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CHAPTER

4

CXCL12 γ immobilized by heparan-sulfate proteoglycans on human bone marrow stromal cells instigates adhesion and drug resistance in multiple myeloma

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Abstract

Multiple Myeloma (MM) is a malignancy of plasma cell expanding in the bone marrow (BM). The survival and proliferation of MM cells critically depends on communication with the BM niche, an interaction involving the chemokine CXCL12 and its receptor CXCR4. We here establish an important role for the recently defined CXCL12 γ isoform, which is characterized by an extended C-terminal domain that binds heparan sulfate (HS) with an extraordinary high affinity. We show that CXCL12 γ is expressed *in situ* by stromal cells in both the normal and MM bone marrow microenvironment and highly expressed by isolated bone marrow stromal cells (BMSCs) as well as by BMSC cell lines. Importantly, upon secretion, CXCL12 γ is retained on the surface of these BMSCs. This membrane immobilization is CXCL12 γ -isoform specific and HSPG-mediated, since it was completely abolished by CRISPR-Cas9 mediated knockout of the HS co-polymerase EXT1. Functionally, surface-immobilized recombinant CXCL12 γ induces strong adhesion of MM cells to vascular cell-adhesion molecule (VCAM)-1 and fibronectin and mediates ERK1/2 activation. Interestingly, BMSCs with a specific CRISPR-Cas9-mediated deletion of the CXCL12 γ isoform, or of HS, showed a reduced ability to mediate MM cell adhesion and an impaired capacity to protect MM cells from bortezomib-induced cell death, indicating that HSPG-bound CXCL12 γ plays an important role in cell adhesion-mediated drug-resistance (CAM-DR). Our data indicate that CXCL12 γ functions as a 'niche chemokine' that, in conjunction with HSPGs, plays a unique role in controlling adhesion, BM retention, and drug resistance of MM cells, and suggest these molecules as potential therapeutic targets in MM.

Introduction

The uncontrolled growth of cancer cells is driven by mutations in essential growth control genes, but their growth and survival is also strongly dependent on signals from the tumor microenvironment. In multiple Myeloma (MM), a clonal expansion of malignant plasma cells in the bone marrow (BM), interaction of the tumor cells with specific niches in the BM plays an important role in proliferation, survival, and progression as well as in drug resistance. This interaction involves signaling via cell-surface receptors, including adhesion molecules, in addition to signals from multiple soluble factors secreted by various cells in the BM niche.¹⁻³ In the past two decades, major progress has been made in MM treatment due to introduction of proteasome inhibitors, immunomodulatory drugs (IMiDs) and, recently, monoclonal antibodies targeting MM cells.⁴⁻⁶ However, despite these achievements, MM is generally still incurable, which is largely due to the development of therapy resistance. There is ample evidence that interaction of MM cells with the BM niche, in particular with bone marrow stromal cells (BMSCs), plays a key role in the development of this resistance, protecting cells from drug-induced cell death. Hence, targeting this interaction has been proposed as a promising strategy to overcome therapy resistance.^{1,7,8}

Homing of hematopoietic stem cells (HSCs) and plasma cell precursors to the BM is controlled by the chemokine CXCL12.^{9,10} In MM, CXCL12 regulates the adhesion, transendothelial migration, and homing of the malignant plasma cells to the BM by binding its receptor CXCR4 on MM cells.^{11,12} Within the BM, CXCL12 is mainly produced by BMSCs. Several splice variants of CXCL12 have been identified,¹³ which all contain the CXCR4 binding motif but are differentially expressed in various murine and human tissues.¹⁴ To date, the functional differences and biological significance and these distinct isoforms has remained largely unexplored. Virtually all *in vitro* functional studies, including those on MM migration and adhesion, have exclusively employed the CXCL12 α isoform. Moreover, the reported *in vivo* studies do not allow discrimination between the functionality of distinct CXCL12 isoforms, since the mouse models studied either carried a full deletion of CXCL12 or a deletion of CXCR4, the cognate receptor for all isoforms. Interestingly, the recently characterized isoform CXCL12 γ has been shown to promote *in vivo* leukocyte accumulation and angiogenesis with a much higher efficiency than CXCL12 α , despite a reduced CXCR4 agonist activity *in vitro*. This enhanced biological activity of CXCL12 γ is mediated by its extended C-terminal domain, which binds heparan sulfate proteoglycans (HSPGs) with an unprecedentedly high affinity.^{15,16} Notably, in murine BM CXCL12 γ was reported to be the dominant CXCL12 isoform, and deletion of the HSPG-binding motives of CXCL12 gave rise to increased numbers of circulating HSCs.^{15,17}

HSPGs are membrane-bound or matrix proteins decorated by covalently linked HS side-chains composed of repeating disaccharide units, which undergo complex modifications

that determine their binding capacity and specificity.^{18,19} Via their HS chains, HSPGs can bind and present a wide variety of morphogens, growth factor, and chemokines, thereby controlling their spatial distribution and activity.^{20,21} Given these functional properties, HSPGs could play an important role in organizing cellular niches. Indeed, studies in *Drosophila* have demonstrated that the HSPGs Dally and Dally-like play a crucial role in the germ cell- as well as hematopoietic-stem cell niches by controlling activity of bone morphogenetic proteins (BMPs), restricting their diffusion.^{22,23} In addition, HSPGs are known to bind a variety of other factors like Wnts, FGF, Midkine, and CXCL12, which play important roles in intestinal, neural, and HSC niches.^{20,21,24}

The extraordinary properties of CXCL12 γ and its reported strong expression in murine BM, prompted us to investigate the expression of this isoform in the human BM microenvironment and to study its possible functional role in the interaction of MM plasma with BMSCs cells. Our results indicate that CXCL12 γ functions as a ‘niche chemokine’ which, in conjunction with HSPGs, plays a unique role in controlling cell adhesion, BM retention, and cell-adhesion-mediated drug resistance (CAM-DR) of MM cells.

Materials and Methods

Cell culture

The human multiple myeloma cell lines (HMCLs) XG-1, MM1S and L363 were cultured as described previously.²⁴ For XG-1, medium was supplemented with 500 pg/mL IL-6 (Prospec). BMSC lines HS5 and HS27a were cultured in DMEM (Invitrogen Life Technologies) with 10% FBS (Invitrogen Life Technologies), 100 μ g/ml streptomycin, 100 units/ml penicillin (Sigma Aldrich), Bone marrow endothelial cell lines HBMEC60 and 4LHBMEC were cultured in EGM-2MV medium (Lonza). Primary MM cells and BMSCs were derived from MM patients diagnosed at the Academic Medical Center, Amsterdam, the Netherlands. This study was conducted and approved by the AMC Medical Committee on Human Experimentation. Informed consent was obtained in accordance with the Declaration of Helsinki.

Cloning, transfection and transduction

pLentiCrispr-sgEXT1 was constructed by inserting sgRNA-*EXT1* (GACCCAAGCCTGCGACCACG) into pL-CRISPR.EFS.GFP (Addgene plasmid # 57818) as previously described.²⁵ pLentiCrispr-sgCXCL12 γ were constructed by inserting sgRNA-CXCL12 γ #1(TTTAACAACCTGGCCCGTGTAC) and sgRNA-CXCL12 γ #2 (AACTGTGGTCCATCTCGAGG) into pL-CRISPR.EFS.GFP. Pbabe-CXCL12 α and Pbabe-CXCL12 γ were constructed by inserting CXCL12 α or CXCL12 γ cDNA containing C-terminally C9-tagged (TETSQVAPA) sequences into Pbabe-puro (Addgene plasmid # 1764). Lentiviral and retroviral particle production and transduction were as described before.²⁴

RT-PCR and genomic DNA PCR

Total RNA was isolated using TRI reagent (Invitrogen Life Technologies) according to the manufacturer's instructions and converted to cDNA using oligo-dT. PCRs were conducted using SensiFast (Bioline) on the CFX384 RT-PCR detection system (Bio-Rad). Isoform specific primers sequences and housekeeping gene primers are as shown in the table:

CXCL12 α forward	ccaaactgtgcccttcagat
CXCL12 α reverse	cgtctttgcccttcacatc
CXCL12 γ forward	ccaaactgtgcccttcagat
CXCL12 γ reverse	ctttctgggcagccttct
RPLPO forward	gcttcctggagggtgccgc
RPLPO reverse	tccgtctccacagacaaggcca

Genomic DNA was isolated using QIAamp DNA Kit according to the manufacturer's instructions. PCR primers used for detect CXCL12 γ deletion are: forward primer: tccccagtgggaatcaggtt; reverse primer: ctggagctcccaggtattc.

Adhesion assay

CXCL12 α and CXCL12 γ -induced adhesion to vascular cell-adhesion molecule (VCAM) and fibronectin were performed as described before.²⁶ For adhesion to BMSCs and bone marrow endothelial cells, MM cells were added to 96 well plates with confluent BMSCs or bone marrow endothelial cells expressing a GFP fluorescence maker. MM cells were spun-down for 30 second at 400 RPM and subsequently incubated for 20 minutes to allow adhesion of MM cells to BMSCs or bone marrow endothelial cells. Non-adherent cells were removed by washing with RPMI containing 1% BSA. Adherent cells were detached by trypsin and cell numbers were quantified by flow cytometry.

Co-culture assay

For the co-culture assay, BMSCs were seeded in 96-well plates one day in advance to allow cell attachment. On the next day, MM cells were added to the plate and incubated for 2 hour. Subsequently, drugs at the indicated concentrations were added to the wells. After 3 days of culture, cells were collected and analyzed by flow cytometry, using 7-AAD (Thermo Fisher Scientific) to exclude dead cells. In the transwell assay, BMSCs were seeded in the lower compartment, and MM cells were seeded in the transwell insert (Costar, 0.4 μ m; Corning, USA). After culturing the cells for 3 days in the presence or absence of bortezomib, the cell viability was analyzed by flow cytometry, using 7-AAD (Thermo Fisher Scientific) to exclude dead cells.

Immunoblotting

Immunoblotting was performed as described before.²⁴ The antibodies used were: Phospho-ERK1/2 (Cell Signaling), and total ERK2 antibody (C-14) (Santa Cruz).

Cell surface protein staining

HSPG staining was performed as described before,²⁴ Heparitinase used for digestion of cell surface HS was purchased from amsbio (Abingdon, UK). For CXCL12 γ cell surface staining, cells were detached by 2 μ M EDTA, stained with isotype specific antibody 6E9.¹⁵ Primary antibodies were detected with rabbit anti-mouse IgG1-APC (Southern Biotech, Birmingham). For recombinant CXCL12 γ binding assay, HMCL XG1 were incubated with 1 μ g/ml recombinant CXCL12 γ at 4°C for 90 minutes. After washing three times, cells were stained with 6E9 antibody. Primary antibodies were detected with rabbit anti-mouse IgG1-APC (Southern Biotech, Birmingham). Stainings were analyzed by flow cytometry.

Immunohistochemistry

Paraffin-embedded BM biopsies for immunohistochemical investigations were obtained from department of pathology, Academic Medical Center, Amsterdam, the Netherlands. To stain for CXCL12 γ , 4 μ m tissue sections were treated with Tris ethylenediaminetetraacetic acid (EDTA, pH 9) for 20 min at 121°C for antigen retrieval. Sections were incubated overnight at 4°C with the CXCL12 γ isoform specific antibody 6E9.¹⁴⁰ Subsequently, the tissues were washed with PBS and incubated with rabbit-anti-mouse antibody (Southern Biotech) for 30 min at room temperature. CXCL12 γ was detected by poly-HRP-anti-rabbit IgG (DPVR110HRP, Immunologic) followed by Ultra DAB (Immunologic).

Results

CXCL12 γ is expressed by primary human BMSCs and BMSC lines

BMSCs secrete high levels of CXCL12, which has been shown to mediate the homing of both HSCs and MM cells to the BM.^{9,12} However, to date, the expression of specific CXCL12 isoforms, including CXCL12 γ , in the human BM microenvironment and their specific role in the interaction with MM cells has not been defined. To study CXCL12 γ expression *in situ* in human bone marrow, we employed immunohistochemistry using mAb 6E9 recognizing an epitope specific for CXCL12 γ isoform (Figure 1A). Interestingly, this antibody show specifically staining on the population of stromal cells, including cells bordering the perist, lipocytes, and BM capillaries, areas with putative niche functions. Interestingly, in MM BM stroma, an increased CXCL12 γ expression was observed.

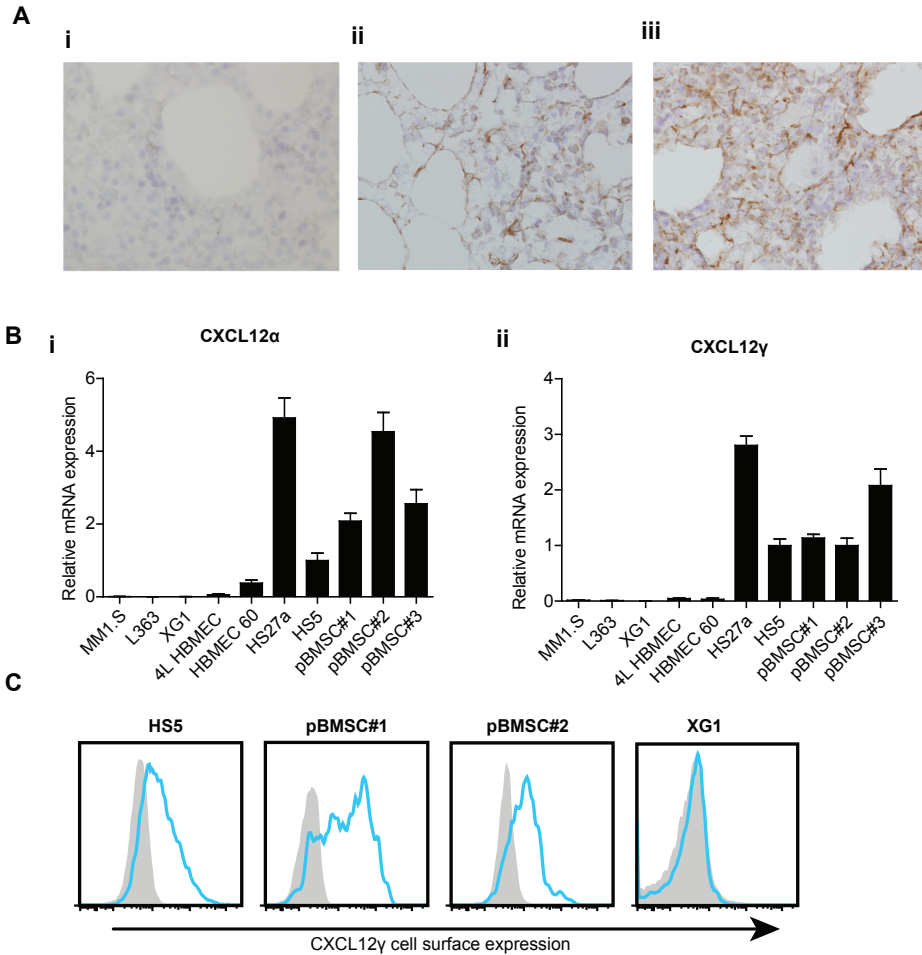


Figure 1. CXCL12 γ expression on BMSC. (A) Immunohistochemical staining of CXCL12 γ with CXCL12 γ isoform-specific 6E9 antibodies in normal (ii) and MM patient (iii) BM biopsy, mouse IgG was used as antibody isotype control (i). (B) mRNA expression of CXCL12 α (i) and CXCL12 γ (ii) in HMCLs MM1.S, L363 and XG1, HBMEC lines 4LHBMEC and HBMEC 60, human BMSC lines HS27a and HS5, and primary BMSC (pBMSC) derived from MM patients, was analyzed by qPCR and expression was normalized to the housekeeping gene RPLPO. (C) Flow cytometry detection of cell surface expression of CXCL12 γ in BMSC line HS5, primary BMSC (pBMSC) derived from MM patients and MM cell line XG1, using CXCL12 γ isotype specific antibody 6E9. Representative plots are shown. (n=3 for HS5 and XG1, n=2 for primary BMSC).

To further assess the expression of CXCL12 γ in different BM-derived cell types, human BMSCs, including primary BMSCs and the BMSC lines HS27a and HS5, the human bone-marrow endothelial cell (HBMEC) lines 4L-HBMEC and HBMEC60, as well as the human myeloma cell lines (HMCLs) XG1, MM1S and L363 were examined. As shown in Figure 1B, primary BMSCs and BMSC lines express both CXCL12 α and CXCL12 γ mRNA, but CXCL12 expression was very low or undetectable in the HBMEC cell lines and in HMCLs.

HSPGs immobilize CXCL12 γ on the cell surface of BMSCs

The C-terminal domain of CXCL12 γ contains three positive-charged HSPG-binding motives.^{15,16} Via this domain, exogenously overexpressed CXCL12 γ in HEK293T cells was shown to strongly interact with cell-surface expressed HSPGs and immobilized on the cell membrane.¹⁵ We hypothesized that CXCL12 γ expressed by BMSCs might similarly be retained by HSPGs on the cell, thereby functioning as a membrane-bound chemokine. By employing the CXCL12 γ -isoform specific mAb 6E9, we indeed detected CXCL12 γ on the cell surface of CXCL12 γ -expressing primary BMSCs and on the BMSC line HS5, but not on the HMCL XG1, which is CXCL12 γ mRNA negative (Figure 1C).

Both primary BMSCs and HS5 cells express high levels of cell-surface HSPGs, as detected by the HS-specific mAb 10E4 (Figure 2A). To establish if HS moieties indeed are responsible for the membrane retention of CXCL12 γ , we employed CRISPR-Cas9 mediated KO to delete *EXT1*, encoding the HS co-polymerase EXT1 which is critically required for the synthesis of HS-chains.²⁴ *EXT1* deletion resulted in a complete loss of cell-surface HS expression in HS5 BMSCs and caused a concomitant loss of cell-surface CXCL12 γ (Figure 2B). Similarly, enzymatic removal of HS (Figure 2C-i) from primary BMSCs by heparitinase treatment resulted in a strong reduction of cell-surface bound CXCL12 γ (Figure 2C-ii). Incubation with recombinant CXCL12 γ of the HMCL XG1, which expresses the HSPG syndecan-1²⁴ but does not express CXCL12 γ (Figure 1B and 1C), resulted in cell-surface binding. This binding was also strongly attenuated by *EXT1* deletion, further illustrating the importance of HSPGs for CXCL12 γ cell surface binding (Figure 2D).

To assess whether membrane retention indeed is a unique feature of CXCL12 γ , not shared with the 'common' CXCL12 α isoform, we exogenously overexpressed C9-tagged CXCL12 γ or CXCL12 α in either wild-type or EXT1KO HS5 cells. Intracellular expression of CXCL12 γ -C9 and CXCL12 α -C9 was detected in all conditions (Figure 2E). Importantly, CXCL12 γ -C9 was detected on the cell surface of wild type, but not of HS5-EXT1KO cells (Figure 2E-i). By contrast, overexpressed CXCL12 α -C9 was not detected on the cell surface of either of these cells (Figure 2E-ii). Taken together, these data demonstrate that BMSC produce CXCL12 γ , which once secreted, is immobilized by HSPGs on the surface of BMSC.

Recombinant CXCL12 γ mediates MM cell adhesion to VCAM and fibronectin

It is well established that CXCL12 α is able to induce integrin-mediated adhesion of MM cells to VCAM and fibronectin.^{26,27} To assess if the γ -isoform of CXCL12 can similarly induce MM cell adhesion, the HMCLs XG1 and MM1S were exposed to various concentration of either recombinant CXCL12 α or CXCL12 γ . As is shown in Figure 3A, both CXCL12 α and CXCL12 γ induced adhesion of these HMCLs to VCAM and fibronectin. Whereas the CXCL12 α -induced adhesion showed a concentration-dependent bell-shape curve, typical for chemokine/CXCL12 α induced responses, with an optimum at 25-50 ng/ml, CXCL12 γ hardly induced

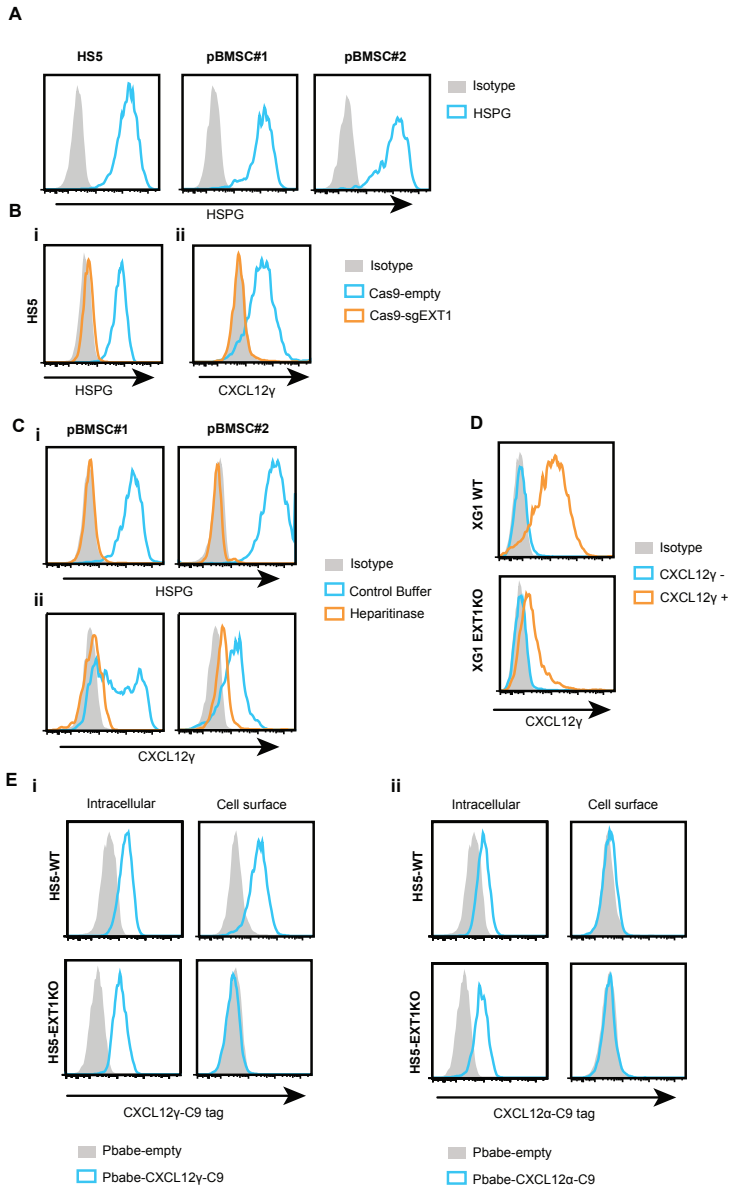
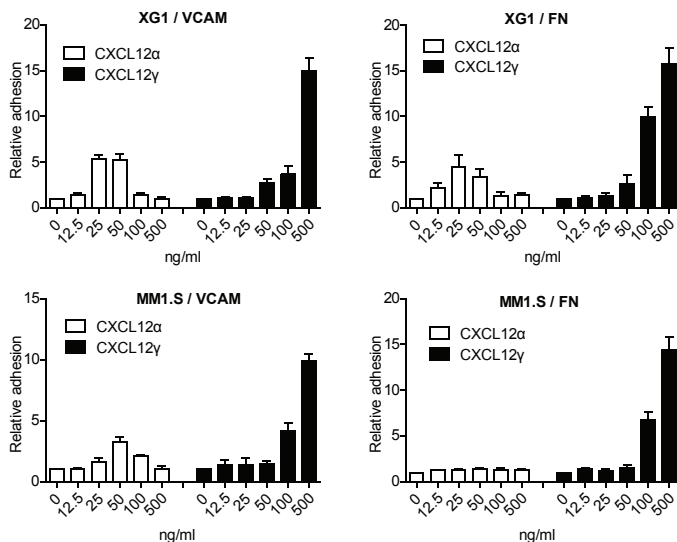
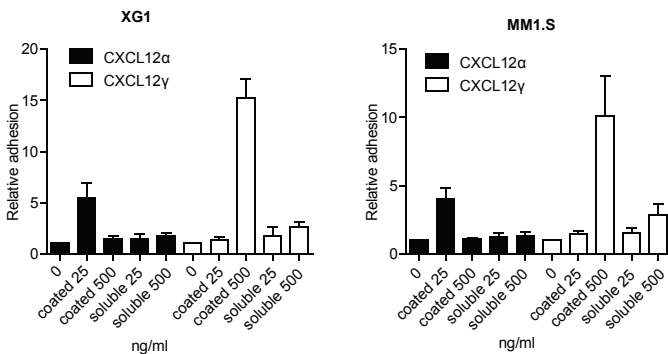


Figure 2. Bone marrow stromal cell HSPGs present CXCL12 γ on the cell surface. (A) Flow cytometry detection of cell surface expression of HSPG in BMSC line HS5 and primary BMSCs. (B) i : Flow cytometry detection of cell surface expression of HSPG in HS5 transduced with CRISPR empty vector or CRISPR sgEXT1. ii: Flow cytometry detect cell surface expression of CXCL12 γ in HS5 transduced with the CRISPR empty vector or CRISPR sgEXT1. (C) i : Flow cytometry detect cell surface expression of HSPG in primary BMSCs treated with heparitinase or control buffer. ii: Flow cytometry detect cell surface expression of CXCL12 γ in primary BMSCs treated with heparitinase or control buffer. (D) Flow cytometry detect cell surface binding of CXCL12 γ in MM cell line XG1 WT (wild type) or XG1 EXT1 KO (knockout) pre-incubated with 1 μ g/ml recombinant CXCL12 γ . (E) HS5-WT and HS5-EXT1KO cells were transduced with Pbabe-CXCL12 γ -C9 (i) or Pbabe-CXCL12 α -C9 (ii). Intracellular and cell surface expression of CXCL12 γ -C9 (i) and CXCL12 α -C9 (ii) was detected with anti-C9 antibody.

A



B



C

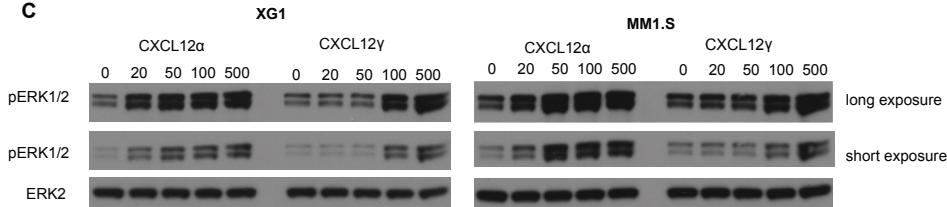


Figure 3. CXCL12 α and CXCL12 γ induced MM adhesion. (A) XG1 and MM1S cells were allowed to adhere to a surface co-coated with VCAM or fibronectin (FN) and CXCL12 (ng/ml) as indicated for 2 minutes. The adhesion in the absence of CXCL12 was normalized to 1. The mean \pm SD of 3 independent experiments, each in triplicate, is shown. (B) XG1 and MM1S cells were allowed to adhere to VCAM, either co-coated with CXCL12 (coated) or in the presence of medium containing soluble CXCL12 (soluble) for 2 minutes. The adhesion in the absence of CXCL12 condition was normalized to 1. The mean \pm SD of 3 independent triplicate experiments is shown. (C) XG1 and MM1S were stimulated with CXCL12 α or CXCL12 γ using indicated concentration (ng/ml) and immunoblotted for phosphorylated ERK1/2. Total ERK2 was used as loading control.

adhesion at these relatively low ligand concentrations. At higher ligand concentrations, however, the CXCL12 γ -induced adhesion was much stronger than the maximum adhesion achieved by CXCL12 α . Notably, both CXCL12 α and CXCL12 γ only induce MM cell adhesion when coated to the adherence surface, soluble ligands failed to do so, indicating that CXCL12 immobilization is crucial for induction of cell adhesion (Figure 3B). Similar to induction of adhesion, ERK1/2 phosphorylation by CXCL12 γ required higher ligand concentrations compared to activation by the CXCL12 α isoform (Figure 3C). These observations are in line with previous studies in T lymphoblastoid cells, showing that CXCL12 γ has reduced agonistic activity, presumably due to interference of its C-terminal region with the activation of CXCR4.²⁸

Endogenous CXCL12 γ and HSPGs promote adhesion of MM cell to BMSCs

In view of the established functions of the CXCL12/CXCR4 axis in cell adhesion as well as the specific physicochemical properties of CXCL12 γ , we hypothesized that this isoform might play a specific role in mediating MM adhesion to and retention in the BM niche. This prompted us to study the role of BMSC-derived CXCL12 γ in MM cell adhesion to BMSCs. To be able to specifically study the biological functions of BMSC-derived CXCL12 γ in MM adhesion to BMSCs, we deleted the HS-binding motif-containing C-terminal tail of CXCL12 γ , which characterizes this specific isoform. This was achieved in HS5 BMSCs by employing two sgRNAs targeting CXCL12 γ upstream and downstream of the fourth exon encoding this C-terminal tail (Figure 4A). Successful deletion yielded a smaller PCR product with a predicted size of approximately 500bp (Figure 4B) and was verified by Sanger sequencing (Figure 4C). Moreover, deletion was confirmed by loss of cell surface CXCL12 γ protein expression (Figure 4D). Notably, the expression of CXCL12 α was not affected by deletion of CXCL12 γ (Supplemental figure 1) and deletion of CXCL12 γ had no effect on BMSC growth (Figure 4E).

As is shown in figure 5A, the HMCLs XG1 and MM1.S showed strong adhesion to BMSCs, but did not adhere to BMECs. Interestingly, deletion of CXCL12 γ significantly reduced the MM cell adhesion to HS5 BMSC (Figure 5B and Supplemental figure 2). Since CXCL12 γ expressed at the surface of BMSCs is HSPG bound, we envisaged that loss of HSPG would also affect adhesion of MM to BMSCs. Indeed, we observed that adhesion of MM cells to HS5-EXT1KO cells was significantly reduced (Figure 5C). Similar to HMCLs, primary MM cells also displayed a reduced adhesion to BMSCs lacking either CXCL12 γ or HSPGs (Figure 5D). Importantly, exogenous overexpression of CXCL12 γ by means of a CXCL12 γ expression construct, completely restored the adhesion defect in the HS5-CXCL12 γ KO BMSCs. Notably, this CXCL12 γ expression construct can not be targeted by CRISPR since the two CXCL12 γ CRISPR sgRNAs target non-coding sequences (Figure 4A). In contrast, in line with the critical role of HSPGs in immobilizing CXCL12 γ , overexpression CXCL12 γ in the HS-deficient BMSCs did not rescue the adhesion defect (Figure 5E). Taken together, our data indicate that

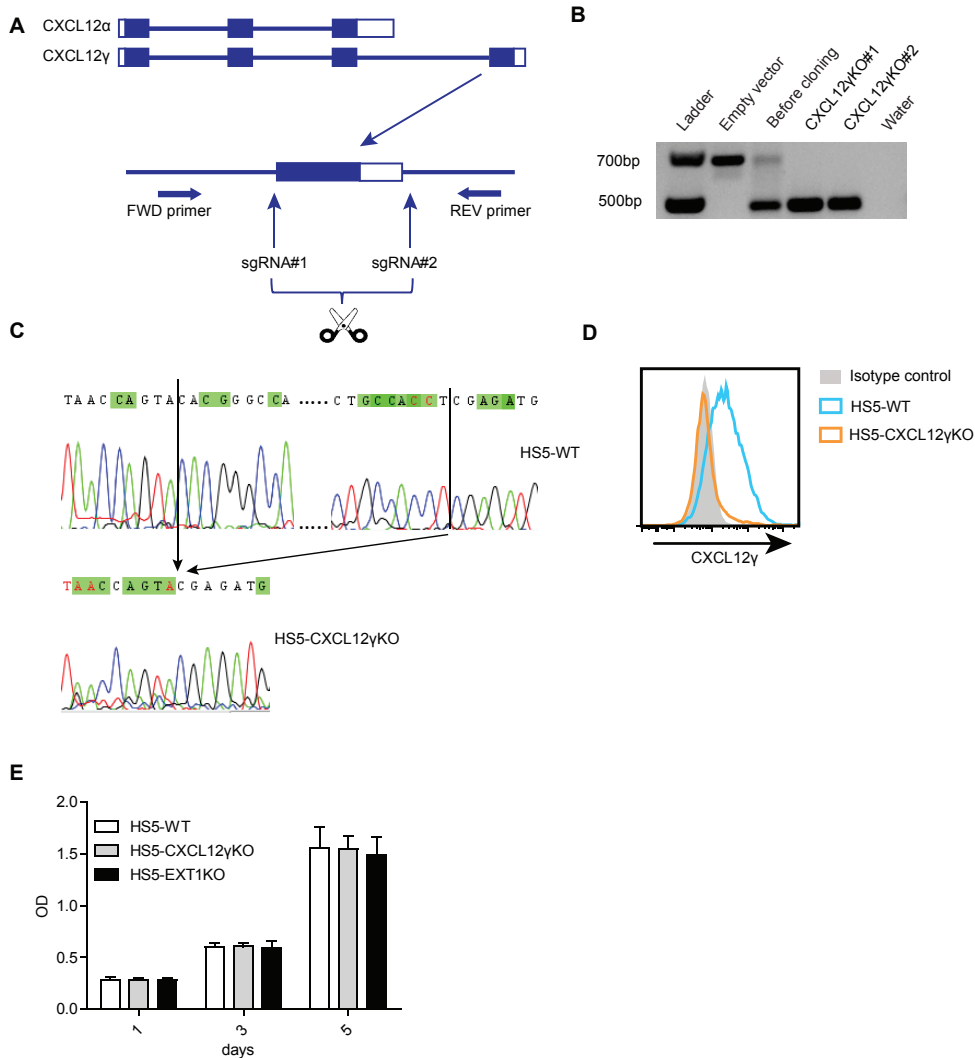


Figure 4. Dual-sgRNA CRISPR-mediated CXCL12 γ KO. (A) schematic depiction of CRISPR-induced deletion of the fourth exon (C-terminal tail) of CXCL12 γ . sgRNA#1 was designed to target upstream of the fourth exon, and sgRNA#2 was designed to target the 3'UTR of the fourth exon. Rectangle represent exon, with filled rectangle represent the coding sequence. Line indicate intron. Forward and reverse PCR primers were used to detect the deletion. (B) PCR analysis of the deletion of CXCL12 γ . PCR primers used are as indicated in figure 4A. Genomic DNA was isolated from HS5 cells treated as follows: Empty vector, HS5 transduced with CRISPR empty vector; Before cloning, HS5 co-transduced with CRISPR sgRNA#1 and CRISPR sgRNA#2, and cells were harvest before single cell cloning; CXCL12 γ KO, HS5 co-transduced with CRISPR sgRNA#1 and CRISPR sgRNA#2, and cells were harvest from two single cell KO clones. Water was used as negative control. DNA ladder size is indicated in the left. (C) Sanger sequencing analysis of the CRISPR-induced deletion. Genomic DNA was isolated from HS5 cells transduced with empty vector CRISPR (CXCL12 γ WT) or transduced with CRISPR sgRNA#1 and CRISPR sgRNA#2(CXCL12 γ KO). The CRISPR cutting sites are indicated with arrows. (D) Flow cytometry analysis the cell surface expression of CXCL12 γ in HS5 cell transduced with empty vector CRISPR (CXCL12 γ WT) or upon CRISPR-induced CXCL12 γ KO. (E) MTT assay analysis of the growth of HS5-WT, HS5-CXCL12 γ KO and HS5-EXT1KO cells. The mean \pm SD of 3 independent experiments, each in triplicate, is shown.

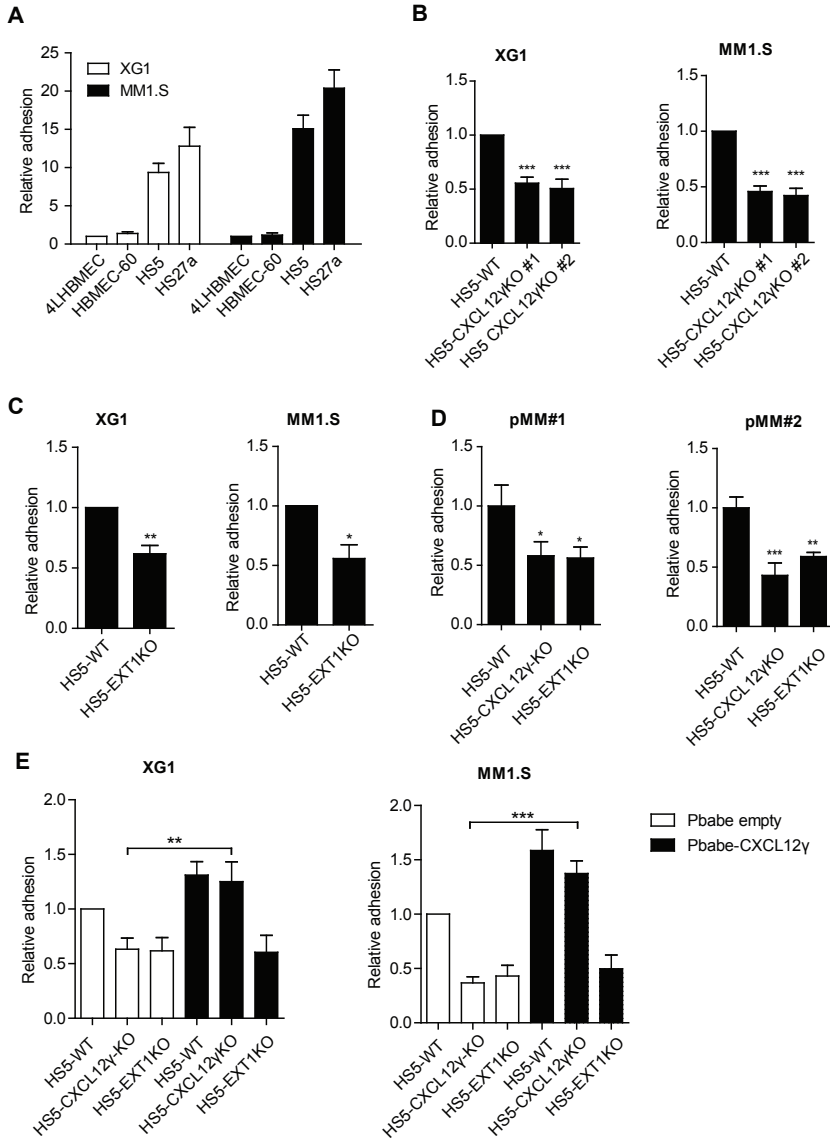


Figure 5. Role of BMSC expressed CXCL12 γ and HSPGs on MM adhesion. (A) Flow cytometry analysis of HMCLs XG1 and MM1S adhesion to HBMEC lines (4LHBMEC and HMMEC-60) and BMSC lines (HS5 and HS27a). The adhesion to 4LHBMEC is normalized to 1. The mean \pm SD of 3 independent experiments in triplicate is shown. (B) XG1 and MM1S cells adhesion to HS5-WT or two independent CXCL12 γ KO single cell clones. The mean \pm SD of 3 independent experiments in triplicate is shown. ***, $P \leq 0.001$ using one-way ANOVA analysis. (C) XG1 and MM1S cells adhesion to HS5-WT or HS5-EXT1KO. The mean \pm SD of 3 independent experiments in triplicate is shown. *, $P \leq 0.05$; **, $P \leq 0.01$ using unpaired student's t test. (D) Primary MM cells from two patients were allowed to adhere to HS5-WT, CXCL12 γ KO, or EXT1KO cells. A representative plot for 2 independent experiment is shown. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$ using one-way ANOVA analysis. (E) XG1 and MM1S cells adhesion to HS5-WT, CXCL12 γ KO, or EXT1KO cells. co-transduced with Pbase-empty vector or Pbase-CXCL12 γ . The mean \pm SD of 3 independent experiments in triplicate is shown. **, $P \leq 0.01$; ***, $P \leq 0.001$ using one-way ANOVA analysis.

CXCL12 γ expressed by BMSC and immobilized by cell-surface HSPGs plays an important role in mediating MM cell adhesion to BMSC.

BMSC-mediated resistance to proteasome inhibitors involves CXCL12 γ and HSPGs

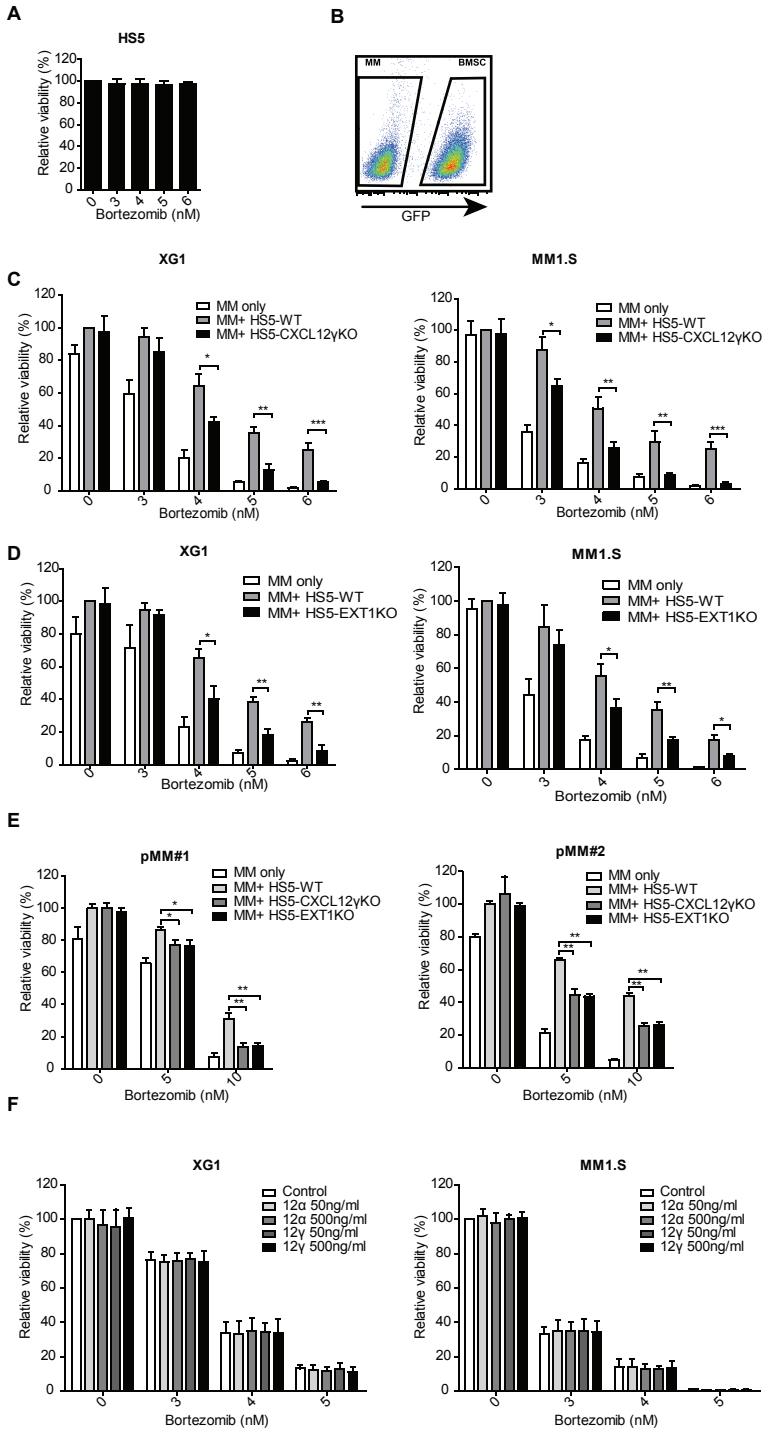
Interaction of MM cells with BMSCs plays a central role the homing/retention, growth, and survival of MM cells as well as in their drug resistance.^{1,2,29,30} Inhibition of the CXCL12/CXCR4 axis, disrupting interaction of MM cells with BMSCs, alleviates the protective effect of BMSCs and enhances the sensitivity of MM cells to drugs.^{1,8}

Given our observation that BMSC-derived CXCL12 γ play an important role in MM adhesion to BMSCs, we next study the possible involvement of CXCL12 γ in the protective effect of BMSCs against drug-induced MM cell death. We focused on bortezomib and the related proteasome inhibitor carfilzomib since these drugs represent a mainstay of current MM therapies. Moreover, unlike MM cells, which are highly sensitive to these drugs, BMSC are bortezomib resistant *in vitro* (Figure 6A), allowing reliable quantification of MM-specific cell death.

To measure MM cell death and the protective effect of BMSCs, XG1 and MM1S HMCLs or pMM cells were co-cultured with HS5 BMSC, expressing green-fluorescent protein (GFP) to distinguish them from the MM cells (Figure 6B). As shown in Figure 6C, 6E, and Supplemental figure 3, co-culture with HS5-wild-type (WT) BMSCs protected both HMCLs and pMMs from bortezomib-induced cell death. Interestingly, this protective effect was significantly reduced in co-cultures with HS5-CXCL12 γ KO cells, indicating involvement of CXCL12 γ in mediating bortezomib resistance.

Since deletion of *EXT-1* resulted in loss of CXCL12 γ from the cell surface of MM cells (Figure 2B), we examined if *EXT-1* KO would also reduce the protective effect of BMSCs. Indeed, similar to HS5-CXCL12 γ KO cells, HS5-EXT1KO cells showed a reduced capacity to protect HMCLs against bortezomib-induced cell death (Figure 6D,E). Furthermore, HS5-CXCL12 γ KO cells and HS5-EXT1KO cells also provided reduced protection to carfilzomib induced MM cell death (Supplemental figure 4). Recombinant CXCL12 γ (or CXCL12 α), in the

Figure 6. > BMSC-mediated resistance of myeloma cells to bortezomib involves CXCL12 γ and HSPGs. (A) BMSC HS5 were co-cultured with XG1 in the presence of bortezomib for 3 days. The viability of BMSC HS5 was analyzed by flow cytometry. (B) Representative plot illustrating the discrimination of MM cells and BMSCs by flow cytometry. (C) HMCLs alone or co-cultured with BMSC HS5-WT or with HS5-CXCL12 γ KO in the presence of bortezomib for 3 days. The viability of MM cells was analyzed by flow cytometry. The mean \pm SD of 3 independent experiments in triplicate is shown. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$ using one-way ANOVA analysis. (D) HMCLs alone or co-cultured with BMSC HS5 WT or with HS5 EXT1KO in the presence of bortezomib for 3 days. The viability of MM cells was analyzed by flow cytometry. The mean \pm SD of 3 independent experiments in triplicate is shown. *, $P \leq 0.05$; **, $P \leq 0.01$ using one-way ANOVA analysis. (E) Primary MM cells from two patients were cultured alone or co-cultured with HS5-WT, HS5-CXCL12 γ KO or HS5-EXT1KO in the presence of bortezomib for 3 days. The viability of MM cells was analyzed by flow cytometry. A representative plot for 2 independent experiment is shown. *, $P \leq 0.05$; **, $P \leq 0.01$ using one-way ANOVA analysis. (F) HMCLs XG1 or MM1.S were cultured on a surface co-coated with CXCL12 α or CXCL12 γ and FN, in the presence of bortezomib, for 3 days. The cell viability was analyzed by flow cytometry. The mean \pm SD of 3 independent experiments in triplicate is shown. >



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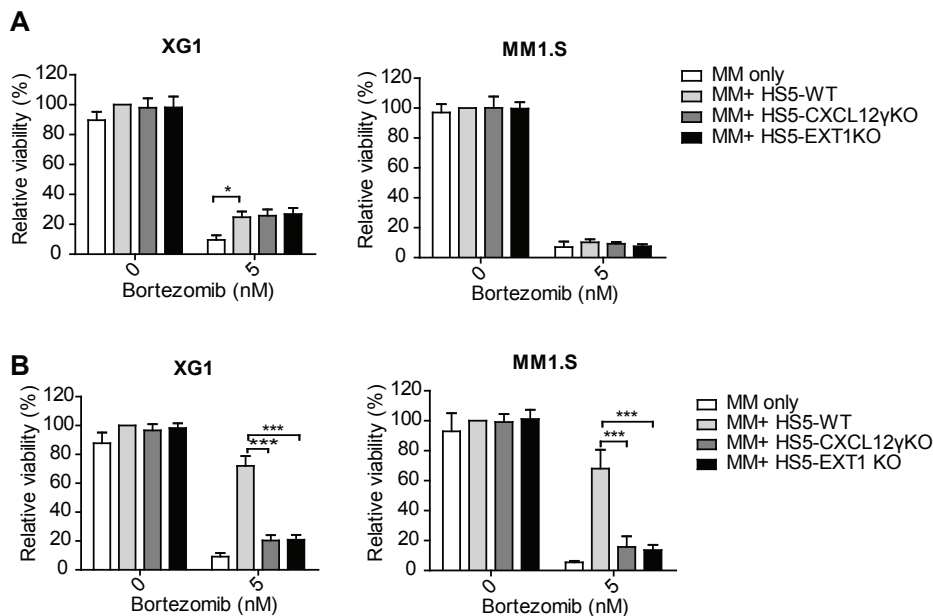


Figure 7. BMSC derived-CXCL12 γ and HSPGs mediate CAM-DR (A) MM cells were cultured in transwells, either alone or, separated by the transwell, in co-culture with HS5-WT, HS5-CXCL12yKO or HS5-EXT1KO, in the presence of bortezomib for 3 days. The viability of MM cells was analyzed by flow cytometry. The mean \pm SD of 3 independent experiments in triplicate is shown. *, $P \leq 0.05$ using one-way ANOVA analysis. (B) MM cells were cultured, either alone or co-cultured with HS5-WT, HS5-CXCL12yKO or HS5-EXT1KO, in the presence of bortezomib for 3 days. The MM cells which is in suspension (i.e., the non-adherent cells) were removed. The viability of the remaining adherent MM cells was analyzed by flow cytometry. The mean \pm SD of 3 independent experiments in triplicate is shown. ***, $P \leq 0.001$ using one-way ANOVA analysis.

absence of BMSCs, did not affect the sensitivity of HMCLs to these drugs (Figure 6F). These findings suggest that BMSCs mediate drug resistance via a mechanism involving cell-surface CXCL12 γ and HSPGs.

BMSC derived-CXCL12 γ and HSPGs mediate CAM-DR.

Drug resistance mediated by the MM BM microenvironment can either be caused by soluble factors or by interactions via cell adhesion molecules, termed soluble factor-mediated drug resistance (SFM-DR) and cell adhesion-mediated drug resistance (CAM-DR), respectively.^{1,31,32} To directly investigate the cell-cell contact dependence of the BMSC-mediated resistance to bortezomib and address whether soluble factors released by BMSCs are (also) involved, we employed transwell co-cultures to physically separate MM cells from BMSCs. As shown in Figure 7A, in the transwell setting, HS5 BMSC weakly but significantly protected the HMCL XG1, but not MM1.S, from bortezomib-induced cell death. This protective effect was not affected by deletion of CXCL12 γ or EXT1. However, in the direct contact setting, BMSCs conferred a much stronger drug resistance to both XG1 and MM1.S. Importantly, this protective effect was largely abrogated by deletion of CXCL12 γ or EXT1 and, hence, was

CXCL12 γ and HSPG-dependent (Figure 7B). Thus, CXCL12 γ and HSPG on the cell surface of BMSCs promote MM cell adhesion to these BMSC and thereby play an important role in cell adhesion-mediated drug resistance (CAM-DR)

Discussion

The CXCL12/CXCR4 axis plays a key role in the homing of normal plasma cell precursors and MM cells to the BM,^{9,10} but the expression and specific role of CXCL12 γ , a recently characterized CXCL12 isoform which bind HSPGs with an extremely high affinity, has not been addressed. Here, we show that CXCL12 γ is expressed *in situ* by stromal cells in the human bone niche as well as by primary BMSC isolates and BMSC cell lines. Upon secretion, CXCL12 γ is immobilized on the cell surface of BMSCs by HSPG, which are highly expressed. Functionally, this BMSC-bound CXCL12 γ promotes adhesion of MM cells to BMSCs, thereby protecting MM cells from drug-induced cell death.

Our immunohistochemical study of the *in situ* expression of CXCL12 γ in the human BM shows that CXCL12 γ is exclusively expressed by the stromal component of the BM. Distinct expression was observed in stromal cells bordering the periosteum, lipocytes, and venules, areas with a putative niche function for plasma cells and/or HSCs.^{33,34} In BM involved by MM, an increased expression was found. A similar expression pattern was also found with a pan-CXCL12 antibody. Study of isolated primary BMSCs and cell lines corroborated these findings, demonstrating that CXCL12 γ (and CXCL12 α) mRNA is expressed by BMSCs but not by endothelial or MM cell lines.

CXCL12 γ has an extremely high affinity for HSPGs due to its unique C-terminal domain.^{15,16} Interestingly, we observed that both primary BMSCs and HS5 cells constitutively express CXCL12 γ on their cell surface, suggesting that this chemokine is bound by HSPGs upon secretion. Indeed, we observed that KO of the HS-chain co-polymerase *EXT1* in HS5 BMSCs results in a complete loss of membrane-bound CXCL12 γ . Importantly, immobilization by cell-surface HSPGs is a specific feature of CXCL12 γ , since overexpression of CXCL12 α in HS5 did not result in detectable membrane expression, notwithstanding substantial cytoplasmic expression.

We observed that isoform-specific CRISPR-Cas9 mediated deletion of CXCL12 γ strongly reduces the capacity of HS5 BMSCs to mediated adhesion of MM cells to their cell surface. This finding extends a previous study, showing that a total (*i.e.* non-isoform-specific) knockdown of CXCL12 leads to reduced adhesion of MM cells to BMSCs.⁸ Like CXCL12 γ deletion, *EXT1* KO also inhibited MM cell adhesion to the BMSCs. Importantly, whereas the defective adhesion to HS5-CXCL12 γ KO cells could be overcome by overexpressing CXCL12 γ , overexpression of CXCL12 γ in HS5-*EXT1*KO cells did not correct the adhesion defect,

suggesting that immobilized by HSPGs is critically required for the CXCL12 γ -controlled adhesion. In line with this, when using recombinant CXCL12 to induced MM cell adhesion to VCAM or fibronectin coated plastic, we observed that only immobilized (*i.e.* coated) CXCL12 was able to induced adhesion (Figure 3B), affirming that CXCL12 immobilization is required for effective adhesion induction.

Interaction of MM cells with BMSCs plays a central role in MM cell homing/retention and can confer drug resistance.^{1,7} We observed that co-culture with HS5 BMSCs of the HMCLs XG1 and MM1.S and of primary MM cells does not affect tumor cell viability *per se*, however, it significantly reduced their sensitivity to the proteasome inhibitors bortezomib and carfilzomib. Interestingly, this resistance was largely annulled by deletion of CXCL12 γ , identifying CXCL12 γ as a major factor in the BMSC-mediated drug resistance. HS5 BMSCs cells with a deletion of *EXT-1* showed a similarly reduced capacity to protect MM cells, indicating that membrane retention of CXCL12 γ by HSPGs is essential for this protective effect.

Drug resistance mediated by BMSCs can either be caused by soluble factors or by interactions via cell adhesion molecules.^{1,31,32} We observed that the protective effect of BMSCs to MM cells was largely abolished by physical separation of the MM and BMSCs, implying that this protection requires direct cell-cell contact. This suggests that BMSCs might convey MM drug resistance via direct integrin-mediated adhesion, rather than by soluble growth and survival factors, although such factors are abundantly expressed by BMSCs.^{24,30,35} However, in contradiction with this scenario, we observed that recombinant CXCL12 γ (or CXCL12 α) induced adhesion to fibronectin or VCAM-coated plastic did not protect MM cells against bortezomib-induced cell death, indicating that integrin-mediated cell adhesion *per se* is not sufficient to instigate bortezomib resistance. Conceivably, the CXCL12 γ -controlled adhesion serves to retain MM cells in close physical contact with the BMSCs. In addition to providing MM cells with growth and survival signals through integrin receptors, this contact with essential niche cells provides MM cell with access to many short-range growth and survival factors, such as Wnts and vascular endothelial growth factor^{36,37}, which may act in concert to mediate drug resistance.

Our data suggest targeting the CXCL12/CXCR4 axis, specifically CXCL12 γ and/or is interaction with HS, as a potential therapeutic strategy. Notably, MM cells express high levels of the HSPG syndecan-1, which is crucial for MM cell survival and promotes Wnt-mediated cell proliferation²⁴ as well as HGF, FGF, EGF and APRIL induced signaling.³⁸⁻⁴⁰ Hence, targeting HSPGs or their biosynthesis machinery could simultaneously hit MM cells and their physical interaction with BM microenvironment. With respect to the CXCL12/CXCR4 axis, previous studies have already shown that disrupting the interaction between MM cells and BMSCs, by using the CXCR4 inhibitor AMD3100, enhances MM sensitivity to multiple therapeutic agents such as bortezomib, dexamethasone and melphalan.^{1,7,30}

In addition, targeting CXCL12, irrespective of the isoform, by olaptesed pegol (ola-PEG), which neutralizes CXCL12, prevented MM progression in a murine model¹⁹⁶. Interestingly, a recent phase IIa clinical trial reported that patients with relapsed/refractory MM respond favorably to a combination of bortezomib or dexamethasone with ola-PEG.⁴¹ Apart from CXCR4, MM cells also express CXCR7, which is alternative receptor of CXCL12. The CXCL12/CXCR7 axis may also be involved in adhesion induced drug resistance in MM⁷ as well as in MM progression⁴². Targeting the ligand CXCL12(γ) might be used to simultaneously inhibit signaling through both CXCR4 and CXCR7.

Taken together, in this study we demonstrate that BMSCs express CXCL12 γ , which tightly binds to cell-surface HSPGs. Loss of BMSC CXCL12 γ or HSPGs disrupt the interaction between MM cells and the BMSCs, alleviating the protective effect of BMSCs against anti-MM agents. Our study supports the concept of targeting the BM microenvironment to treat MM, and advocates combining cytotoxic agents with drugs that disrupt interaction between MM cells and the microenvironment as promising therapeutic strategy.

Acknowledgments

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Authorship Contributions

Z.R. designed the research, performed experiments, analyzed the data, designed the figures and wrote the paper; H.L., A.K., and W.K., performed experiments. F.A.S provide materials. M.S. and S.T.P. supervised the study, designed the research and analyzed the data. S.T.P wrote the paper.

Conflict of interest

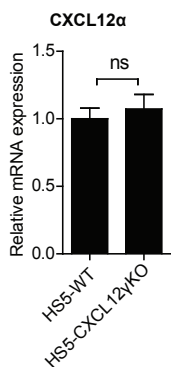
The authors declare no conflict of interest.

Reference list

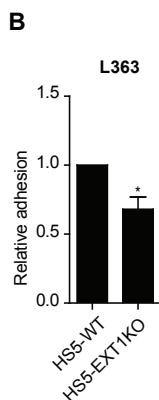
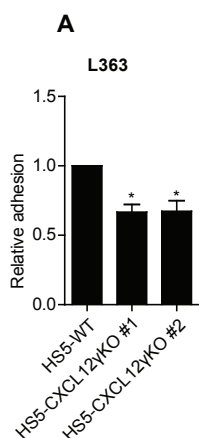
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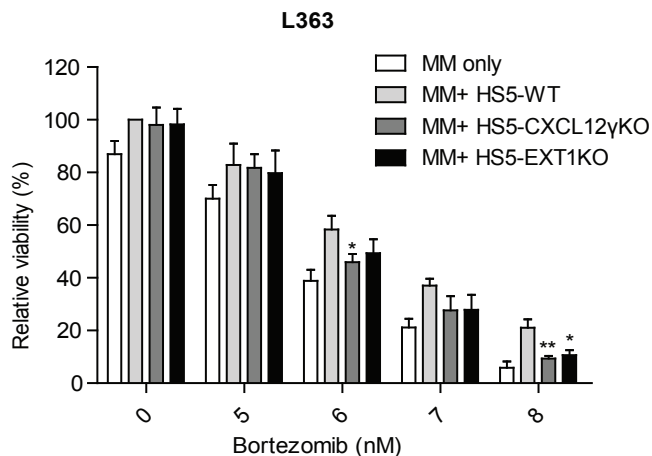
Supplemental data



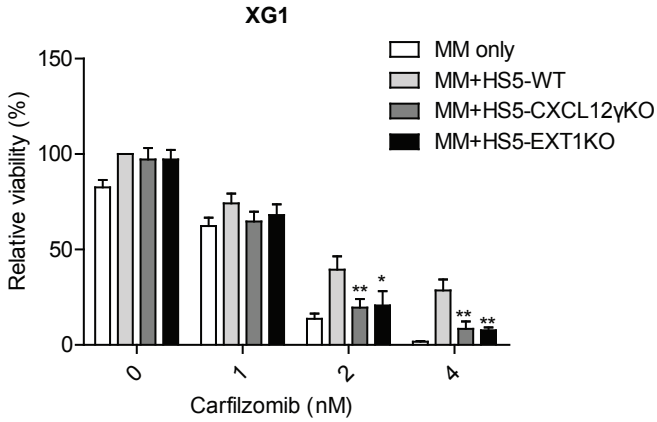
1. mRNA expression of CXCL12α in HS5-WT and HS5-CXCL12γ KO cells. A representative plot for 2 independent experiment is shown. ns, $P > 0.05$; using unpaired student's t test.



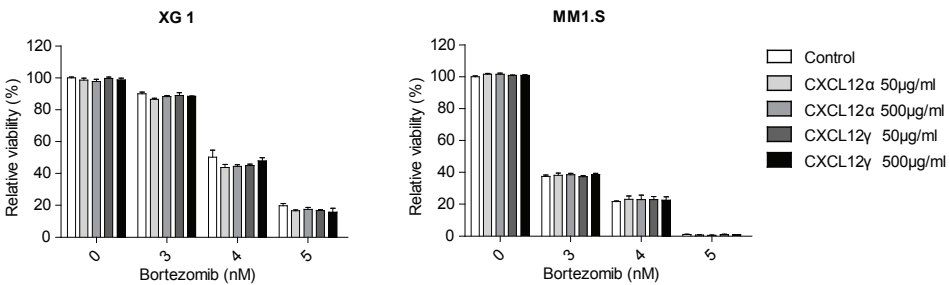
2. (A) HMCL L363 adhesion to HS5-WT or two CXCL12γKO single cell clones. The mean \pm SD of 3 independent experiments in triplicate is shown. *, $P \leq 0.05$ using one-way ANOVA analysis. (B) HMCL L363 cells adhesion to HS5-WT or HS5-EXT1KO. The mean \pm SD of 3 independent experiments in triplicate is shown. *, $P \leq 0.05$ using unpaired student's t test.



3. HMCL L363 was cultured alone or co-cultured with HS5-WT, HS5-CXCL12γKO or HS5-EXT1KO in the presence of bortezomib for 3 days. The viability of L363 cells was analyzed by flow cytometry. The mean \pm SD of 3 independent experiments in triplicate is shown. *, $P \leq 0.05$; **, $P \leq 0.01$ using one-way ANOVA analysis.



4. HMCL XG1 was cultured alone or co-cultured with HS5-WT, HS5-CXCL12 γ KO or HS5-EXT1KO in the presence of carfilzomib for 3 days. The mean \pm SD of 3 independent experiments in triplicate is shown. *, $P \leq 0.05$; **, $P \leq 0.01$ using one-way ANOVA analysis.



5. HMCLs XG1 or MM1.S were allowed to adhere to 96 well plate co-coated with CXCL12 α or CXCL12 γ and VCAM, and subsequently cultured in the presence of bortezomib for 3 days. The cell viability was analyzed by flow cytometry. A representative plot for 2 independent experiment is shown.