significantly different for p values less than 0.05.
RESULTS

PSGL-1 is highly expressed on MM Cells

The expression of PSGL-1 on MM cells was examined in BM biopsies from 17 MM patients; we used immunohistochemistry, or flow cytometry on fresh MM samples. In agreement with previous findings, we confirmed that PSGL-1 was highly expressed on MM cells in all 17 MM patients tested (Figure 1A). The expression of PSGL-1 on MM cells was also confirmed by flow cytometry for 7 MM cell lines: again, high levels of expression of PSGL-1 were detected on all 7 cell lines tested (Figure 1B). PSGL-1 was also differentially expressed on plasma cells isolated from patients at different stages of MM. PSGL-1 gene expression increases with disease progression from MGUS to smoldering MM, and then to active disease, as shown by analysis of the published gene expression datasets by Chng et al\textsuperscript{30} (Figure 1C). Together, these observations indicate that PSGL-1 is highly expressed on all MM cells.

Moreover, we examined the expression of selectin on MM cells, relative to plasma cells obtained from the bone marrow (BM) of healthy donors. Unlike the high expression of the selectin ligand, we found no expression of E-, L- and P- selectins on any of the MM cells tested (primary samples and cell lines) (Supplementary Figure 1).

The role of PSGL-1 in the interaction of recombinant selectins with MM cells

When we tested the interaction of recombinant selectins with plasma cells derived from normal-subjects, from MM patients, or from MM cells lines, we observed that that E-selectin did not interact with MM cells and normal plasma cells; L- and P-selectins, on the other hand, were highly interactive with MM cell lines and MM primary samples, but not
with normal plasma cells (Figure 1D). This is in agreement with previous reports showing that PSGL-1 interacts with both L- and P-selectins\textsuperscript{31}. Moreover, the differences in the interaction between of MM cells and normal plasma cells are apparently due to lower expression of PSGL-1 in normal plasma cells, or potentially due to different glycosylation patterns of the PSGL-1, which is known to alter binding of selectins to the ligand \textsuperscript{32,33}.

To confirm the role of PSGL-1 in the interaction of selectins with MM cells we knocked down PSGL-1 in MM1s cells using siRNA (as verified by flow cytometry in Supplementary Figure 2). Knockdown of PSGL-1 decreased the high level of interaction of L-and P-selectins with MM cells (the effect was stronger for interaction of P-selectin on MM cells). While E-selectin did not bind with MM cells, and the knockdown of PSGL-1 did not alter this effect (Figure 1E). We further confirmed that GMI-1070, a specific selectin inhibitor, which is currently being used in clinical trials, inhibits the interaction of L and P selectins with MM cells (Figure 1F). Supplementary Figure 3 shows that GMI-1070 does not induce cytotoxicity in MM cells and HUVECs.

**PSGL-1 regulates adhesion of MM cell to ECs.**

We first determined the expression of selectins on ECs in 5 BM aspirates collected from patients with MM, and in HUVECs. E- and P-selectins (but not L-selectin) were highly expressed on ECs isolated from the BM of MM patients, and HUVECs (Figure 2A). To investigate the role of each of selectin in the interaction of ECs with MM cells we knocked down each of the selectins in HUVECs (as confirmed by flow cytometry in Supplementary Figure 4). As shown in Figure 2Bi, knockdown of P-selectin, but not of E- or L-selectins, decreased the adhesion of MM cells to HUVECs. Similarly, blocking
antibodies for P-selectin (but not for E- or L selectins) inhibited the adhesion of MM cells to HUVECs (Figure 2Bii). Moreover, increasing the expression of P-selectin (by stimulation with IL4 and histamine) enhances the adhesion of MM cells to HUVECs, while increasing the expression of E-selectin (by stimulation with TNFα) did not increase this adhesion (Figure 2C). These results indicate that P-selectin regulates adhesion of MM cells to ECs.

We next investigated role of PSGL-1 in the adhesion of MM cells to ECs. As shown in Figure 2D, knock-down of PSGL-1 in MM cells decreased the adhesion of MM cells to HUVECs. Similarly inhibition of the interaction between P-selectin in HUVECs and PSGL-1 in MM cells decreased the adhesion of MM cells to HUVECs in a dose-dependent manner (Figure 2E). These results were reconfirmed using a blocking antibody which showed that blocking PSGL-1 decreased the adhesion of MM cells to HUVECs (Figure 2F). Together, these findings indicate that interactions between selectins and selectin ligand regulate adhesion of MM tumor cells with ECs.

**PSGL-1 regulates trans-endothelial migration of MM cell**

To understand the physiological process of extravasation of MM cells through ECs, we tested the role of the interaction PSGL-1 with P-selectin during migration of MM cells through a monolayer of ECs in response to the chemokine SDF1α. Figure 3A shows that blocking P-selectin (by siRNA - Figure 3Ai; or blocking antibodies - Figure 3Aii), significantly reduced the trans-endothelial migration of MM cells by 70%, while blocking E- or L-selectin did no such effect.
Moreover, down-regulation of PSGL-1 on MM cells by siRNA decreased the trans-endothelial migration of MM cells through the endothelial cell monolayer in vitro (Figure 3B). Similar results were obtained when the interaction between selectin on the ECs and PSGL-1 on MM cells was inhibited using the selectin inhibitor GMI-1070 (Figure 3C). To confirm that the effect on trans-endothelial migration was through the specific interaction between selectins in ECs and PSGL-1 in MM cells, we eliminated the endothelial cell component from the experiment, and tested the effect of down-regulation of PSGL-1 in MM cells and of GMI-1070 on the trans-well (through the filter) migration of MM cells in response to SDF1 through. Neither down-regulation of PSGL-1(Figure 3D), nor treatment with GMI-1070 (Figure 3E), had an effect on migration of MM cells in response to SDF1α. These results were reconfirmed using a blocking antibody which showed that blocking PSGL-1 decreased the trans-endothelial migration of MM cells (Figure 3F). These observations indicate that the interaction of P-selectin in ECs with PSGL-1 in MM cells play a critical role in the transendothelial migration, which represents the first step of the extravasation process and homing of MM cells into the BM in vivo.

**PSGL-1 regulates extravasation and homing of MM cells to BM in vivo.**

To examine the role of the interaction of PSGL-1/P-selectin on homing of MM cells to the BM in vivo we used in vivo flow cytometry which measures the number of circulating cells as number of cells which pass in an appropriate artery in the mouse ear per minute 29. Figure 4A shows that in control non-treated mice, more than 90% of the MM cells had extravasated within 25 min post-injection, while in mice pre-treated with blocking
P-selectin antibody or with GMI-1070 (25mg/kg), MM cells displays delayed extravasation: more than 50% of the cells were still circulating at 35 min post-injection.

**PSGL-1 regulates homing of MM cells to BM in vivo.**

We also used *in vivo* live confocal imaging to examine homing of MM cells to the BM, in which we detect the presence of MM cells in the BM niches of the skull of the mouse. PSGL-1 in MM cells was knocked down, or mice were treated with GMI-1070, to interfere with the interaction ECs and MM cells. Figures 4Bi shows that knock-down of PSGL-1 in MM cells decreased the number of MM cells that had homed to the BM an average of 18 images taken from 3 different mice, while Figure 4Bii shows representative images of the BM in the calvaria of mice: showing less homing of Calcein-labeled MM cells (Green), in MM cells transfected with PSGL-1 siRNA compared to scramble siRNA. Blood vessels are labeled with Evans-Blue (Red). Similarly, Figures 4Ci shows that inhibition of selectins in the host environment by GMI-1070 (25 mg/kg) decreased the number of MM cells that homed to the BM and Figure 4Cii shows representative images of homing MM cells in mice treated with GMI-1070 or vehicle: fewer MM cells were present in the BM vascular niches in the GMI-1070-treated mice compared to control mice.

**The interaction of P-selectin with PSGL-1 regulates adhesion signaling and activates β1-integrin.**

To further understand the nature of the interaction between P-selectin and PSGL-1 in adhesion to ECs, we studied the molecular events and adhesion-related cell signaling in MM cells. Treatment of MM cells with recombinant P-selectin activated cell-adhesion
signaling, evidenced by increased phosphorylation of FAK, Src, coflin, AKT and GSK-3α/β. Maximal activation of most proteins was achieved at 30min (Figure 5A). Inhibition of P-selectin by GMI-1070 (500 μM, 1hr) reversed the activation of these kinases induced by recombinant selectin (Figure 5B). Similarly, co-culture of HUVECs with MM cells induced activation of the same adhesion-related signaling in MM cells; which was inhibited by GMI-1070 in a dose-dependent manner (Figure 5C). Given that selectin ligands activate adhesion-related kinases, we tested the effect of P-selectin on the activation of integrins in MM cells. Recombinant P-selectin increased the activation and clustering of β1-integrin on the MM cell surface, which was inhibited by blocking P-selectin with GMI-1070 (Figure 5 Di). Representative immunofluorescent images of β1-integrin clustering are shown in Figure 5 Dii.

**PSGL1/P-selectin interaction regulates proliferation of MM cells in the context of the BM microenvironment in vitro and in vivo.**

Previous studies show that adhesion of MM cells to cells in the BM microenvironment leads to increased proliferation of MM cells. We hypothesized that PSGL-1/P-selectin axis plays a role in the proliferation of MM cells induced by BMSCs and ECs. To examine this possibility, we first determined the expression of selectins on BMSCs derived from the BM of 5 MM patients. Figure 6A shows that, as for ECs derived from BM of MM patients, BMSCs express E- and P-selectins (but not L-selectin). Inhibition of the interaction of PSGL-1 in MM and P-selectin with BMSCs and cells by knock-down of PSGL-1 in MM cells (Figure 6Bi) or GMI-1070 (Figure 6Bii) reduced the adhesion of MM cells to BMSCs.
Next we studied the role of PSGL-1/P-selectin axis in MM cell proliferation induced by BMSCs and ECs. Knock-down of PSGL-1 on MM cells reduced the proliferation of MM induced by BMSCs (Figure 6Ci) and ECs (Figure 6Cii). Similarly, inhibition of P-selectin in BMSCs (Figure 6Di) or ECs using GMI-1070 had similar effects (Figure 6Dii). In addition, we found that the mitochondria of MM1s cells incubated with stroma were less primed for apoptosis compared with those cultured in the absence of stroma. Notably, this reduction in priming was reversed by selectin inhibition with GMI-1070 (Supplementary Figure 5).

To determine the role of PSGL-1/P-selectin in tumor initiation \textit{in vivo}, we examined the level of MM cells in the BM of mice one week after injection of MM cells intravenously or directly into the BM of the tibia as models of tumor initiation. Figure 6E shows that, when injected intravenously, knockdown of PSGL-1 in MM cells decreased the tumor initiation \textit{in vivo} to 40% compared to scramble siRNA transfected cells. Figure 6F shows that, when injected directly to the tibia, knockdown of PSGL-1 in MM cells decreased tumor initiation \textit{in vivo} to 65% compared to scramble siRNA transfected cells.

**PSGL-1/P-selectin regulates resistance of MM cells to chemotherapy in the context of the BM niches.**

We have shown that the interaction of MM cells with the BM microenvironment induces drug resistance, we thus examined the role of PSGL-1/P-selectin in this effect \textit{in vitro} and \textit{in vivo}.

Co-culture of MM cells with either BMSCs or ECs induced resistance to bortezomib and dexamethasone, as demonstrated by reduced inhibition of proliferation...
(compared to MM cells cultured alone). Importantly, inhibition of P-selectin by GMI-1070 restored the sensitivity of MM cells to bortezomib and dexamethasone, even when co-cultured with BMSCs (Figure 7A) and ECs (Figure 7B).

We next confirmed the effect of GMI-1070 on sensitivity of MM cells to bortezomib in vivo. We examined the effect of GMI-1070, bortezomib used either alone or in combination on MM tumor progression in vivo. MM tumors were established in SCID mice and treatment was initiated after tumor detection by bioluminescence imaging at 4 weeks after the MM cell injection. Unlike the effect on tumor initiation, inhibition of PSGL-1/P-selectin axis by treatment of mice with GMI-1070 alone did not affect tumor growth compared to the vehicle-receiving group (Figure 7C). Expectedly, treatment with bortezomib alone delayed MM tumor growth; however, tumor progression was detected even when the animals were still on treatment with bortezomib. Interestingly, the combination of bortezomib and GMI-1070 inhibited tumor growth completely while animals were on treatment (first 28 days). Furthermore, tumor growth was delayed after stopping therapy in the mice treated with the combination of GMI-1070 and bortezomib (Figure 7C).

Similarly, GMI-1070 did not affect the survival of animals compared to the vehicle-treated group (50% of the animals died at 28 days in both groups). Bortezomib alone increased the survival of the animals, in which 50% of the mice died at day 38). The combination of bortezomib and GMI-1070 significantly enhanced the survival, in which, up to day 42, 100% of the animals in this group were alive, while only 40%, 10% and 0% of the bortezomib group, vehicle group, and the GMI-1070 group were alive at this time.
point, respectively. Moreover, the animals in the combination group reached 50% survival at day 52 (Figure 7D).
DISCUSSION

One of the crucial steps in the early phase of tumor dissemination appears to be the interaction of tumor cells with the endothelium for extravasation and homing, which leads to the formation of new metastatic lesions. Selectins are molecules expressed on the cell surface of ECs and were shown to promote the first interaction between an extravasating cell and the blood-vessel wall. Selectins were previously shown to play an important role in seeding of tumor cells in distant organs and facilitating metastasis. Metastasis was dramatically reduced in mice with double deficiency of P and L selectin, suggesting a synergistic action of P-and L-selectin in this process.

In this study, we examine the role of PSGL-1 and its interaction with P-selectin in tumor progression in MM. We found that PSGL-1 was highly expressed in MM cells and that P-selectin was highly expressed on cells in the BM microenvironment including BMSCs and ECs. Functionally, in vitro studies showed that the interaction of PSGL-1 with P-selectin was the most critical in regulating adhesion to ECs as well as trans-endothelial migration. These results were confirmed by downregulation of PSGL-1 in the MM cells and by down regulation of selectins in the ECs. Similar results were obtained using the pan-selectin inhibitor GMI-1070, which is currently in clinical trials for vaso-occlusive crisis in sickle cell disease. These findings were confirmed in vivo, in which downregulation of PSGL-1 in the MM cells or inhibition of the selectin in the mice, using blocking antibodies and GMI-1070, decreased the extravasation and homing to the BM of MM cells. The interaction between P-selectin and PSGL-1 mediated adhesion through activation of adhesion-related cell signaling and activation of integrins in MM cells. Future

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studies to examine mechanisms of interaction of selectins and integrins in MM are warranted but were beyond the scope of this study.

We have previously showed that the interaction of MM cells with the BM microenvironment, including BMSCs and ECs induces MM proliferation and drug resistance. Although selectins are supposed to regulate the early steps of homing, here we show that they are also critical regulators of the interaction of MM with the BM microenvironment, which is a critical for MM proliferation and drug resistance. Indeed, we showed that E and P-selectins were expressed in ECs as well as BMSCs isolated from the BM of MM patients. Inhibition of the PSGL-1/P-selectin interaction, by down regulation of PSGL-1 in MM cells or by inhibition of selectin with GMI-1070, reversed the proliferative effects and drug resistance induced by BMSCs and ECs in vitro. These findings were confirmed in vivo, in which inhibition of the PSGL-1/P-selectin interaction decreased tumor initiation, sensitized MM cells to bortezomib and enhanced the survival of the animals. Tumor initiation was decreased in vivo due to knockdown of PSGL-1 after both intravenous injection and direct injection to the BM in the tibia; however, the decrease after intravenous injection was more than after tibial injection. This indicates that the decrease in tumor initiation is not only due to prevention of homing of MM cells, but also due to inhibition of proliferation induced by the interaction with the BM microenvironment. These results confirm our hypothesis that disruption of the interaction of MM cell with the BM microenvironment sensitizes MM cell to therapy, and suggests the interaction between PSGL-1 and P-selectin as a therapeutic target.

In summary, these studies delineate the important role of PSGL-1 in MM interaction with the BM microenvironment as a regulator of adhesion of MM cells to ECs
and BMSCs. In addition, it highlights the interaction between PSGL-1 in MM cells and P-selectin in the microenvironment as a therapeutic target for prevention of tumor progression and drug resistance in MM.
ACKNOWLEDGEMENTS

We would like to thank Sonal Jhaveri for editing this paper. This study was supported in part by Multiple Myeloma Research Foundation (MMRF), R01CA125690 and 1R01CA152607.

AUTHOR CONTRIBUTION

AKA: Designed research, performed research, analyzed data, and wrote the paper.
PQ, FA, CP, BT, TC, JTP, RC, VM and LMF: Performed research and analyzed data.
PM, AS, HTN, JR, CPL, JLM, AL and ALK: Analyzed data.
AMR,: Analyzed data and wrote the paper.
IMG: Designed research and wrote the paper.

CONFLICT OF INTEREST DISCLOSURE

Dr. Ghobrial is on the advisory board of Millennium/Takeda, Genzyme, Celgene, Onyx and Novartis.

Dr. Roccaro is on the advisory board of Roche.

Dr. Patton and Dr. Magnani are employees of GlycoMimetics, Inc, Gaithersburg, MD
REFERENCES

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FIGURE LEGENDS

Figure 1: PSGL-1 is highly expressed on MM Cells and regulates interaction with P-selectins.

(A) Expression of PSGL-1 detected in BM biopsies from MM patients (n=17) and healthy individuals (n=3) using immunohistochemistry. Images are showing 20X magnification and inserts are showing 100X magnification. All MM patients present with higher expression of PSGL-1 compared to healthy individuals. (B) Expression of PSGL-1 has been evaluated on MM cell lines (MM.1S, OPM1, OPM2, LR7, RPMI.8226, U266, H929) using flow cytometry and expressed as ratio between mean fluorescence intensity (MFI) of PSGL-1 and MFI of isotype control. (C) Gene expression profiling of PSGL-1 from available dataset series No. GSE 6477. The expression level of PSGL-1 significantly increases with MM tumor progression from MGUS to smoldering MM to newly diagnosed MM. Significant differences are observed between healthy subject and MM patients (both smoldering MM and newly diagnosed MM) (P <.01).

(D) CD138+ cells isolated from either normal BM (n=3) or MM BM (n=6), and MM cell lines (MM.1S, OPM1, OPM2, RPMI.8226, U266, LR7, H929) have been incubated with free human Fc-chain (isotype control) or with chimeras of human-Fc chain and recombinant human E, L or P-selectin (10μg/ml) for 1hr followed by FITC-conjugated mouse-anti human Fc. The interaction of the selectins with MM cells was analyzed by flow cytometry and quantified as the ratio of the MFI of each selectin/MFI of isotype control. P and L-selectins (but not E-selectin) interacted with MM primary cells and cell lines, while none of the selectins interacted with the normal plasma cells. (E) MM1s cells were transfected with either PSGL-1 siRNA or scramble siRNA. Cells were exposed to
recombinant human E-, L- and P-selectin (10μg/ml) for hr. The interaction of selectins with MM cells was analyzed by flow cytometry and quantified as ratio of MFI of each selectin/MFI of isotype control. Downregulation of PSGL-1 reduced the interaction of both L and P-selectins with MM cells. (F) MM.1S cells were treated with increasing concentrations of GMI-1070 (0, 250 and 500μM) for 1hr then exposed to recombinant E-, L-, and P-selectin (10μg/mL, for 1hr). The interaction of selectins with MM cells was analyzed by flow cytometry and quantified as a ratio of MFI of each selectin/MFI of isotype control. Dose-dependent inhibition of the interaction of L- and P-selectins was observed. All data represent mean (± SD) of triplicate experiments.

**Figure 2: PSGL-1 regulated adhesion of MM cells to endothelial cells (ECs).**

(A) Expression of E-, L- and P-selectins has been evaluated on HUVECs and primary MM BM-derived ECs (n=5) using flow cytometry and expressed as ratio between MFI of selectin/MFI of isotype control. ECs present with higher expression of E- and P-selectins. (B) HUVECs were transfected with siRNAs for either E-, L- or P-selectin. Scramble siRNA has been used as control (panel i); or treated with anti-E-, L-, or P-neutralizing antibodies. Isotype control antibody was used as control (panel ii). Adhesion of MM cells on HUVECs has been evaluated: significant inhibition of MM cells to HUVECs was observed in P-selectin knockdown cells (panel i) and in HUVECs treated with neutralizing antibody for P-selectin (panel ii), P<0.01. All (C) HUVECs were exposed to TNFα (30U/ml, 3 hrs); or to IL-4 (3ng/ml, 24 hrs) and histamine (2.25 mM, 4 hrs). Expression of E-, L-, and P-selectins was evaluated by flow cytometry. Induction of E- and P-selectin was observed upon activation with TNFα or IL-4/histamine, respectively (panel i). Adhesion of
non-treated MM cells to HUVECs was evaluated. MM cells showed increased adhesion to HUVECs with activation of P-selectin (panel ii), \( P < 0.01 \). (D) MM1s cells were transfected with either PSGL-1 siRNA or scramble siRNA. Adhesion of MM cells to HUVECs was evaluated: significant inhibition of MM cells to HUVECs was observed in PSGL-1 knockdown cells \( (P < 0.05) \). (E) HUVEC cells were treated with increasing concentrations of GMI-1070 (0, 100, 250, and 500 \( \mu \)M) for 1 hr, and adhesion of non-treated MM cells (H929, OPM1, MM.1S) to HUVECs was evaluated: dose-dependent inhibition of MM cells adhesion to HUVECs was observed, \( (P < 0.05) \). All data represent mean (± SD) of triplicate experiments. (F) MM1s cells were treated with either isotype control or PSGL-1 blocking antibody. Adhesion of MM cells to HUVECs was evaluated: significant inhibition of MM cells to HUVECs was observed in cells treated with PSGL-1 blocking anti-body \( (P < 0.01) \).

Figure 3: PSGL-1 regulates trans-endothelial migration of MM cells.

(A) HUVECs were transfected with siRNAs for either E-, L- or P-selectin. Scramble siRNA has been used as control (panel i); or treated with anti-E-, L-, or P-neutralizing antibodies. Isotype control antibody has been used as control (panel ii). Transendothelial migration of MM1s cells in response to SDF-1\( \alpha \) (30nM) was tested. Significant inhibition of MM cell transendothelial migration was observed in HUVECS with P-selectin knock-down (panel i) and in HUVECs treated with neutralizing antibody for P-selectin (panel ii), \( P < 0.01 \). (B) MM1s cells were transfected with either PSGL-1 or scramble siRNA, and transendothelial migration of MM cells in response to SDF-1\( \alpha \) (30nM) was tested. Significant inhibition of MM cell transendothelial migration was observed in MM cells
with PSGL-1 knock-down. (C) HUVEC cells were treated with increasing concentrations of GMI-1070 (0, 100, 250 and 500μM) for 1 hr, and transendothelial migration of non-treated MM1s cells in response to SDF-1α (30nM) was evaluated: dose-dependent inhibition of MM cells transendothelial migration was observed (P <0.02). (D) MM1s cells were transfected with either PSGL-1 or scramble siRNA, and migration of MM cells (with no presence of HUVECs) in response to SDF-1α (30nM) was tested. No difference in cell migration was observed in MM cells transfected with PSGL-1 or scramble siRNA. (E) MM1s cells were treated with increasing concentrations of GMI-1070 (0, 100, 250 and 500μM) for 1 hr, and migration of MM cells (with no presence of HUVECs) in response to SDF-1α (30nM) was tested: No difference in cell migration was observed in MM cells treated with or without GMI-1070. (F) MM1s cells were treated with either isotype control or PSGL-1 blocking antibody. Trans-endothelial migration of MM cells was evaluated: significant inhibition of MM cells to HUVECs was observed in cells treated with PSGL-1 blocking antibody.

Figure 4: The interaction of PSGL-1 with P-selectin regulates extravasation and homing to the BM of MM cells in vivo.

(A) Calcein-labeled MM1s cells were injected iv into SCID mice which were treated with either vehicle (Ctrl), anti-P-selectin antibody (250 μg/kg, iv) or GMI-1070 (25 mg/kg, ip) for 1 hour before injection of MM1s cells (n=3 per group). The number of circulating cells was followed over time using in vivo flow cytometry. Cells were counted every 5 minutes for 40 minutes. Fluorescence signal was detected on an artery in the ear and digitized for analysis with Matlab software. Inhibition of P-selectin using GMI-1070 or neutralizing
antibody delayed the extravasation of MM cells (B) MM1s cells were transfected with either PSGL-1 or scramble siRNA, labeled with Calcein, and injected iv into SCID mice (n=3 per group), followed by iv injection of Evans Blue. Homing to the BM of mice was imaged by in vivo confocal microscopy at 30 min after injection. Inhibition of the homing of MM cells to the BM was observed with knockdown of PSGL-1, shown as an average of number of MM cells in 18 images taken from 3 different mice (P<0.01) (i), and in representative images of the BM (ii) (Green: MM cells, Red: Blood vessels). (C) Calcein-labeled MM1s cells were injected iv into SCID mice, which were treated with either vehicle (Ctrl), or GMI-1070 (25 mg/kg, ip) for 1 hour before injection of MM1s cells (n=3 per group), followed by iv injection of Evans Blue. Homing to the BM of mice was imaged by in vivo confocal microscopy at 30 min after injection. Inhibition of the homing of MM cells to the BM was observed in mice treated with GMI-1070, shown as an average of number of MM cells in 18 images taken from 3 different mice (P<0.01) (i), and in representative images of the BM (ii) (Green: MM cells, Red: Blood vessels).

Figure 5: The interaction of PSGL-1 and P-selectin regulates adhesion-related signaling and \( \beta \)-integrin activation in MM cells.

(A) MM1s cells were treated with recombinant P-selectin 10\( \mu \)g/ml for different durations (0, 5, 10, 20, 30 and 60 min), lysed and whole cell lysates were subjected to western blotting for pFAK, pAKT, pCofilin, pSRC and p-GSK3\( \alpha /\beta \). Increased adhesion related signaling was observed after activation with recombinant P-selectin with maximal activation at 30 min. (B) Recombinant P-selectin was incubated with or without GMI-1070 (500mM, 1hr) and then applied to MM1s cells, and non-treated MM cells were used as a
control. Cells were then lysed and whole cell lysates were subjected to western blotting for pFAK, pAKT, pCofilin, pSRC and p-GSK3α/β. GMI-1070 reversed the induction of adhesion related signaling in MM cells induced by recombinant P-selectin (C) HUVECs were treated with or without GMI-1070 (500mM, 1hr) and non-treated MM1s cells were co-cultured with the HUVECs for 1 hr; and MM1s cells which were not co-cultured with HUVECs served as a control. MM cells were then separated from the HUVECs, lysed and whole cell lysates were subjected to western blotting for pFAK, pAKT, pCofilin, pSRC and p-GSK3α/β. Co-culture of MM cells with HUVECs induced adhesion related signaling in MM cells which was reversed by GMI-1070. (D) Recombinant P-selectin was incubated with or without GMI-1070 (500mM, 1hr) and then applied to MM1s cells, and non-treated MM cells were used as a control. Cells were fixed, and the expression of activated β1-integrin was detected using FITC-labeled antibody under immuno-fluorescence microscopy. Increased activation of β1-integrin was observed after activation with recombinant P-selectin which was reversed by GMI-1070; shown as an average of percent of cell with activated integrins and normalized to control (P<0.001) (i), and in representative fluorescent images X20, and insert X100 (ii).

**Figure 6:** The interaction of PSGL-1 and P-selectin regulates proliferation of MM cells induced by BMSCs and ECs *in vitro*, and tumor initiation *in vivo*.

(A) Expression of E-, L- and P-selectins has been evaluated primary BMSCs (n=5) using flow cytometry and expressed as ratio between MFI of selectin/MFI of isotype control. BMSCs present with higher expression of E- and P-selectins. (B) MM1s cells were transfected with either PSGL-1 siRNA or scramble siRNA. Adhesion of MM cells to
BMSCs has been evaluated: significant inhibition of MM cell adhesion to BMSCs was observed in PSGL-1 knockdown cells ($P = 0.006$) (i). BMSCs were treated with or without GMI-1070 (500 μM) for 1 hr, and adhesion of non-treated MM1s cells to BMSCs was evaluated: inhibition of MM cells adhesion to BMSCs was observed in HUVECs treated with GMI-1070 ($P < 0.001$) (ii). Data represent mean (± SD) of triplicate experiments. (C) MM1s cells were transfected with either PSGL-1 siRNA or scramble siRNA and cultured with or without the presence of BMSCs (i) and HUVECs (ii). Cell proliferation was measured at 24 hours by BrdU incorporation and ELISA. Co-culture of MM1s with HUVECs and BMSCs increased the proliferation of MM1s cells transfected with scramble siRNA, an effect which was reversed by PSGL-1 siRNA. Data represent mean (± SD) of triplicate experiments. (D) HUVECs and BMSCs were treated with or without GMI-1070 and non-treated MM cells were cultured with or without the presence of BMSCs (i) and HUVECs (ii). Cell proliferation was measured at 24 hours by BrdU incorporation and ELISA. Co-culture of MM1s with non-treated HUVECs and BMSCs increased the proliferation of MM1s cells, an effect which was reversed by GMI-1070. Data represent mean (± SD) of triplicate experiments. (E) MM1s cells were transfected with either PSGL-1 or scramble siRNA, and injected iv into SCID mice (n=4 per group), and after one week the BM was extracted from the femurs of the mice and tumor initiation was determined as the percent of CD138+ in the BM. Inhibition of tumor initiation in the BM of the mice was observed with knockdown of PSGL-1, ($P<0.001$). (F) MM1s cells were transfected with either PSGL-1 or scramble siRNA, and injected to the BM of the tibia of SCID mice, and after one week the BM was extracted from the tibia of the mice and tumor initiation was determined as the percent of CD138+ in the BM. Inhibition of tumor initiation in the BM
of the mice was observed with knockdown of PSGL-1 but not to the same extent observed after iv injection (P=0.02).

**Figure 7: The interaction of PSGL-1 and P-selectin regulates drug resistance of MM cells induced by BMSCs and ECs *in vitro*, and tumor progression *in vivo*.

(A) MM1s cells (treated with vehicle, bortezomib 5nM or dexamethasone 250 nM) were cultured with or without the presence of BMSCs (treated with or without GMI-1070 500 μM). Cell proliferation was measured at 24 hours by BrdU incorporation and ELISA. Co-culture of MM1s with BMSCs induced resistance to both bortezomib and dexamethasone in MM1s cells which was reversed by GMI-1070. Data represent mean (± SD) of triplicate experiments. (B) MM1s cells (treated with vehicle, bortezomib 5nM or dexamethasone 250 nM) were cultured with or without the presence of HUVECs (treated with or without GMI-1070 500 μM). Cell proliferation was measured at 24 hours by BrdU incorporation and ELISA. Co-culture of MM1s with HUVECs induced resistance to both bortezomib and dexamethasone in MM1s cells which was reversed by GMI-1070. Data represent mean (± SD) of triplicate experiments. (C) The effect of inhibition of the interaction of PSGL-1 with P-selectin by GMI-1070 on the sensitivity of MM tumors to Bortezomib *in vivo*. SCID mice (N=10 per group) were injected with MM1s cells engineered to express luciferase and the tumor growth was determined by bioluminescence imaging. Mice were divided in 4 groups: (1) control group, received weekly ip injection of vehicle and implanted with pumps loaded with vehicle every two weeks (for 4 weeks); (2) GMI-1070 treated group, which received weekly ip injection of vehicle and was implanted with a pump loaded with 200 μl of 150mg/ml GMI-1070 which was changed every two weeks.
(for 4 weeks); (3) bortezomib treated group, which received weekly ip injection of bortezomib 1.5 mg/Kg and implanted with a pump loaded with vehicle which was changed every two weeks (for 4 weeks), and (4) combination group, which received weekly ip injection of bortezomib 1.5 mg/Kg and was implanted with a pump loaded with 200 µl of 150mg/ml GMI-1070 which was changed every two weeks (for 4 weeks). Tumor progression was detected using bioluminescence. Treatment with GMI-1070 alone did not affect the tumor progression, but it increased the sensitivity of MM cell to bortezomib and decreased tumor progression was observed in the group treated with combination of GMI-1070 and bortezomib, compared to bortezomib alone \((P<0.01)\). (D) Kaplan Meier curves of survival of Groups 1, 2, 3 and 4 (described in C). Increased survival was observed in mice treated with the combination of GMI-1070 and bortezomib, compared to bortezomib alone, \((P=0.012)\).
Figure 1

A
Figure 1

B

Expression of PSGL-1 (MFI Selectin/MFI Isotype)

C

Gene Expression of PSGL-1

D

Interaction with recombinant selectins (MFI Selectin/MFI Isotype)

E

Interaction with recombinant selectins (MFI Selectin/MFI Isotype)

F

Interaction with recombinant selectins (MFI Selectin/MFI Isotype)
Figure 2

A

![Expression of Selectins](image)

B

i

![Adherent cells](image)

ii

![Adherent cells](image)

C

i

![Expression of selectins](image)

ii

![Number of Adherent MM cells](image)
Figure 2

D

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P<0.05

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P<0.05

F

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P<0.01

For personal use only.
Figure 4

A

![Graph showing cell distribution over time with different treatments.]

B

1. i

![Bar graph showing homing to BM with siRNA.]

2. ii

![Images showing blood vessels and MM cells with different treatments.]

C

1. i

![Bar graph showing homing to BM with different treatments.]

2. ii

![Images showing blood vessels and MM cells with different treatments.]

Legend:
- Ctrl
- Anti-P Selectin (250 μg/Kg) (IV)
- GMI-1070 (25 mg/Kg) (IP)
- P=0.03
- P=0.01
- NS
- Scr siRNA
- PSGL-1 siRNA
Figure 5

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D

i

Activation of Integrins (% of control)

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![Graph showing activation of integrins](image)

p<0.001

ii

Ctrl

P-selectin

P-Selectin + GMI-1070

![Images showing fluorescence under X100 and X20 magnification](image)
Figure 6

A

Expression of Selectins (MFI Selectin / MFI Isotype) vs. BMSC

B

Adhesion to BMSCs (% of Control)

i

P<0.001

ii

P=0.006

P<0.001

C

Proliferation (% of Control)

i

P=0.04

P<0.01

P<0.01

ii

P<0.001

P=0.002

P<0.001

D

Proliferation (% of Control)

i

P<0.001

P<0.001

P<0.001

ii

P=0.002

P=0.02

P<0.001

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Figure 6

E

![Bar chart for tumor initiation and MM cell amount in BM](image)

- **Scr siRNA**: Tumor initiation and MM cell amount (% of Scramble)
- **PSGL-1 siRNA**: Tumor initiation and MM cell amount (% of Scramble)

- **P < 0.001**

F

![Bar chart for tumor initiation and MM cell amount in BM](image)

- **Scr siRNA**: Tumor initiation and MM cell amount (% of Scramble)
- **PSGL-1 siRNA**: Tumor initiation and MM cell amount (% of Scramble)

- **P = 0.02**
Figure 7

A

B

C

D

Proliferation (% of Control)

Proliferation (% of Control)

Bioluminescence (10^9 ph/s/ROI)

Survival (%)
P-selectin glycoprotein ligand regulates the interaction of multiple myeloma cells with the bone marrow microenvironment