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Original Articles

System Xc^- inhibition blocks bone marrow-multiple myeloma exosomal crosstalk, thereby countering bortezomib resistance

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ABSTRACT

Multiple myeloma (MM) cells derive proliferative signals from the bone marrow (BM) microenvironment via exosomal crosstalk. Therapeutic strategies targeting this crosstalk are still lacking. Bortezomib resistance in MM cells is linked to elevated expression of xCT (the subunit of system Xc^-). Extracellular glutamate released by system Xc^- can bind to glutamate metabotropic receptor (GRM) 3, thereby upregulating Rab27-dependent vesicular trafficking. Since Rab27 is also involved in exosome biogenesis, we aimed to investigate the role of system Xc^- in exosomal communication between BM stromal cells (BMSCs) and MM cells. We observed that expression of xCT and GRMs was increased after bortezomib treatment in both BMSCs and MM cells. Secretion of glutamate and exosomes was simultaneously enhanced which could be countered by inhibition of system Xc^- or GRMs. Moreover, glutamate supplementation increased exosome secretion by increasing expression of Alix, TSG101, Rab27a/b and VAMP7. Importantly, the system Xc^- inhibitor sulfasalazine reduced BMSC-induced resistance to bortezomib in MM cells *in vitro* and enhanced its anti-MM effects *in vivo*. These findings suggest that system Xc^- plays an important role within the BM and could be a potential target in MM.

1. Introduction

Multiple myeloma (MM) is a hematological malignancy, characterized by expansion of monoclonal plasma cells in the bone marrow (BM) [1]. Bortezomib (BZ) was the first proteasome inhibitor approved by the US Food and Drug Administration (FDA) for the treatment of MM and is currently one of the standard-of-care agents for first-line treatment [2]. However, drug resistance (DR) to BZ ultimately develops in the majority of MM patients [3]. This resistance can be intrinsic or extrinsically mediated by the BM, but the exact mechanisms remain unclear. Exosomes are a subset of extracellular vesicles with a size range of ~50–150 nm in diameter secreted by a wide range of cell types and contain many constituents derived from the original cells [4,5]. We have previously demonstrated that exosomes play important roles in the crosstalk between bone marrow stromal cells (BMSCs) and MM cells which promotes MM progression by supporting MM proliferation and survival, as well as inducing DR, immune suppression, angiogenesis and osteolysis [6–11]. It has become increasingly evident that therapies targeting the

Abbreviations: BM, Bone marrow; BMMC, Bone marrow mononuclear cell; BMSC, Bone marrow stromal cell; BZ, Bortezomib; CM, Conditioned medium; CPPG, (RS)-α-Cyclopropyl-4-phosphonophenylglycine; DR, Drug resistance; EFS, Event-free survival; EX, Exosome; FDA, US Food and Drug Administration; Glu, Glutamate; GRM, Glutamate metabotropic receptor; H-BMSC, Primary human bone marrow stromal cell; KO, Knockout; MM, Multiple myeloma; MMRF, Multiple Myeloma Research Foundation; MVB, Multivesicular body; M-BMSC, Primary mouse bone marrow stromal cell; NTA, Nanoparticle tracking analysis; OS, Overall survival; PFS, Progression-free survival; SASP, Sulfasalazine; TSG101, Tumor susceptibility gene 101 protein.

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communication between cancer cells and their microenvironment have higher therapeutic success [12–14]. Therefore, the bone marrow-MM exosomal crosstalk is a promising therapeutic target.

System Xc^- which is composed of a specific light chain, xCT (solute carrier family 7 member 11, SLC7A11), and a heavy chain, 4F2, can export intracellular Glu in exchange for equimolar extracellular cystine [15]. Extracellular Glu released by system Xc^- can activate glutamate metabotropic receptor (GRM) 3, thereby promoting invasiveness of breast cancer cells by upregulating Rab27-dependent vesicular trafficking [16]. As Rab27 also plays important roles in the intracellular trafficking of multivesicular bodies which promotes exosome secretion [17], we hypothesized that system Xc^- mediated Glu secretion could promote exosome release, contributing to exosomal crosstalk in the bone marrow. A recent study showed that the tumor-promoting effect of cancer-associated fibroblasts, derived from pancreatic ductal adenocarcinoma, is dependent on their high expression of xCT [14]. Though the xCT involvement in the pro-MM effect of BMSCs remains unexplored, upregulation of xCT expression in MM cells by BZ treatment is reported to promote DR by protecting MM cells from oxidative stress through increased glutathione production [18,19]. However, if and how system Xc^- mediated Glu release is involved in xCT mediated DR in MM is still unclear. Therefore, we investigated whether system Xc^- mediated Glu secretion could promote exosome release in both BMSCs and MM cells, leading to DR by mediating intercellular communication.

In this study, we evaluated whether BZ triggers exosome secretion by upregulating xCT and enhancing Glu release, both in MM cells and BMSCs *in vitro*. We investigated which signaling pathways are activated by binding of Glu to its receptors. Finally, we determined whether inhibiting system Xc^- using sulfasalazine (SASP) could block exosome exchange and reduce tumor growth both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Dataset analysis

The survival analysis is based on the TT2 cohort (GSE2658) and the Multiple Myeloma Research Foundation (MMRF) CoMMpass Study of patients with MM.

2.2. Drugs and reagents

BZ was obtained from Selleckchem (Munich, Germany), Sulfasalazine and Glu from Sigma-Aldrich (Darmstadt, Germany) and (RS)-α-Cyclopropyl-4-phosphonophenylglycine (CPPG) was obtained from Tocris Bioscience (Bristol, UK).

2.3. Exosome isolation

The concentrated conditioned medium (CM) was prepared as described in supplemental methods and was filtered again using 0.22 μm pore filters, followed by incubation with ExoQuick-TC exosome precipitation solution (System biosciences, Mountain View, CA, USA) at 4 °C overnight. Exosomes were harvested by centrifugation at 1500*g* for 30 min and then suspended in 50 μl PBS or RPMI1640 medium without serum, followed by centrifugation at 10000*g* for 2 min to remove contaminating cell debris. Concentrations of exosome proteins were quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

2.4. Nanoparticle tracking analysis (NTA)

The number of exosomes in the 10 × CM or mouse serum was determined using ZetaView (Particle Matrix, Germany). The data for each sample were processed using 8.4.2 SP2 analytical software.

2.5. Measurement of glutamate concentration

The concentration of Glu in the cell-exposed supernatants or mouse serum was determined using the Glutamine/Glutamate-Glo™ Assay kit (Promega, Madison, WI, USA).

2.6. Mice and 5T33MM model

Female C57BL/KaLwRij mice were purchased from Envigo (Horst, the Netherlands). Housing and maintenance were following conditions approved by the Ethical Committee for Animal Experiments of Vrije Universiteit Brussel (license No LA1230281, project No 20-281-6). The 5T33MM model was maintained as previously described [20] and was propagated by intravenous injection of 5×10^5 BM cells of diseased mice in young (6–10 weeks old) syngeneic mice. End-stage 5T33MM mice were used for primary BMSC isolation or *in vivo* experiments.

2.7. In vivo experiment

5T33MM mice (n = 9/group) were randomly selected for each group and were treated with vehicle, SASP (200 mg/kg/day, ad libitum in drinking water), or with BZ (0.6 mg/kg twice weekly, subcutaneous), or with the combination of both for 3 weeks. 2% orange extract (Arcadie, Méjannes-lès-Alès, France) was added to increase the palatability of the drug solutions. All mice were sacrificed when the first mouse showed signs of morbidity. Tumor burden in the BM was assessed by evaluating plasmacytosis on BM cytospins stained with May–Grünwald–Giemsa. Additionally, serum M spike was measured by means of serum protein electrophoresis. Serum exosome levels and serum Glu concentrations were measured as described before.

2.8. Statistical analysis

All data represent the mean ± standard deviation (SD) of at least three experiments and were analyzed with the GraphPad Prism 7.0 software. Statistical significance was determined by using the Mann-Whitney *U* test and the One-way ANOVA test, and was set at $P < 0.05$. (ns: not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$).

Details about the cell purification and culture, quantitative real-time PCR (qPCR), western blot (WB) analysis, cell viability assay and preparation of CM are included in the supplemental materials.

3. Results

3.1. High expression of xCT is correlated with poor survival in MM patients

To determine whether xCT plays a role in MM development, we first investigated expression of xCT in primary MM cells as well as the relationship between xCT expression and prognosis in patients with MM. Gene expression analysis of BM plasma cell samples from the TT2 cohort showed that primary MM cells had higher expression of xCT compared with normal plasma cells (Fig. 1A). Survival analysis for the TT2 cohort and the MMRF cohort showed that high expression of xCT in MM cells

correlates with significantly lower EFS, PFS and OS of MM patients (Fig. 1B and C). WB analysis showed that xCT was more highly expressed in CD138-bone marrow mononuclear cells (BMMCs) compared to CD138+ MM cells obtained from patients with MM (Fig. 1D), leading us to hypothesize that high expression of xCT in BMSCs could play an important role in supporting MM cell proliferation in the BM microenvironment.

3.2. Bortezomib induces enhanced expression of xCT and GRMs in BMSCs and MM cells

We next investigated whether BZ treatment upregulated xCT expression in BMSCs and MM cells. Different doses of BZ that reduced cell viability about 30% (Fig. S1) were used to treat the human stromal cell line (HS-5) and the human MM cell lines (LP1 and ANBL-6) for 24h. qPCR and WB analysis showed that BZ enhanced expression of xCT in all cells tested (Fig. 2A–C).

Exporting Glu into the extracellular milieu is one of the important functions of the system Xc^- and extracellular Glu can activate downstream signaling pathways by binding to GRMs. GRMs are a family of G-protein-coupled receptors, which are subclassified into three groups based on sequence homology, G-protein coupling and ligand selectivity [21]. Group I includes GRM1 and GRM5, group II includes GRM2 and GRM3, and group III includes GRM4, GRM6, GRM7 and GRM8. These receptors have recently been linked to transformation and maintenance of various cancer types including glioma, breast cancer and melanoma skin cancer [16,22]. Among them, group II and III GRMs were reported to be expressed in human MM cells [23], and group III GRMs were expressed in primary BMSCs [24]. Gene expression analysis of the MMRF cohort showed that the basic expression of GRMs is lower than that of xCT and expression of GRM4 and GRM8 is higher compared to the other GRMs in primary MM cells (Fig. S2). As glutamate is reported to trigger expression of GRMs in mast cells [25], BZ may regulate expression of GRMs through system Xc^--mediated glutamate secretion. Thus, we tested the expression of group II and III GRMs in BMSCs and MM cells after treatment with BZ. qPCR and WB analysis revealed that BZ induced expression of GRMs in a cell type-specific manner. Expression of GRM3, GRM7 and GRM8 was mainly increased in MM cells, while GRM2 and GRM7 expression levels were elevated in HS-5 cells (Fig. 2A–C). The expression of GRM6 was increased in LP1 cells (Fig. S3) but was too low to detect in HS-5 and ANBL-6 cells. Survival analysis of the MMRF cohort showed that positive expression of GRM7 in MM cells correlated with significantly lower PFS and a tendency of lower OS of MM patients, however, high expression of GRM8 did not show correlation with prognosis of MM patients (Fig. 2D). As GRM7 was the most highly increased among GRMs after treatment with BZ in both HS-5 and MM cell lines, we detected the expression in primary cells by WB and found that GRM7 expression is much higher in CD138+ MM cells than in CD138- BMMCs (Fig. S4).

3.3. Secretion of exosomes and Glu is increased in BMSCs and MM cells after treatment with BZ, which can be reduced by targeting system Xc^-

As xCT expression is elevated in both BMSCs and MM cells after treatment with BZ, we evaluated whether expression of xCT influenced exosome secretion. To this end, we concentrated 10 × CM from supernatant obtained from LP1, ANBL-6 and HS-5 cells after treatment with BZ and measured the number of extracellular vesicles in the 10 × CM using NTA by Zetaview (Fig. 3A). Furthermore, exosomes were also isolated from CM after treatment with BZ; their presence was confirmed

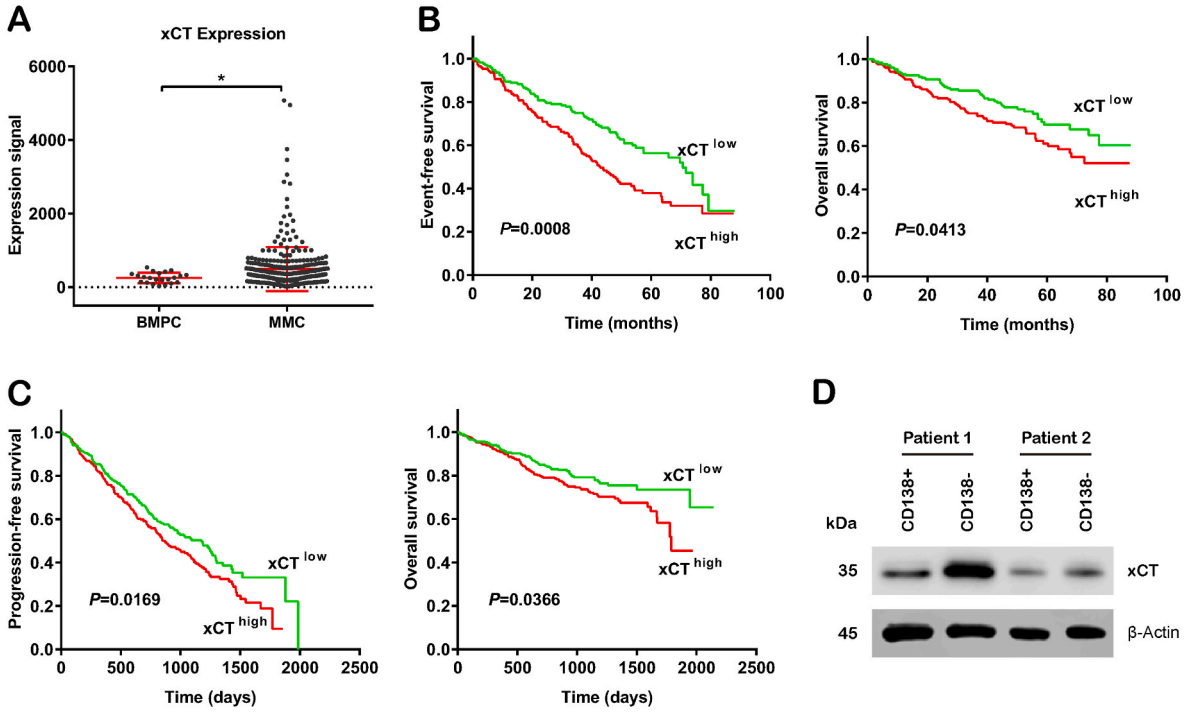

Fig. 1. xCT mRNA expression is increased in MM cells, correlating with poor survival in MM patients. **(A)** xCT mRNA expression levels of bone marrow plasma cell samples from healthy donors (BMPC, n = 22) and plasma cell samples from pre-treated multiple myeloma patients (MMC, n = 345) were obtained from the publicly available microarray dataset GSE2658 (TT2 cohort). Mean expression ± SD is shown in red. Statistical significance was determined by a two-tailed, unpaired Mann-Whitney test. *$P < 0.05$. **(B)** Event-free survival (EFS) and overall survival (OS) according to high xCT expression (n = 172) and low xCT expression (n = 173) on MM cells were determined in MM patients from the TT2 cohort using Log-rank (Mantel-Cox) test (cut-off points used is the median value). **(C)** Progression-free survival (PFS) and OS according to high xCT expression (n = 327) and low xCT expression (n = 326) on MM cells were determined in MM patients from the MMRF cohort using Log-rank (Mantel-Cox) test (cut-off points used is the median value). **(D)** CD138-bone marrow mononuclear cells (BMMCs) and CD138+ MM cells were obtained from 2 MM patients. Expression of xCT in BMMCs and MM cells was detected by WB. β-Actin was used as a loading control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

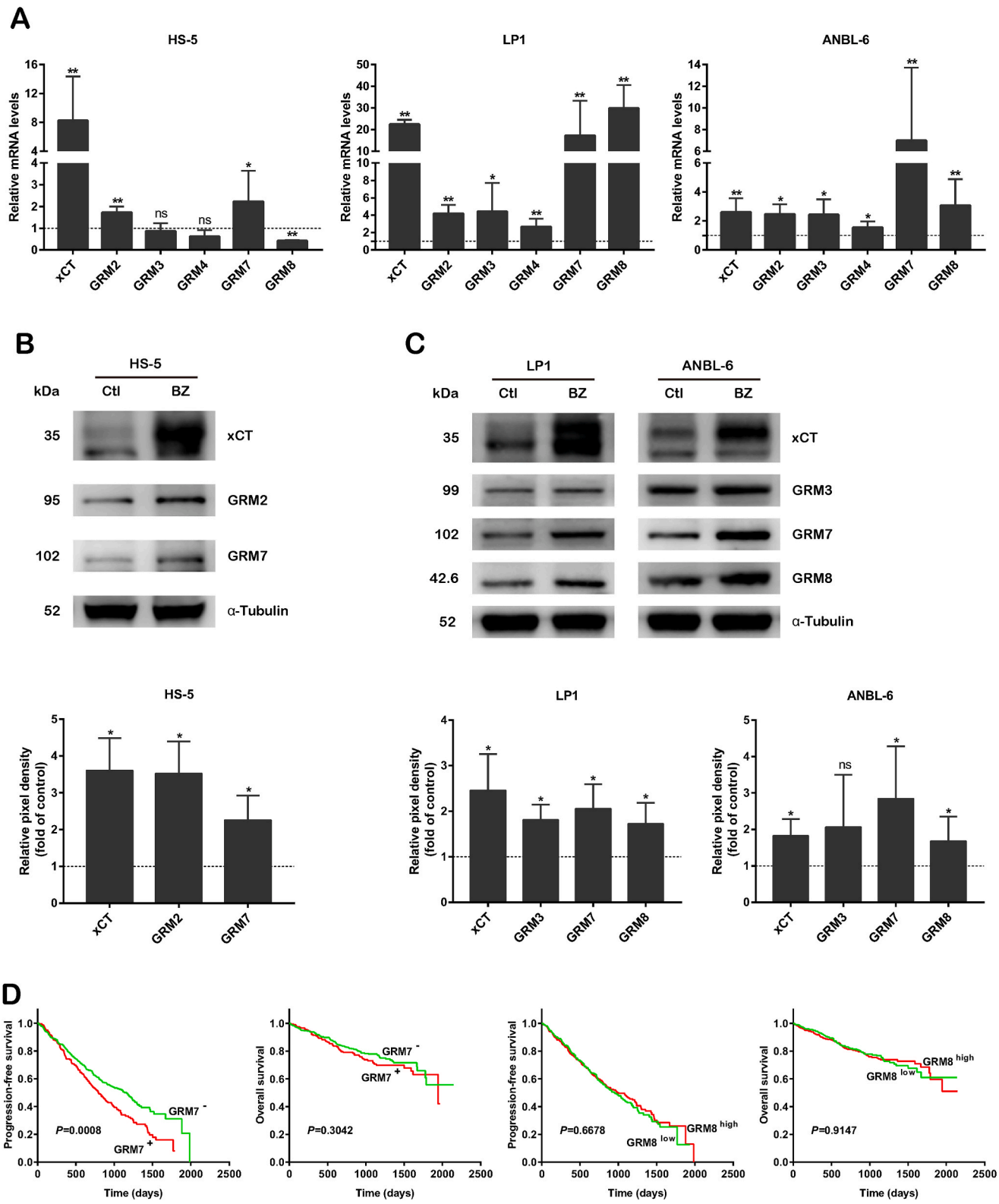

Fig. 2. BZ induces expression of xCT and GRMs in both BMSCs and MM cells. **(A–C)** HS-5, LP1 and ANBL-6 cells were treated with BZ (12 nM, 12 nM and 6 nM, respectively) for 24h. **(A)** mRNA levels of xCT and GRMs were determined using qPCR and normalized according to the housekeeping gene *ABL1*. The fold-change for each gene was relative to mRNA levels in the control group. Results shown are mean ± SD of at least three independent experiments. Statistical significance compared to the control group was determined by a two-tailed, unpaired Mann-Whitney test. $*P < 0.05$; $**P < 0.01$; ns: not significant. **(B, C)** xCT and GRMs protein levels were analyzed by WB. α-Tubulin was used as a loading control. One experiment representative of four is shown. The pixel densities of proteins were quantified and normalized to the control group. Results shown are mean ± SD of four independent experiments. Statistical significance compared to the control group was determined by a two-tailed, unpaired Mann-Whitney test. $*P < 0.05$; ns: not significant. **(D)** PFS and OS according to positive GRM7 expression (n = 210) and negative GRM7 expression (n = 443), high GRM8 expression (n = 327) and low GRM8 expression (n = 326) (cut-off points used is the median value) on MM cells were determined in MM patients from the MMRF cohort using Log-rank (Mantel-Cox) test.

by testing exosomal markers using WB (Fig. 3B). Results showed that BZ increased exosome release (Fig. 3A and B) but did not affect the diameter of exosomes (Fig. S5A) in both BMSCs and MM cells.

We next investigated whether enhanced expression of xCT contributed to increased secretion of exosomes. We used SASP as a system Xc^- inhibitor and evaluated its impact on secretion of exosome and Glu. SASP dampened most of the BZ-induced elevation of exosome secretion in HS-5, LP1 and ANBL-6 cells (Fig. 3A and B) without affecting their viability (Fig. S5B). Similar to the observations on exosome release, the BZ-induced increase in Glu secretion in HS-5, LP1 and ANBL-6 cells was also abolished by SASP treatment (Fig. 3C). Moreover, SASP decreases secretion of exosomes and Glu in primary mouse BMSCs (M-BMSC) isolated from 5T33MM mice and primary human BMSCs (H-BMSC) isolated from MM patients (Figs. S6A and B).

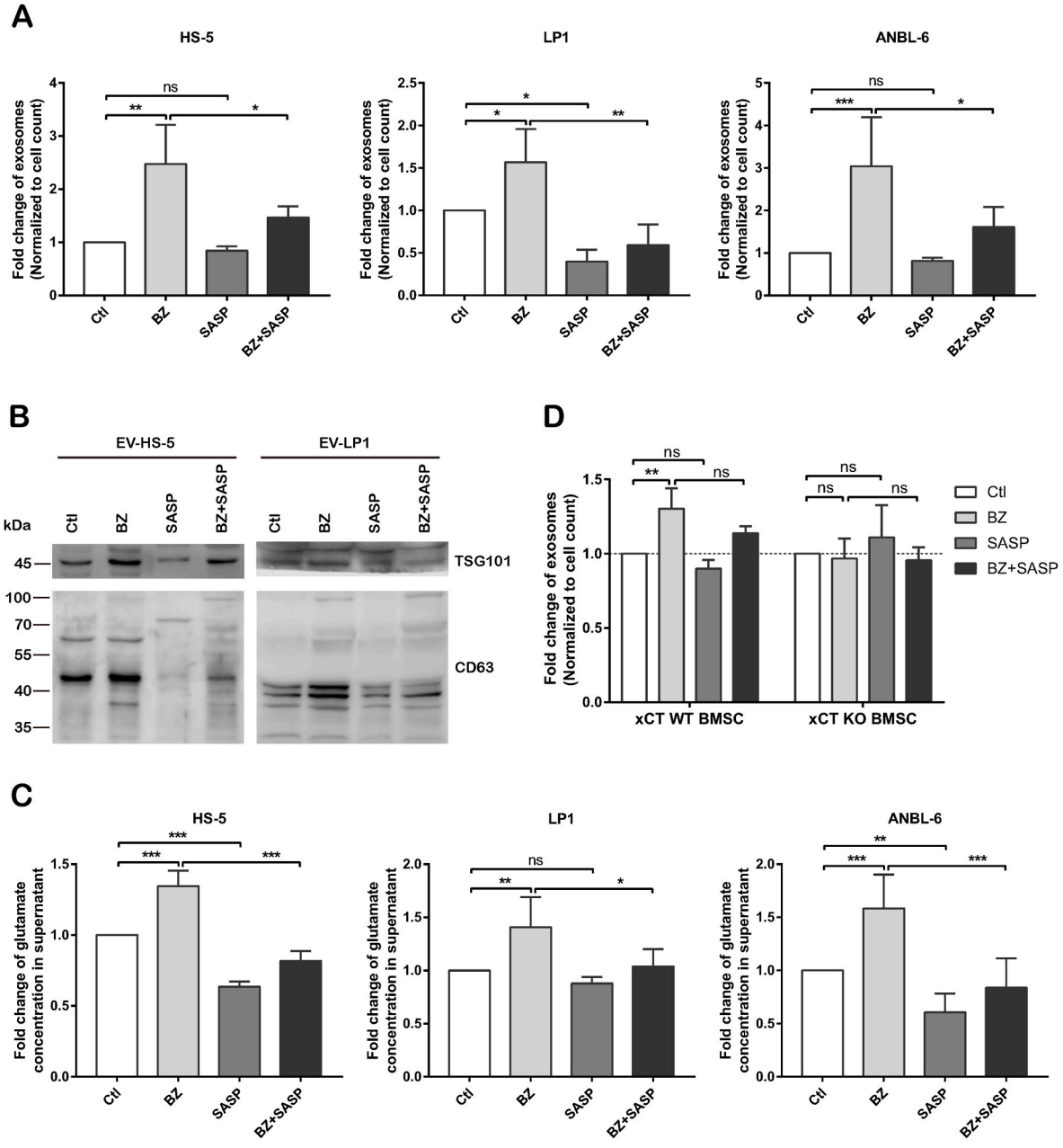

Fig. 3. Inhibition of system Xc^- reduces BZ enhanced glutamate and exosome release. **(A–C)** HS-5, LP1 and ANBL-6 cells were treated with BZ (12 nM, 12 nM and 6 nM, respectively) with or without SASP (100 μM) for 24h. **(A)** Nanoparticle tracking analysis (NTA) by Zetaview showed the concentration of exosomes in the supernatant. The data were normalized to cell count for each condition and were relative to the exosome concentration in the control group. Results shown are mean ± SD of at least three independent experiments. Statistical significance was measured by One-way ANOVA test with Tukey's multiple comparisons test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns: not significant. **(B)** The same number of cells was seeded in each group and exosomes were isolated from the supernatant after 24h. WB analysis of exosomal markers TSG101 and CD63 showed changes of exosome concentration in the supernatant. One experiment representative of three is shown. **(C)** The concentration of glutamate in the supernatant was detected by Glutamine/Glutamate-Glo™ Assay kit and normalized to the control group. Results shown are mean ± SD of at least three independent experiments. Statistical significance was measured by One-way ANOVA test with Tukey's multiple comparisons test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns: not significant. **(D)** Primary mouse BMSCs isolated from xCT knockout (KO) mice and xCT wild-type (WT) mice were treated with BZ (4 nM) with or without SASP (100 μM) in the presence of 2-Mercaptoethanol (2-ME, 50 μM) for 24h. NTA showed concentration of exosomes in the supernatant. The data were normalized to cell count for each condition and were relative to exosome concentration in the control group. Results shown are mean ± SD of three independent experiments. Statistical significance was measured by One-way ANOVA test with Tukey's multiple comparisons test. $^{**}P < 0.01$; ns: not significant.

To further confirm the role of system Xc^- in exosome secretion, we investigated whether xCT knockout (KO) abrogated the changes of exosome secretion induced by BZ and SASP using primary BMSCs isolated from wild-type (xCT+/+) mice (xCT WT BMSCs) and xCT-deficient (xCT−/−) mice (xCT KO BMSCs) [26]. The same changes of exosome release induced by BZ and SASP were observed in xCT WT BMSCs as in HS-5 cells, while both BZ and SASP lose the effect on exosome release in xCT KO BMSCs (Fig. 3D).

3.4. Glutamate is involved in changes of exosome secretion in both BMSCs and MM cells after treatment with BZ

The above data indicate that system Xc^- is closely linked with quantitative changes of exosomes in both BMSCs and MM cells. In order to assess whether Glu is involved in this, we next evaluated the effect of blocking GRMs on exosome secretion. As BZ-induced elevated GRMs in BMSCs and MM cells belong to group II/III GRMs, we chose the non-selective group II/III GRM antagonist, CPPG, for the experiment. When comparing NTA profiles, CPPG, like SASP, also hampered the increase in exosome secretion caused by BZ in HS-5, LP1 and ANBL-6 cells (Fig. 4A) without affecting their viability (Fig. S7). The influence of CPPG on exosome secretion was again confirmed by detecting exosomal markers in the CM (Fig. 4B). Furthermore, as a positive control, we evaluated the effect of Glu on exosome secretion by supplementing the medium with this amino acid. Results showed that Glu increased the number of extracellular vesicles in the supernatant of HS-5, LP1, ANBL-6 cells (Fig. 4C) and the primary mouse BMSCs (Fig. S8A), without affecting cell viability (Fig. S8B). Exosomal markers were also measured

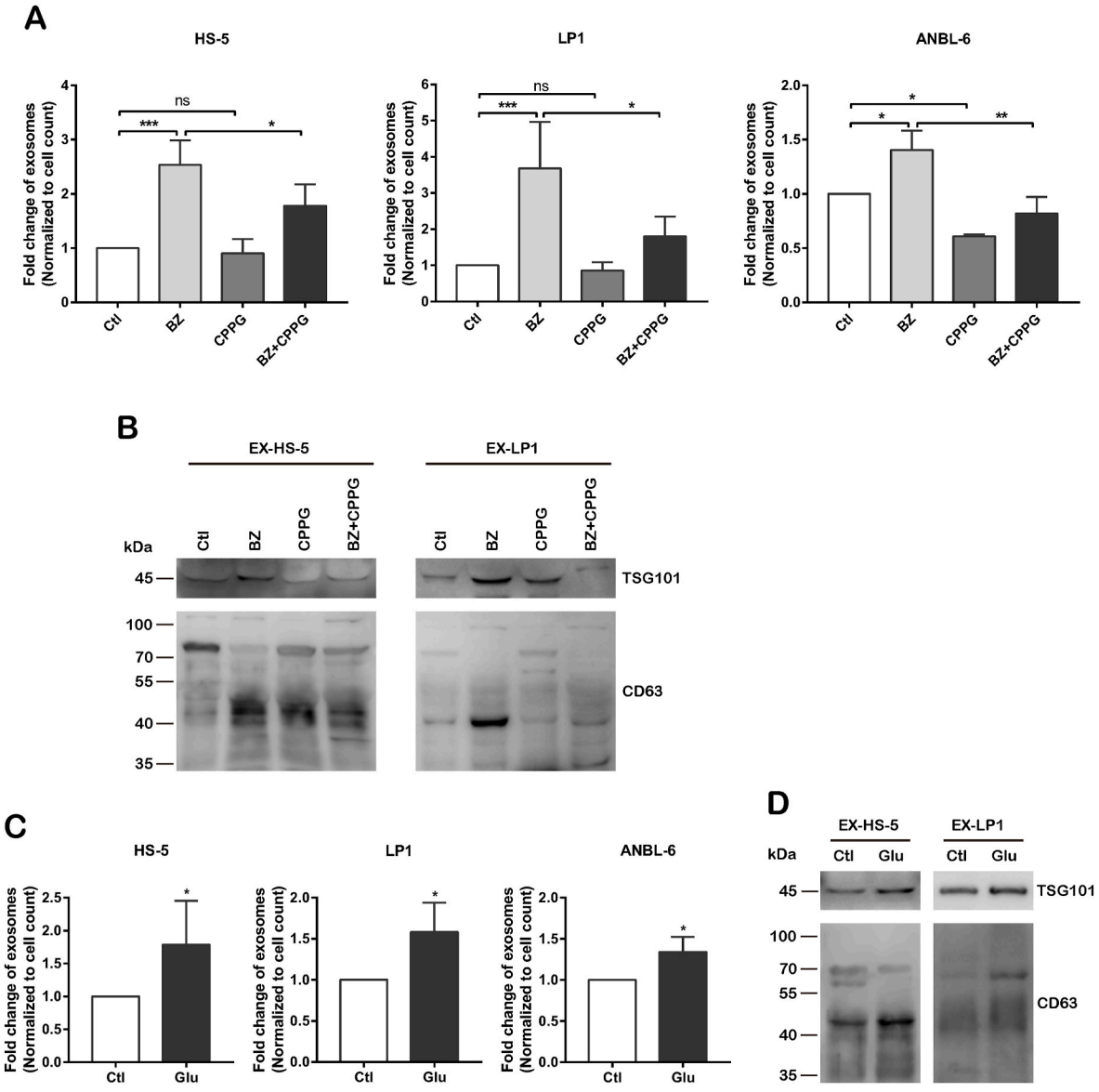

Fig. 4. Glu is involved in BZ-induced exosome secretion in BMSCs and MM cells. **(A, B)** HS-5, LP1 and ANBL-6 cells were treated with BZ (12 nM, 12 nM and 6 nM, respectively) with or without CPPG (20 nM) for 24h. **(C, D)** HS-5, LP1 and ANBL-6 cells were supplemented with Glu (250 μM) in the medium for 24h. **(A, C)** NTA showed the concentration of exosomes in the supernatant. The data were normalized to cell count for each condition and were relative to exosome concentration in the control group. Results shown are mean ± SD of at least three independent experiments. Statistical significance was measured by One-way ANOVA test with Tukey's multiple comparisons test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns: not significant. **(B, D)** The same number of cells was seeded in each group and exosomes were isolated from the supernatant after 24h. WB analysis of exosomal markers TSG101 and CD63 showed changes of exosome concentration in the supernatant. One experiment representative of three is shown.

to confirm the increased exosome secretion induced by Glu in HS-5 and LP1 cells (Fig. 4D). Moreover, addition of glutamate not only rescued the inhibitory effects of SASP on BZ-induced exosome secretion in HS-5 and LP1 cells (Fig. S9), but also rescued the abrogation of elevated exosome secretion induced by BZ in xCT KO BMSCs (Fig. S10).

3.5. System Xc^- mediated glutamate release contributes to exosome secretion by activating exosome secretion pathways

It is reported that tumor susceptibility gene 101 protein (TSG101) and Alix contribute to regulation of exosome biogenesis [27], Rab27a and Rab27b are involved in intracellular trafficking of multivesicular bodies [17], and VAMP7 is specifically required for fusion of multivesicular bodies with the plasma membrane and therefore is involved in exosome secretion [28]. To define the molecular mechanisms underlying the exosome secretion mediated by the system Xc^--GRM loop, we measured the expression of Alix, TSG101, Rab27a/b and VAMP7 in HS-5, LP1 and ANBL-6 cells after treatment with SASP or Glu. Results showed that expression of Alix, TSG101 and VAMP7 was decreased in all cells tested after treatment with SASP (Fig. 5A–D). However, expression of Rab27b was decreased in HS-5 cells (Fig. 5A, C), while expression of Rab27a was decreased in LP1 and ANBL-6 cells (Fig. 5B, D). To further assess whether expression changes of those proteins are induced by system Xc^- mediated Glu secretion, we detected their expression levels in HS-5, LP1 and ANBL-6 cells after supplementation with Glu in the medium. Consistent with the above results, Glu supplementation increased expression of Alix, TSG101, Rab27a/b and VAMP7 in the cells measured (Fig. 5A–D). Furthermore, survival analysis for the MMRF cohort showed that high Alix, TSG101 and VAMP7 expression in MM cells significantly correlated with poor survival of MM patients, though high Rab27a expression only showed a tendency of low OS (Fig. 5E).

3.6. SASP decreased BMSC-induced drug resistance in MM cells by modulating exosome-mediated communication between them

We have previously reported that BMSC-derived exosomes promote survival and DR in MM cells [29]. A reduction in exosome secretion should counter the supportive role of BMSCs on MM cell survival. To test this hypothesis, primary mouse MM cells (5T33vv) were treated with or without BZ, in the presence of an equal volume of exosomes enriched from the same number of M-BMSCs treated with either vehicle or SASP. We tested their viability by CellTiter-Glo and found that exosomes derived from SASP-treated BMSCs had reduced pro-survival effect and even reduced pro-DR effects compared with the exosomes derived from vehicle-treated BMSCs (Fig. 6A). To certify that the above effects were caused by the quantitative changes of exosomes induced by SASP, 5T33vv cells were treated with or without BZ, in the presence of an equal amount of exosomes (50 μg/ml) enriched from M-BMSCs treated with either vehicle or SASP. The cell viability assay showed that an equal amount of exosomes had the same direct pro-survival effect on 5T33vv cells; however, an equal amount of exosomes derived from SASP- treated BMSCs tended to have reduced pro-DR effects (Fig. 6B).

3.7. SASP enhances the anti-MM effect of BZ in vivo

We finally wanted to evaluate whether the observed anti-MM effects *in vitro* could be translated *in vivo*. We treated 5T33MM bearing mice with either low doses of BZ or SASP as single agent or a combination of both and evaluated effects on tumor burden (Fig. 6C). While SASP alone had no direct anti-tumor effects, it strongly enhanced the anti-tumor effect of low doses of BZ, as demonstrated by both reducing MM cells in BM and lowering serum M protein (Fig. 6D). Moreover, SASP combined with BZ significantly reduced tumor invasion in spleen (Fig. 6D) and had a tendency to reduce tumor invasion in liver (Fig. S10) compared with BZ monotherapy. Consistent with results of the *in vitro* experiments, mice treated with BZ had higher serum exosome levels than the vehicle group, and SASP tended to decrease this effect (Fig. 6E). Finally, serum Glu concentrations were significantly lower in the combination treated group versus vehicle group (Fig. 6F).

4. Discussion

In this study, we have shown for the first time that system Xc^- mediated Glu release contributes to DR in MM by modulating exosome exchange between BMSCs and MM cells both *in vitro* and *in vivo* (Fig. 7). High expression of xCT in MM cells correlates with poor survival in MM patients. Upregulation of xCT in MM cells has been reported to block the BZ-induced stress response by increasing uptake of cystine to support elevated glutathione synthesis [19]. As the system Xc^- antiporter also exports Glu, an additional consequence of high xCT expression would be accumulation of extracellular Glu. Whether this accumulation contributes to DR in MM has not been investigated yet. Here we found that the increased xCT expression in MM cells after treatment with BZ, is accompanied by an increased secretion of both Glu and exosomes, which is reduced by system Xc^- inhibition with SASP. By contrast, supplementation with Glu increased exosome release, indicating a link between system Xc^- mediated Glu release and exosome secretion. This is similar to exosome secretion in the nervous system whereby both glial and neuronal cells secrete exosomes in response to glutamatergic synaptic activation [30]. Next, we investigated how system Xc^- mediated Glu release impacts exosome secretion. Though GRM subtypes have also been reported to be expressed in human MM cells [23], it is not clear whether activation of GRM downstream signaling pathways is linked to exosome secretion. We show for the first time that expression of GRMs is also upregulated in MM cells after treatment with BZ and targeting GRMs using CPPG blocks the increased secretion of exosomes induced by BZ, indicating that system Xc^- mediated Glu release promotes exosome secretion through activating GRMs. Furthermore, we identified that TSG101, Alix, Rab27a/b and VAMP7 are involved in the process.

Importantly, our previous studies have shown that exosomes can mediate communication between MM cells and BMSCs, leading to DR [7,9,10]. Due to the important role of BMSCs in inducing MM cell DR, agents targeting not only MM cells but also BMSCs would be more effective in the treatment of MM. It is clear that, not only MM cells but also BMSCs are affected by BZ in the clinical application of BZ in MM therapy. However, the expression of xCT in BMSCs and whether it is linked to BZ resistance in MM has not been studied. We observed that expression of xCT and GRMs is also increased in HS-5 cells after treatment with BZ, accompanied by increased secretion of Glu and exosomes. Similarly to MM cells, treating BMSCs with SASP or CPPG reduced exosome secretion. More importantly, SASP reduced BMSC-induced DR to BZ in MM cells by modulating exosome secretion.

Though SASP was reported to directly suppress glioma growth by inhibiting nuclear factor-κB activity, concentrations of SASP greater than 500 μM and incubation duration longer than 48h were required [31]. Low concentration of SASP (250 μM) had minimal effect on the activity of nuclear factor-κB, but preferentially affected Glu release by acting on xCT [31]. Moreover, SASP (250 μM) was also reported to potentiate BZ-induced cytotoxicity in ANBL-6 cells [19], while the combination of SASP and BZ did not show direct synergistic anti-MM effects in our study. This may be due to the fact that lower doses of SASP (100 μM) were used in our study to focus on its inhibitory effect on xCT.

To further validate the potential clinical application of our findings, we next used the 5T33MM mouse model to show that while SASP alone has no anti-MM effect, SASP significantly decreases BM tumor burden and spleen infiltration when combined with BZ. We used suboptimal concentrations of BZ in both *in vitro* and *in vivo* experiments to underline the combination effect of SASP and BZ. Though the suboptimal concentration of BZ did not show a significant effect on plasmacytosis in the mouse experiment, it decreased the serum M protein levels which is an important indicator of tumor burden. While the serum exosome levels

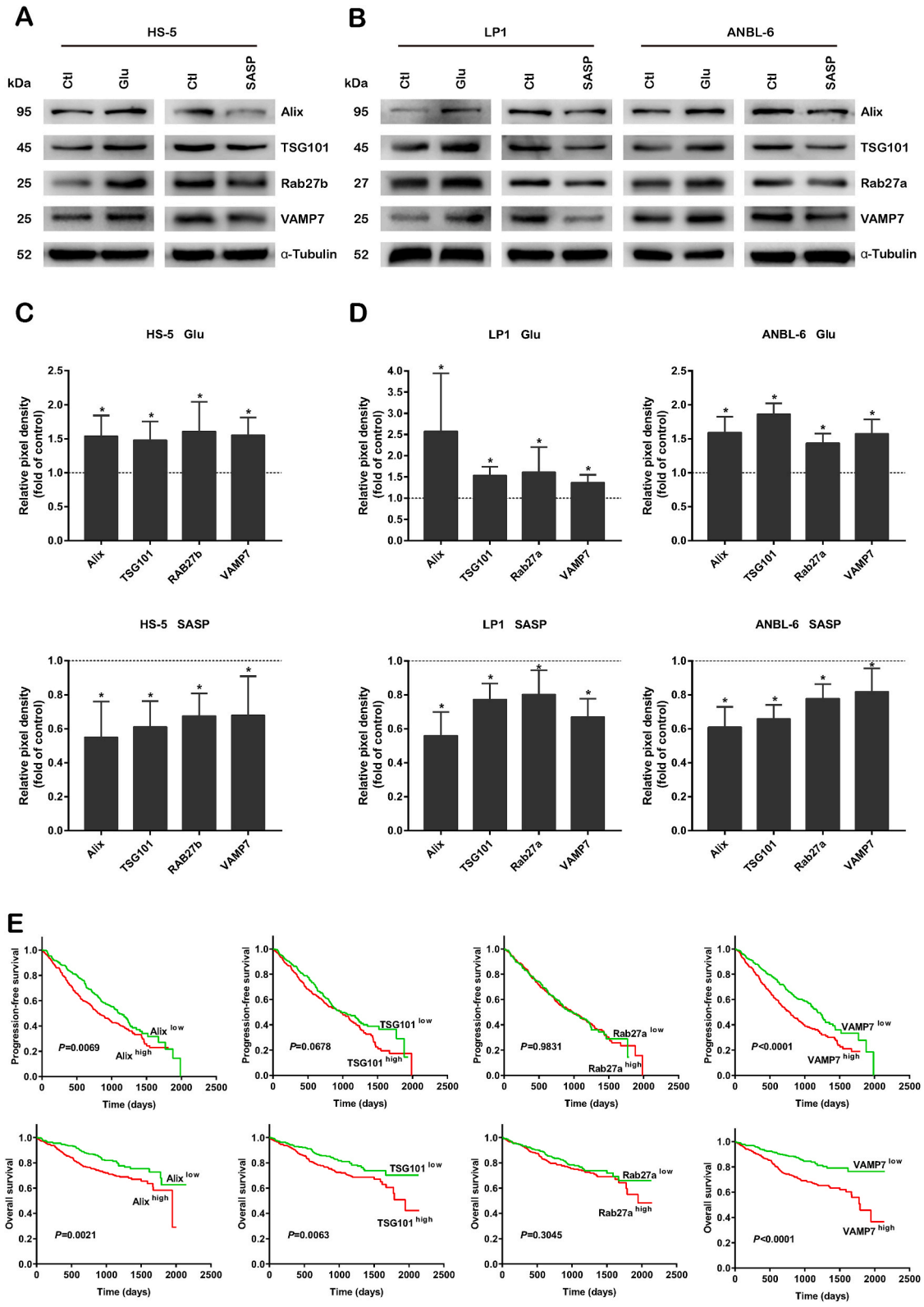

Fig. 5. System Xc^- mediated glutamate release increases expression of proteins involved in exosome secretion pathways. **(A–D)** HS-5, LP1 and ANBL-6 cells were treated with glutamate (Glu, 250 μM) or SASP (100 μM) for 12h. **(A, B)** Expression of exosome secretion associated proteins Alix, TSG101, Rab27a/b and VAMP7 was detected by WB. α-Tubulin was used as a loading control. One experiment representative of four is shown. **(C, D)** The pixel densities of proteins were quantified and were normalized to the control group. Results shown are mean ± SD of four independent experiments. Statistical significance compared to the control group was determined by a two-tailed, unpaired Mann-Whitney test. $*P < 0.05$. **(E)** PFS and OS according to high expression (n = 327) and low expression (n = 326) of Alix, TSG101, Rab27a and VAMP7 on MM cells were determined in MM patients from the MMRF cohort using Log-rank (Mantel-Cox) test (cut-off points used is the median value).

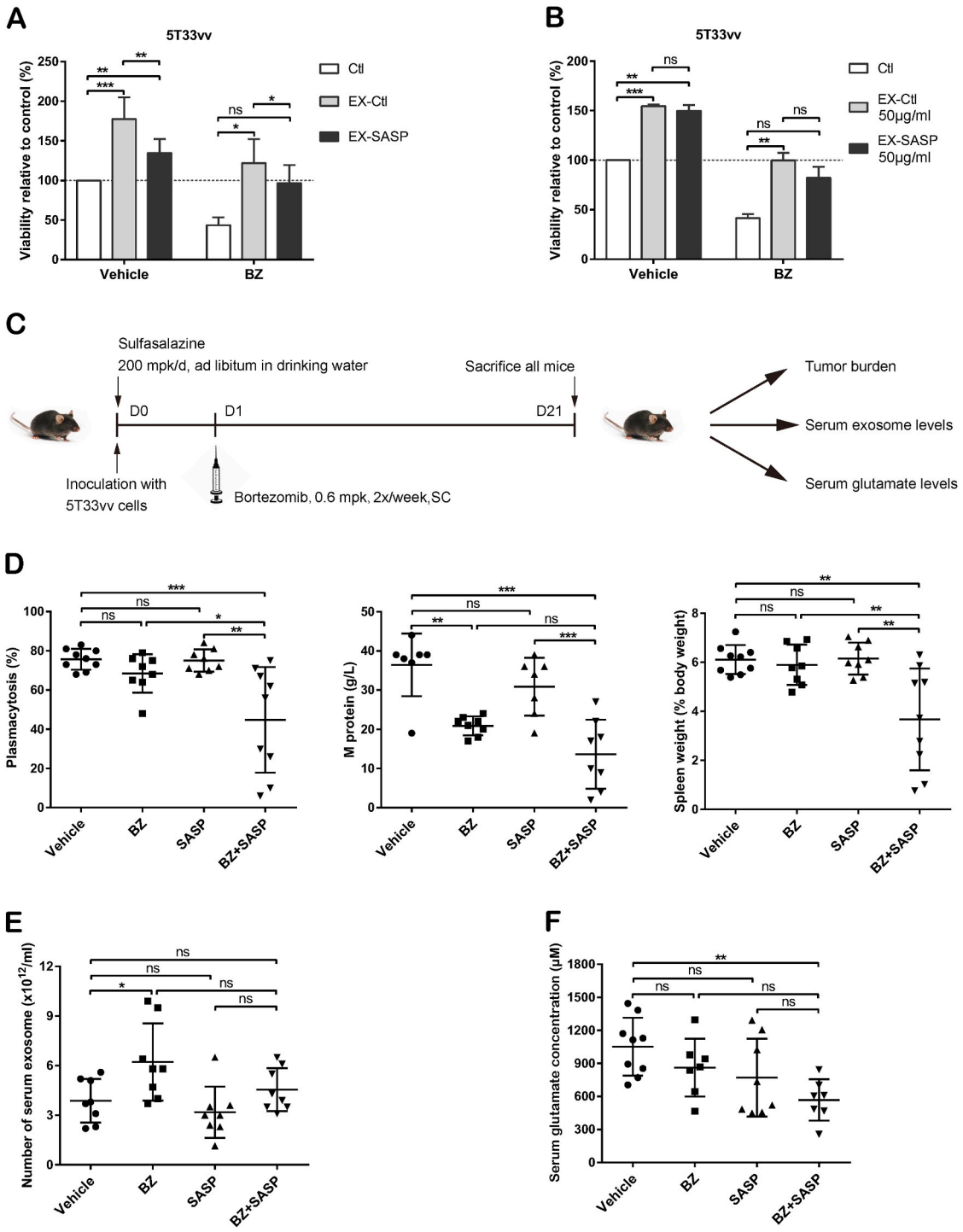

Fig. 6. System Xc^- inhibition increases the anti-MM effect of BZ both *in vitro* and *in vivo*. **(A)** Exosomes (EXs) were isolated from the same number of M-BMSCs treated with or without SASP for 24h. 5T33vv cells were treated with the same volume of EXs with or without BZ (1 nM) and cell viability was detected after 24h. Results shown are mean ± SD of at least four independent experiments. Statistical significance was measured by paired One-way ANOVA test with Tukey's multiple comparisons. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns: not significant. **(B)** EXs were isolated from M-BMSCs treated with or without SASP for 24h. 5T33vv cells were treated with an equal amount of EXs (50 μg/ml) with or without BZ (1 nM) and cell viability was detected after 24h. Results shown are mean ± SD of three independent experiments. Statistical significance was measured by paired One-way ANOVA test with Tukey's multiple comparisons. $^{**}P < 0.01$; $^{***}P < 0.001$; ns: not significant. **(C)** Treatment schedule of 5T33MM mice. 5T33MM mice were treated with SASP (200 mpk/d, ad libitum in drinking water), with BZ (0.6 mpk, 2 times per week, SC), with the combination of both or with vehicle. All mice were sacrificed at day 21. **(D)** BM was collected for analysis of plasmacytosis. M protein was determined by serum electrophoresis. Spleen weight was normalized to body weight. Results shown are mean ± SD. Statistical significance was measured by One-way ANOVA test with Tukey's multiple comparisons test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns: not significant. **(E)** Serum exosome concentration was measured by NTA. Statistical significance was measured by One-way ANOVA test with Tukey's multiple comparisons test. $^*P < 0.05$; ns: not significant. **(F)** Serum glutamate concentration was detected by Glutamine/Glutamate-Glo™ Assay kit. Statistical significance was measured by One-way ANOVA test with Tukey's multiple comparisons test. $^{**}P < 0.01$; ns: not significant.

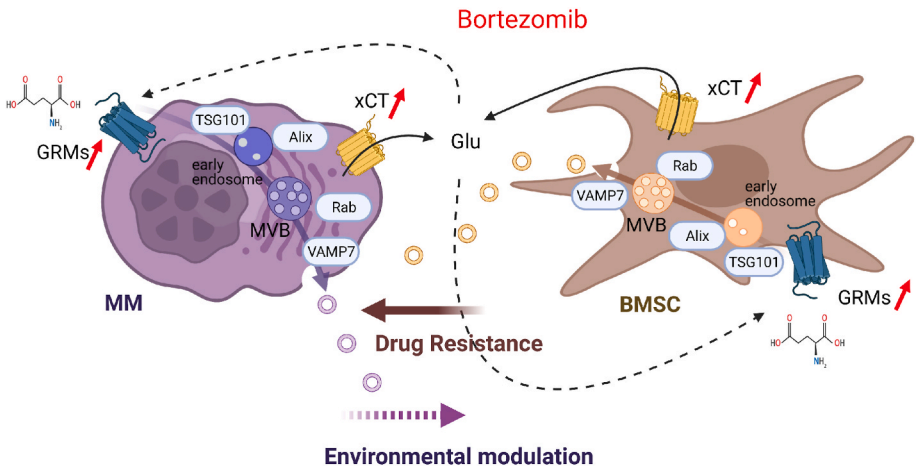

Fig. 7. Graphical summary of the involvement of system Xc^- mediated exosome exchange in MM drug resistance. After treatment with BZ, both BMSCs and MM cells increase expression of xCT and GRMs. Enhanced xCT expression increases the release of Glu which can bind to GRMs and thus activate the multivesicular body (MVB)-derived exosome secretion pathway, including activation of Alix, TSG101, Rab27a/b and VAMP7. Altered exosome secretion in BMSCs contributes to drug resistance of MM cells, while altered exosome secretion in MM cells may promote MM progression by modulating bone marrow environment. Therefore, targeting system Xc^- can enhance the anti-myeloma effect of bortezomib. The figure was created with BioRender.com.

were elevated in the BZ group compared with the vehicle group, serum Glu levels did surprisingly not increase in the BZ group. This may in part be attributed to the complex regulatory mechanisms to maintain homeostasis of Glu in the body, which makes it difficult to reflect the dynamic Glu dysregulation by measuring serum Glu levels at a single time point [32].

Oral SASP was chosen in this study to be as close to its existing clinical application as possible. Bioavailability studies in humans suggest that only approximately 12% of orally ingested SASP escapes colonic cleavage and enters the systemic circulation [33]. An oral dose of approximately 8 mg/kg/day of SASP has been reported to reduce serum Glu levels in tumor-bearing mice, primarily due to the inhibitory effect of absorbed intact SASP on system Xc^- [34]. A higher dose of SASP (200 mg/kg/day) was administered in this study to ensure that enough SASP entered the systemic circulation and reached the BM to exert the inhibitory effect on system Xc^-. The decreasing trend of serum Glu levels in mice treated with SASP also indicated the inhibitory effect of absorbed intact SASP on system Xc^-. Further studies are needed to determine whether the metabolic byproducts of SASP, 5-aminosalicylic acid and 5-aminopyridine, are also involved in the combinatory anti-MM effect *in vivo*.

In addition to the combinatory anti-MM effect, the combination of BZ and SASP may have some other benefits in the treatment of patients with MM. It has been reported that aberrant system Xc^- mediated Glu release in breast cancer cells stimulates nociceptors on peripheral endings, possibly by activating GRMs, and thus contributes to cancer-induced bone pain which could be significantly reduced by SASP in cancer cells [35,36]. Elevated Glu concentration is also reported to be involved in BZ-induced peripheral neuropathy which is a dose-limiting toxicity [37]. Our results offer the possibility that combination therapy with SASP may prevent BZ-induced peripheral neuropathy. Furthermore, exosomes have been reported to be involved in the crosstalk between MM cells and cells in the BM microenvironment, which plays important roles in inducing immune suppression, angiogenesis and osteolysis [8–11]. Hence, targeting system Xc^- may exert anti-MM effects in many aspects by affecting the communication between MM cells and cells in the BM microenvironment. As SASP is already an FDA-approved clinical drug, it can be readily used for clinical trials. Clinical trials have been registered to assess whether the addition of SASP can enhance the anti-tumor efficacy of stereotactic radiosurgery for recurrent glioblastoma and decrease opioids requirements in breast cancer patients (NCT04205357, NCT03847311).

In conclusion, our data show that system Xc^- mediated Glu release is involved in BMSC-MM exosomal crosstalk, contributing to BZ resistance. We shed light on a new link between exosome secretion and system Xc^- mediated Glu release as well as the GRM downstream signaling pathways. Most importantly, we indicate that targeting system Xc^- not only in MM cells but also in BMSCs is useful in enhancing the anti-myeloma effect of BZ in MM by partially blocking the cell-cell communication mediated by exosomes. Our study further supports the clinical development of strategies that target system Xc^- in both MM cells and BMSCs to improve therapeutic efficacy in MM.

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Authors' contributions

Fang Wang and Eline Menu conceived and designed the experiments. Fang Wang, Eline Menu, Philip Vlummens performed the experiments and analyzed the data. Fang Wang and Eline Menu wrote the manuscript. Inge Oudaert, Chenggong Tu, Anke Maes and Arne Van der Vreken performed the experiments added during the revision. Peng Shang, Elke De Bruyne, Kim De Veirman, Anke Maes, Philip Vlummens, Yanmeng Wang, Inge Oudaert, Rong Fan, Ann Massie and Karin Vanderkerken helped in experimental set-up and provided crucial suggestions on the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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