Research paper

Expression of the chemokine receptor CCR1 decreases sensitivity to bortezomib in multiple myeloma cell lines

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ABSTRACT

Background: The proteasome inhibitor bortezomib is one of the primary therapies used for the haematological malignancy multiple myeloma (MM). However, intrinsic or acquired resistance to bortezomib, via mechanisms that are not fully elucidated, is a barrier to successful treatment in many patients. Our previous studies have shown that elevated expression of the chemokine receptor CCR1 in MM plasma cells in newly diagnosed MM patients is associated with poor prognosis. Here, we hypothesised that the poor prognosis conferred by CCR1 expression is, in part, due to a CCR1-mediated decrease in MM plasma cell sensitivity to bortezomib.

Methods: In order to investigate the role of CCR1 in MM cells, CCR1 was knocked out in human myeloma cell lines OPM2 and U266 using CRISPR-Cas9. Additionally, CCR1 was overexpressed in the mouse MM cell line 5TGM1. The effect of bortezomib on CCR1 knockout or CCR1-overexpressing cells was then assessed by WST-1 assay, with or without CCL3 siRNA knockdown or addition of recombinant human CCL3. NSG mice were inoculated intratibially with OPM2-CCR1^{KO} cells and were treated with 0.7 mg/kg bortezomib or vehicle twice per week for 3 weeks and GFP$^+$ tumour cells in the bone marrow were quantitated by flow cytometry. The effect of CCR1 overexpression or knockout on unfolded protein response pathways was assessed using qPCR for *ATF4*, *HSPA5*, *XBP1*, *ERN1* and *CHOP* and Western blot for IRE1α and p-Jnk.

Results: Using CCR1 overexpression or CRIPSR-Cas9-mediated CCR1 knockout in MM cell lines, we found that CCR1 expression significantly decreases sensitivity to bortezomib *in vitro*, independent of the CCR1 ligand CCL3. In addition, CCR1 knockout rendered the human MM cell line OPM2 more sensitive to bortezomib in an intratibial MM model in NSG mice *in vivo*. Moreover, CCR1 expression negatively regulated the expression of the unfolded protein response receptor IRE1 and downstream target gene *XBP1*, suggesting this pathway may be responsible for the decreased bortezomib sensitivity of CCR1-expressing cells.

Conclusions: Taken together, these studies suggest that CCR1 expression may be associated with decreased response to bortezomib in MM cell lines.

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1. Background

Multiple myeloma (MM) is a largely incurable haematological malignancy characterised by the uncontrolled growth of clonal plasma cells (PC) within the bone marrow (BM) frequently accompanied by end organ damage including osteolytic bone disease, anaemia and renal impairment [1]. In Europe [2], the USA [3] and Australia [4,5], the proteasome inhibitor bortezomib is a common frontline treatment, used in over 50% of MM patients. Resistance to bortezomib represents a major hurdle in the successful treatment of a proportion of MM patients. To this end, approximately 20% of patients present with intrinsic resistance to frontline bortezomib treatment [6,7]. In addition, acquired resistance is common in patients treated with bortezomib, with approximately 40–50% of patients relapsing or becoming unresponsive to bortezomib retreatment [8,9]. Despite this, therapeutic strategies to overcome bortezomib resistance are lacking, highlighting the need to fully understand the mechanisms involved.

Proteasome inhibitors, like bortezomib, exert anti-tumour effects through the accumulation of misfolded proteins leading to activation of the unfolded protein response (UPR), and stabilisation of pro-apoptotic molecules that induce apoptosis [10,11]. Bortezomib inhibits the catalytic activity of the 26 S proteasome, preventing degradation of ubiquitinated proteins and therefore inducing endoplasmic reticulum (ER) stress [12]. This, in turn, induces the UPR, which is activated when the accumulation of unfolded or misfolded proteins leads to the dissociation of the ER chaperone binding immunoglobulin protein (BiP, also known as heat shock protein family A member 5 [HSPA5]) from the three UPR transmembrane stress sensors: inositol-requiring kinase 1 (IRE1; also known as ER to nucleus signalling 1 [ERN1]), protein kinase R-like ER kinase (PERK) and activating transcription factor 6 (ATF6). Activation of IRE1, PERK and ATF6 signalling induces the expression of genes responsible for adaptation to stress conditions [13,14]. However, prolonged ER stress induced by bortezomib treatment leads to the induction of pro-apoptotic signalling pathways in what is termed the terminal UPR [15]. MM PCs are thought to be particularly susceptible to proteasome inhibitors as the synthesis of abundant secreted immunoglobulin by PCs renders these cells highly sensitive to disruption of protein handling pathways [15,16].

The C-C motif chemokine receptor 1 (CCR1) is a G protein-coupled receptor whose most potent ligand is C-C motif chemokine ligand 3 (CCL3; also known as macrophage inflammatory protein 1 alpha [MIP-1α]). We have previously shown that elevated BM MM PC expression of *CCR1* is an independent predictor of poor prognosis in newly diagnosed MM patients treated with bortezomib-based treatment regimens [17, 18]. Moreover, we found that, in approximately one quarter of MM patients with low CCR1 at diagnosis, *CCR1* expression was increased at the time of disease relapse, and that this elevation in *CCR1* expression at relapse was associated with poor overall survival [18]. Furthermore, we have previously shown that CCR1 expression plays a pivotal role in MM PC dissemination *in vivo*, without affecting cell proliferation [17,18]. Notably, a previous study suggests that CCR1/CCL3 signalling may play a role in bortezomib and melphalan resistance in transformed B-cell lines [19], suggesting a further potential mechanism which could account for the association between elevated CCR1 and poor prognosis in MM. In this study, we investigated whether CCR1 affected survival of MM cell lines following bortezomib therapy *in vitro* and *in vivo*, and whether endogenous or exogenous CCL3 influenced this response. In addition, we assessed the role of CCR1 in modulating the expression of key factors involved in the UPR.

2. Methods

2.1. Cell culture

Cell culture reagents were sourced from Sigma-Aldrich (St Louis, USA) unless otherwise stated. Media were supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate and 10 mM HEPES. The human myeloma cell line U266 was originally obtained from the American Type Culture Collection (ATCC); OPM2 cells were originally kindly provided by Prof. Andrew Spencer (Monash University, Melbourne, Australia). Human MM cell lines were maintained in RPMI-1640 medium with 10% foetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, USA). The mouse MM cell line 5TGM1 was originally kindly provided by Assoc. Prof. Claire Edwards (University of Oxford, Oxford, UK) and was maintained in Iscove's modified Dulbecco's medium (IMDM) with 20% FBS.

2.2. Overexpression and knockdown studies

CRISPR-Cas9-mediated homozygous knockout of CCR1 in the human MM cell lines OPM2 (OPM2-CCR1-KO-1) and empty vector controls (OPM2-EV-1) (hereafter called OPM2-CCR1^{KO} and OPM2-EV respectively), and U266 cells (U266-CCR1^{KO} and U266-EV) has been described previously [17,18]. Overexpression of CCR1 in 5TGM1 cells (5TGM1-CCR1) and empty vector controls (5TGM1-EV), have been described previously [18]. Knockout and overexpression of CCR1 protein in these cell lines has been confirmed by flow cytometry and Western blot, as described previously [17,18]. In order to achieve siRNA-mediated knockdown of CCL3, Silencer Select *CCL3*-targeting siRNAs (Assay ID s12568 [siRNA#1]; Assay ID s199846 [siRNA#2]) or negative control siRNA (Cat. 4390843; Invitrogen, Waltham, USA) in Opti-minimal essential medium (Opti-MEM) with Lipofectamine RNAi-MAX (Invitrogen) were added dropwise to U266 or OPM2 cells (2.5×10^5 cells/mL in Opti-MEM), for a final concentration of 25 nM siRNA and 2.5 μL/mL Lipofectamine. After 6 hours at 37°C, cells were cultured in antibiotic-free RPMI-1640 containing 10% FBS and supplements for two (OPM2) or three (U266) days. Cells were then used for RNA isolation or WST-1 assays.

2.3. In vitro bortezomib sensitivity assays

Where indicated, cells were seeded in media containing bortezomib (Selleck Chemicals, Houston, USA) in 0.002% DMSO vehicle with, or without, recombinant human (rh) CCL3 (100 ng/mL; R&D Systems, Minneapolis, USA). After 24 hours, relative numbers of viable cells per well were quantitated using WST-1 reagent (Roche, Basel, Switzerland) as previously described [20,21].

2.4. Real-time quantitative PCR (qPCR)

Total RNA was isolated using TRIzol reagent (Thermo) and DNase treated using RQ1 DNase (Promega, Madison, USA) as per manufacturers' instructions. cDNA was synthesized and qPCR was performed as described previously [22] using primers outlined in Table 1. Gene expression was calculated relative to *ACTB* using the $2^{-\Delta\Delta Ct}$ method [23].

2.5. Western blotting

MM cell lines were treated with bortezomib or vehicle (0.002% DMSO) for 6 hours and cell lysates were prepared as previously described [22]. Proteins (50 μg) were resolved under reducing conditions on 10% SDS-PAGE gels, transferred to 0.45 μm nitrocellulose and immunoblotting was performed with phosphorylated JNK (Thr183/Tyr185), total IRE1α (Cell Signaling Technologies, Danvers, USA; both at 1:1000) and α-tubulin (Abcam, Cambridge, UK; 1:5000) antibodies and Dylight™-680 or 800 secondary antibodies (Invitrogen; 1:20,000). Proteins were visualised using an Odyssey Infrared Imaging system (LI-COR Bioscience, Lincoln, USA).

Table 1
Real-time qPCR primers.

Gene	Species	Forward primers	Reverse primer
ACTB	Human	5'-GATCATTGCTCCTCCTGAGC-3'	5'-GTCATAGTCCGCCTAGAAGCAT-3'
ActB	Mouse	5'-TTGCTGACAGGATGCAGAAG-3'	5'-AAGGGTGTAAACGCAGTTC-3'
ATF4	Human	5'-TCTCATTCAGGCTTCTCACGGCAT-3'	5'-AAGCTCATTTCGGTCATGTTGCGG-3'
Atf4	Mouse	5'-CCTAGGTCTCTTAGATGACTATCTGGAGG-3'	5'-CCAGGTCATCCATTCGAAACAGAGCATCG-3'
CCL3	Human	5'-CTGGTTTCAGACTTCAGAAGGAC-3'	5'-GTAGTCAGCTATGAAATTCTGTGG-3'
CHOP	Human	5'-AGAACCAGGAAACGGAAACAGA-3'	5'-TCTCCTTCATGCGCTGCTTT-3'
Chop	Mouse	5'-CCACCACACCTGAAAGCAGAA-3'	5'-AGGTGAAAGGCAGGGACTCA-3'
ERN1	Human	5'-CGGGAGAACATCACTGTCCC-3'	5'-CCCGGTAGTGGTGCTTCTTA-3'
Ern1	Mouse	5'-CCCTGATAGGTTGAATCCTGGCTATGTG-3'	5'-AATCTATGCGCTAATCTGCTGGCCTCTG-3'
HSPA5	Human	5'-TGTTCAACCAATTATCAGCAAACTC-3'	5'-TTCTGCTGTATCCTCTTCACCAGT-3'
Hspa5	Mouse	5'-TTCAGCCAATTATCAGCAAACTCT-3'	5'-TTTTCTGATGTATCCTCTTCACCAGT-3'
XBP1	Human	5'-TTGTCACCCCTCCAGAACATC-3'	5'-TCCAGAATGCCCA ACAGGAT-3'
Xbp1	Mouse	5'-TGGCCGGGTCTGCTGAGTCCG-3'	5'-GTCCATGGGAAGATGTTCTGG-3'
XBP1s	Human	5'-TGCTGAGTCCGCAGCAGGTG-3'	5'-GCTGGCAGGCTCTGGGGAAG-3'
Xbp1s	Mouse	5'-CTGAGTCCGAATCAGGTGCAG-3'	5'-GTCCATGGGAAGATGTTCTGG-3'

2.6. NOD-scid gamma (NSG) murine model of myeloma

All animal studies were performed in accordance with the SAHMRI Animal Ethics Committee approval number SAM286 and with the Australian code for the care and use of animals for scientific purposes and are reported in accordance with ARRIVE guidelines (https://arrive guidelines.org). At the start of the study, mice were randomised into different treatment groups, with groups being evenly distributed across different cages. Female 5–6-week-old NOD scid gamma (NSG) mice [24] (sourced from the SAHMRI Bioresources facility) were inoculated intratibially with 5×10^5 OPM2-EV or OPM2-CCR1^{KO} cells in 10 μL PBS. Mice received either intravenously administered bortezomib (0.7 mg/kg) or vehicle (0.33% DMSO in PBS) on days 7, 11, 14, 18, 21 and 25 after tumour injection. On day 28, mice tumour-injected tibiae were flushed with PBS containing 2% FBS and 2 mM EDTA (PFE), and isolated GFP-positive tumour cells were enumerated using a LSRFortessa flow cytometer (BD Biosciences).

2.7. Statistical Analyses

Statistical analyses were performed using GraphPad Prism 9 (GraphPad, San Diego, USA) using an unpaired t-tests, one-way ANOVA with Tukey's or Sidak's post-tests or two-way ANOVA with Tukey's post-test, as appropriate.

3. Results

Expression of CCR1 decreases sensitivity of MM cell lines to bortezomib *in vitro* and *in vivo*

We have previously shown that elevated BM PC expression of *CCR1* is associated with poor prognosis in newly diagnosed and relapsed MM patients [17,18]. Here, we hypothesised that the prognostic disadvantage of elevated CCR1 expression is, in part, due to decreased sensitivity to the proteasome inhibitor, bortezomib. Initially, we assessed the effect of bortezomib treatment on CCR1 knockout OPM2 cells (OPM2-CCR1^{KO}) [18] and empty vector controls *in vitro*. CCR1 knockout increased the response of OPM2 cells to bortezomib, with bortezomib exposure leading to a greater reduction in cell number in OPM2-CCR1^{KO} cells compared with OPM2-EV cells (OPM2-CCR1^{KO} IC$_{50}$: 8.1 nM; OPM2-EV IC$_{50}$: 19.1 nM; Fig. 1A). Similar results were observed with CCR1 knockout in U266 cells cells (U266-CCR1^{KO} IC$_{50}$: 3.3 nM; U266-EV IC$_{50}$: 4.3 nM; Supplementary Figure 1). Furthermore, enforced expression of CCR1 in the murine MM cell line 5TGM1 [18] (5TGM1-CCR1) decreased the effect of bortezomib in reducing cell number when compared with 5TGM1-EV controls (5TGM1-CCR1 IC$_{50}$: 14.5 nM; 5TGM1-EV IC$_{50}$: 7.2 nM; Fig. 1B).
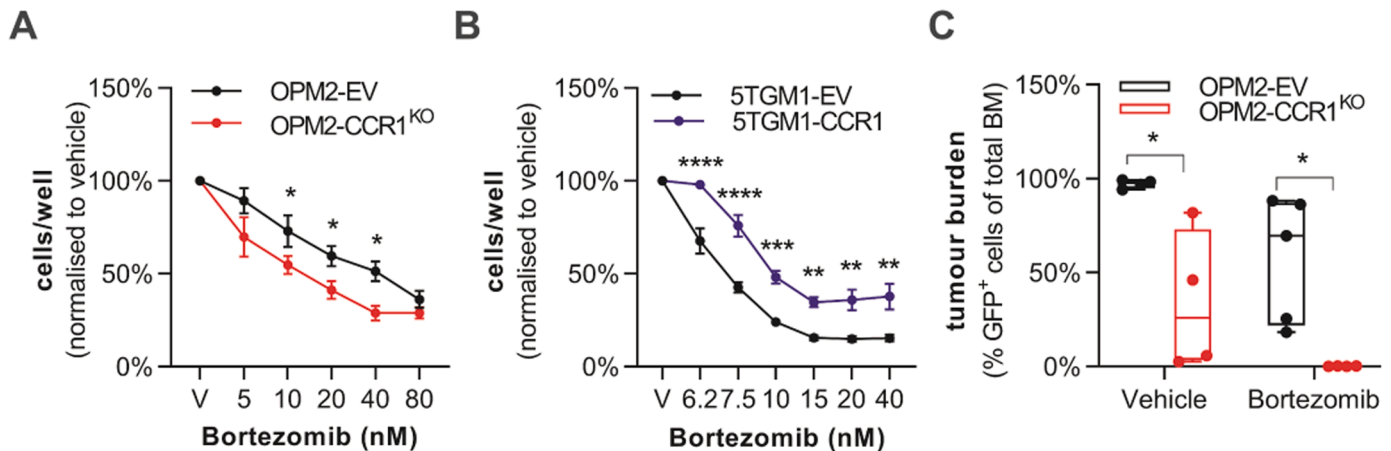
Fig. 1. Expression of CCR1 decreases sensitivity to bortezomib in MM cell lines *in vitro* and *in vivo*. OPM2 CRISPR-Cas9 CCR1 knockout (OPM2-CCR1^{KO}) or empty vector (OPM2-EV) control cells (**A**) or 5TGM1 cells expressing CCR1 (5TGM1-CCR1) or 5TGM1-EV controls (**B**) were treated with bortezomib or 0.002% DMSO vehicle control (V) for 24 hours and relative cell number was assessed by WST-1. NSG mice were inoculated intratibially with OPM2-EV or OPM2-CCR1^{KO} cells and, after allowing tumours to establish for 7 days, were treated with bortezomib (0.7 mg/kg) or vehicle control (0.33% DMSO in saline) intravenously twice per week for 3 weeks. GFP$^+$ tumour cells in the BM of the injected tibiae were quantitated by flow cytometry on day 28 post-tumour cell injection (**C**). Graphs depict mean ± SEM for 4–6 independent experiments (**A,B**) or median and interquartile ranges for n=4–5 mice/group (**C**). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 relative to EV control two-way ANOVA with Sidak's multiple comparisons test.

Next, we assessed the effect of CCR1 expression on the sensitivity of OPM2 cells to bortezomib *in vivo* using an intratibial xenograft MM model. In keeping with our previous findings [18], we observed a significant reduction in tumour burden in NSG mice injected with OPM2-CCR1^KO cells compared with OPM2-EV cells in the vehicle-treated control groups (p<0.05, Fig. 1C). Notably, OPM2-CCR1^KO tumours were significantly more sensitive to bortezomib therapy than OPM2-EV tumours (p<0.05, Fig. 1C). In mice injected with OPM2-CCR1^KO cells, there was a 99% reduction in mean tumour burden following bortezomib treatment compared with vehicle controls (Fig. 1C), while only a 29% reduction was seen in OPM2-EV-bearing mice with bortezomib treatment (Fig. 1C).

CCR1-mediated insensitivity to bortezomib in MM cell lines is independent of exogenous or endogenous CCL3

Previous studies have suggested an association between CCR1 signalling and bortezomib resistance, with treatment with a neutralising antibody against the CCR1 ligand CCL3 increasing the cytotoxic effect of bortezomib in a human immortalised B-cell line *in vitro* [19]. To determine the role of CCL3 in MM PC sensitivity to bortezomib therapy, we initially assessed the effects of exogenous CCL3 on sensitivity to bortezomib *in vitro*. Consistent with our previous findings [18], the addition of rhCCL3 had no effect on the proliferation of OPM2 (Fig. 2A) or 5TGM1 (Fig. 2B) cells. Furthermore, the addition of rhCCL3 showed no effect on the sensitivity of OPM2 (Fig. 2A) or 5TGM1 (Fig. 2B) cells to bortezomib treatment.

We next assessed whether endogenous production of CCL3 could be

responsible for the resistance to bortezomib treatment mediated by CCR1. To investigate this, we knocked down *CCL3* expression using two independent siRNAs in OPM2 and U266 cells. Greater than 50% knockdown of *CCL3* was achieved in both cell lines with each siRNA in comparison to negative control (NC) siRNA (Fig. 2C-D). *CCL3* knockdown in OPM2 and U266 cells had no effect on their sensitivity to bortezomib treatment *in vitro* compared with their NC siRNA controls (Fig. 2E-F). Taken together, these studies demonstrate that neither exogenous nor endogenous CCL3 modulate the response of MM cell lines to bortezomib *in vitro*.

CCR1 negatively regulates IRE1 expression in MM cell lines

Previous studies have shown that decreased expression of ER stress response pathway genes is associated with resistance to bortezomib [16, 25–27]. As such, we postulated that elevated CCR1 expression could affect bortezomib sensitivity of MM cells by modulating response to ER stress and subsequent induction of the UPR. Initially, we assessed the effect of CCR1 on expression of activating transcription factor 4 (*ATF4*), BiP (*HSPA5*) and X-box binding protein 1 (*XBP1*): which are transcriptionally activated via signalling downstream of the UPR receptors PERK, ATF6 and IRE1, respectively. Neither *ATF4* or *HSPA5* were differentially expressed in OPM2-CCR1^KO (Fig. 3A) or 5TGM1-CCR1 (Fig. 3B) cells, compared with EV controls. However, total and spliced *XBP1* were significantly increased in OPM2-CCR1^KO, and decreased in 5TGM1-CCR1 cells, compared with their respective controls (p<0.05, Fig. 3A-B). Consistent with the modulation of IRE1 targets by CCR1, expression of *ERN1* (the gene encoding IRE1) was significantly increased
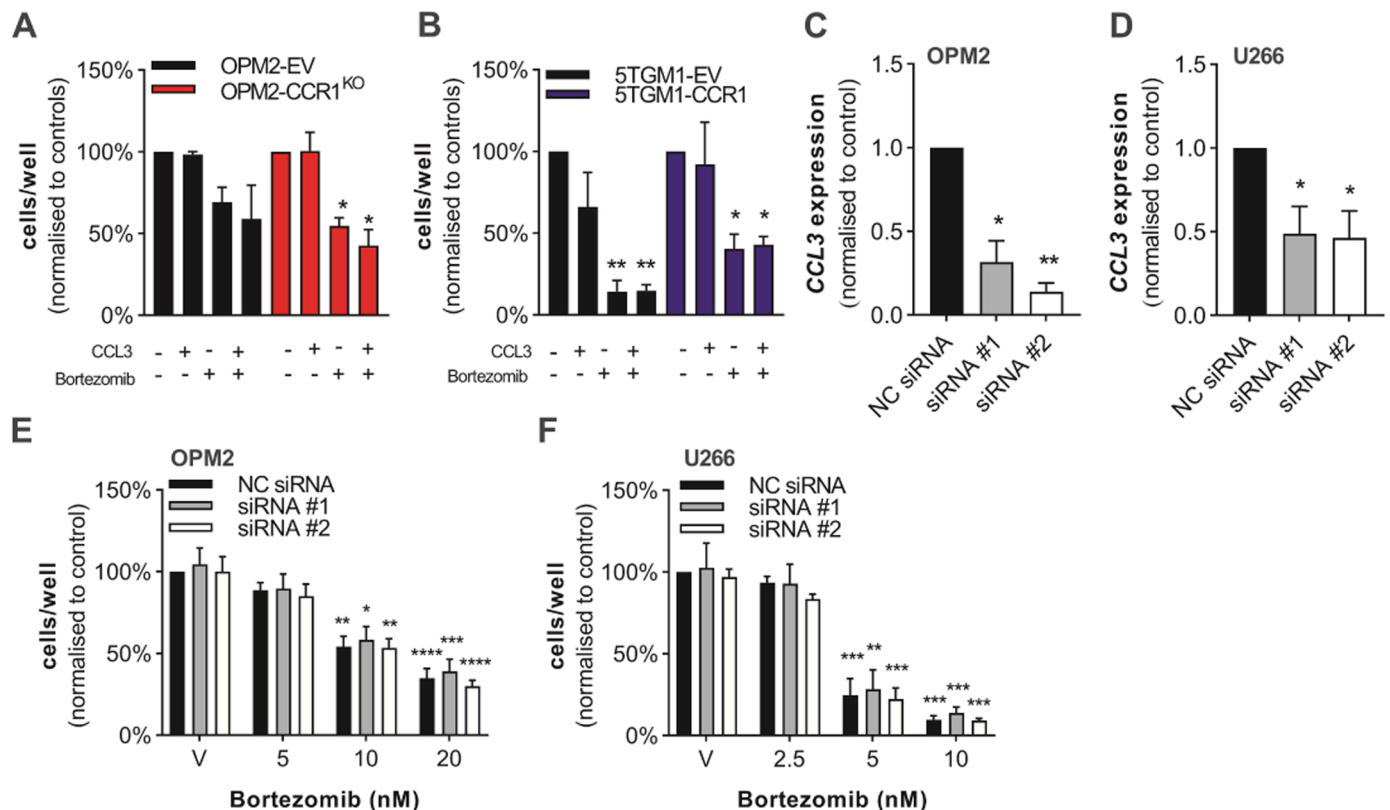
Fig. 2. Decreased sensitivity to bortezomib conferred by CCR1 expression is independent of CCL3/CCR1 signalling. OPM2-CCR1^KO or OPM2-EV cells were treated with either bortezomib (10 nM) or vehicle (0.002% DMSO) with, or without, the addition of recombinant human rhCCL3 (100 ng/mL), for 24 hours and relative cell numbers were assessed by WST-1 (**A**). 5TGM1-CCR1 or 5TGM1-EV cells were treated with either bortezomib (7.5 nM) or vehicle (0.002% DMSO) with, or without, the addition of recombinant human rhCCL3 (100 ng/mL) for 24 hours and relative cell numbers were assessed by WST-1 (**B**). OPM2 (**C**) or U266 (**D**) cells were transfected with CCL3-targeting siRNA (siRNA #1 and siRNA #2) or non-targeting control (NC) siRNA. *CCL3* expression, normalised to *ACTB*, was determined by qPCR after 48 hours (OPM2; **C**) or 72 hours (U266; **D**). Cells were then treated with bortezomib or vehicle alone for a further 24 hours and relative OPM2 (**E**) and U266 (**F**) cell numbers were assessed by WST-1. Graphs depict mean + SEM for 3 or more independent experiments (**A,B,E,F**) or mean range of 2 independent experiments (**C,D**). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, two-way ANOVA with Sidak's multiple comparisons test (**A,B,E,F**) or one-way ANOVA with Tukey's multiple comparisons test (**C,D**) compared with vehicle-treated controls (**A,B,E,F**) or NC siRNA controls (**C,D**).
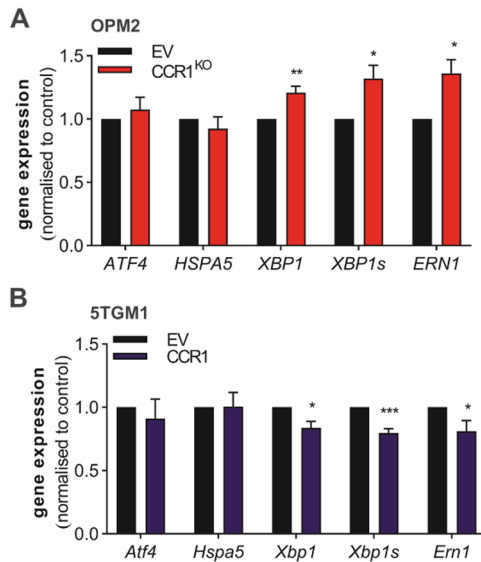
Fig. 3. CCR1 expression negatively regulates a target gene downstream of the IRE1 pathway but not genes downstream of the PERK and ATF6 pathways. Basal expression of PERK target gene *ATF4*, ATF6 target gene *HSPA5*, IRE1 target genes *XBP1* and *XBP1s*, and IRE1 (*ERN1*) in OPM2-CCR1^{KO} or OPM2-EV control cells (**A**) and 5TGM1-CCR1 or 5TGM1-EV cells (**B**) as assessed by qPCR. Gene expression is normalised to *ACTB*. Graphs depict mean + SEM of 4 or more independent experiments expressed relative to EV controls. *p<0.05, **p<0.01, ***p<0.001, unpaired t-test.

in OPM2-CCR1^{KO} cells, and decreased in CCR1-expressing 5TGM1 cells, compared with their requisite controls (p<0.05, Fig. 3A-B). Furthermore, there was a concomitant increase in IRE1 protein expression in OPM2-CCR1^{KO} cells, and a decrease in Ire1 in 5TGM1-CCR1 cells, when compared with EV controls either basally or following bortezomib treatment (Fig. 4A).

Under sustained ER stress, IRE1 also activates a cell death pathway via phosphorylation of c-Jun N-terminal kinase (JNK), leading to activation of caspase-dependent apoptosis [28]. In addition, the terminal UPR leads to increased expression of the pro-apoptotic transcription

factor CCAAT/enhancer binding protein homologous protein (CHOP) [15]. In both OPM2 and 5TGM1 cells, bortezomib treatment led to a dose-dependent induction of p-JNK and *CHOP* expression (Fig. 4A-B). In 5TGM1-CCR1 cells there was a significant decrease in the magnitude of the effect of bortezomib on p-JNK and *Chop* levels when compared with EV controls; however, there was no difference in bortezomib-induced p-JNK or *CHOP* levels between the OPM2-EV and OPM2-CCR1^{KO} cell lines (Fig. 4A-B). Taken together, these data suggest that CCR1 can negatively regulate expression of IRE1 and, in some cases, downstream apoptotic pathways in MM cell lines.

4. Discussion

As bortezomib is commonly used as the backbone to MM therapeutic regimens [2–9], resistance to bortezomib poses a challenge for the successful treatment of MM patients. Several mechanisms have been identified that may contribute to bortezomib resistance in MM. For example, somatic mutations in the β5 proteasome subunit (PSMB5), which have been shown to interfere with bortezomib binding in cell lines, have been identified in bortezomib-resistant MM cell lines, although are rare in bortezomib-resistant myeloma patients [29,30]. Additionally, altered expression of components of the ER stress response pathway (including elevated expression of heat shock proteins like BiP and decreased expression of IRE1 or XBP1) and activation of cell survival signalling pathways have been associated with bortezomib resistance in preclinical studies [31].

We previously showed that CCR1 protein is expressed on MM PCs from more than 50% of newly diagnosed MM patients [17,18]. Additionally, we demonstrated that high MM PC expression of *CCR1* at diagnosis is associated with poor survival, independent of other poor prognostic indicators [17,18]. Furthermore, we have found that elevated CCR1 expression at the time of relapse is associated with poor overall survival [18]. Here, we report, for the first time, a role for CCR1 in decreasing the response of MM cell lines to bortezomib treatment. Using CCR1-expression or CCR1-knockout in murine and human MM cell lines, we identified that CCR1 expression decreased sensitivity, while CCR1-knockout increased sensitivity, to bortezomib *in vitro*. Furthermore, using an orthotopic mouse model of MM, we showed that OPM2-CCR1^{KO} cells are more sensitive to bortezomib therapy compared
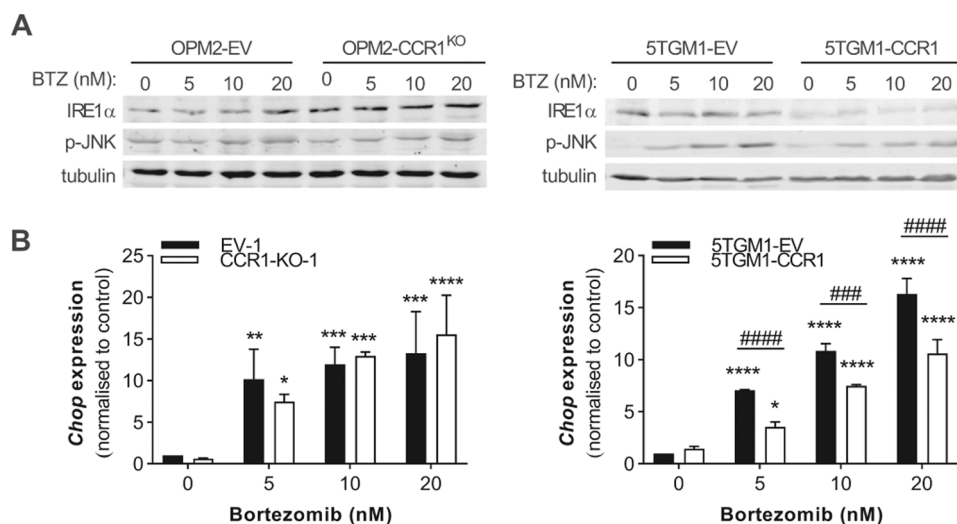
Fig. 4. CCR1 expression modulates IRE1 protein expression and downstream cell death pathways following bortezomib treatment in MM cell lines. OPM2-EV and OPM2-CCR1^{KO} cells or 5TGM1-EV and 5TGM1-CCR1 cells were treated with bortezomib or vehicle alone for 6 hours and IRE1α and p-JNK protein levels were detected by Western blot (**A**). Tubulin was used as a loading control. A representative of 3–4 independent experiments is shown. Images depict cropped Western blots; full-length, uncropped blots are presented in Supplementary Figure 2 and 3. Graphs depict *CHOP* expression, normalised to *ACTB*, as assessed by qPCR analysis (**B**). Graphs depict mean + SEM of 4 or more independent experiments expressed relative to vehicle-treated EV controls. *p<0.05, ***p<0.001, ****p<0.0001 relative to vehicle-treated control; ###p<0.001, ####p<0.0001 relative to EV, as indicated; two-way ANOVA with Sidak's multiple comparisons test.

with EV controls cells. These studies suggest high CCR1 expression may play a functional role in response to treatment with bortezomib-based therapeutic regimens.

The primary ligands for CCR1 are CCL3 and CCL5 [32], with CCL3 being both the most potent and abundant CCR1 ligand in the MM BM [33–35]. CCR1 expression has been suggested to play a role in prostate cancer resistance to chemotherapy, with a taxane-resistant PC3 prostate cancer cell line expressing CCR1 at high levels compared with taxane-sensitive controls *in vitro* [36]. Additionally, a previous study suggests that CCR1/CCL3 signalling may play a role in resistance to bortezomib and the chemotherapeutic melphalan in transformed B-cell lines [19]. Either siRNA-mediated CCL3 knockdown or treatment with a CCL3-neutralising antibody increased the cytotoxic effect of bortezomib in the human immortalised B-cell line IM-9 *in vitro* [19]. Furthermore, a melphalan-resistant sub-line of the MM PC line RPMI-8226 was found to express abundant CCL3, and treatment with a CCL3-neutralising antibody re-sensitised the cells to melphalan treatment [19]. In contrast, addition of recombinant CCL3, even at high concentrations, has been found to have no effect on the response to dexamethasone or melphalan in the human MM cell line MM.1 S *in vitro* [37]. Here, we found that MM PC sensitivity to bortezomib is influenced by CCR1 expression in a CCL3-independent manner. To this end, we observed that neither siRNA-mediated knockdown of CCL3 nor addition of recombinant CCL3 influenced the sensitivity of human and murine MM cell lines to bortezomib. As 5TGM1, U266 and OPM2 cells do not express the alternate CCL3 receptor CCR5, and only produce low or undetectable levels of the CCR1 ligand CCL5 (data not shown), the lack of effect of CCL3 knockdown is unlikely to be due to redundancy in chemokine ligand-receptor binding. Notably, previous studies suggest that certain G-protein coupled receptors, including CCR1, can adopt an active conformation and trigger downstream signalling in the absence of ligand [38,39]. In human and murine leukocytes, CCR1 has been found to take on an active conformational state in the absence of ligand, enabling G-protein-dependent signalling that leads to increased F-actin polymerisation and cell migration, which can be blocked using a CCR1 inhibitor [39]. This suggests that the CCL3-independent effects of CCR1 expression observed here may be attributable to constitutive CCR1 signalling in the absence of ligand.

Bortezomib induces MM cell death through sustained activation of pathways, downstream of the UPR transmembrane stress sensors IRE1, PERK and ATF6 [15,28]. Here, we identified an association between CCR1 expression, decreased IRE1 expression and bortezomib resistance in MM cell lines. In MM cell lines that express CCR1 (OPM2-EV and 5TGM1-CCR1), there was considerably lower basal expression of UPR proteins IRE1α and XBP1, which may allow the MM PCs to better tolerate subsequent stresses and therefore be more resistant to bortezomib. Furthermore, decreased IRE1 expression in 5TGM1-CCR1 cells was found to be associated with decreased activation of apoptosis-associated signalling pathways in response to bortezomib treatment (as seen by decreased induction of *CHOP* and p-JNK levels). Our data is consistent with previous studies showing that siRNA- or shRNA-mediated knockdown of either XBP1s [16,25] or IRE1 [25] in human and murine MM cell lines increases resistance to bortezomib *in vitro*. Furthermore, two independent studies have also demonstrated that lower levels of expression of XBP1 [26] or XBP1 target genes [25, 26] are associated with poor response to bortezomib therapy in MM patients. Mechanistically, it can be postulated that CCR1 may regulate IRE1 expression via AKT signalling. As we have previously shown, CCR1 activation results in the phosphorylation and activation of the PI3K/AKT signalling pathway in MM cells [18], and inhibition of AKT has been shown to increase IRE1 levels in a dose-dependent manner in human MM cell lines [40–42]. Furthermore, AKT inhibition increases the sensitivity of MM cell lines to bortezomib *in vitro* and *in vivo* [42–44]. Taken together, these data suggest a potential mechanism whereby CCR1 may lead to decreased sensitivity of MM PCs to bortezomib therapy via activation of the AKT signalling pathway and, subsequently,

decreased IRE1 and XBP1 expression [40,41].

The chromosomal translocations t(14;16) and t(14;20), which lead to constitutive expression of the transcriptions factors MAF and MAFB, are associated with poor prognosis in MM patients [45,46]. Notably, this is associated with poorer response to bortezomib than that seen in other MM subtypes [47–50]. In overexpression and knockdown studies in human MM cell lines, MAF or MAFB expression was shown to increase resistance to bortezomib *in vitro* [49,50]. Interestingly, expression of CCR1 is known to be upregulated by MAF and MAFB in human MM cell lines [49,50]. The data presented here suggest the hypothesis that elevated CCR1 could potentially contribute to the bortezomib-resistance and prognostic disadvantage associated with elevated MAF/MAFB in these previous studies.

5. Conclusions

This study has identified, for the first time, a novel role for CCR1 in the decreased response of MM cell lines to bortezomib therapy, through a mechanism that involves, in part, decreased expression of IRE1. These results are of potential importance as bortezomib, and related proteasome inhibitor carfilzomib and ixazomib, are commonly used in MM therapeutic regimens. Future studies are warranted to examine whether CCR1 may be a useful biomarker to predict the response to bortezomib therapy and whether CCR1 inhibition can re-sensitise resistant MM cells to bortezomib therapy.

Ethics approval and consent to participate

Consent for publication

Not applicable

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CRediT authorship contribution statement

Andrew C.W Zannettino: Conceptualization, Funding acquisition, Methodology, Writing – original draft. **Kate Vandyke:** Conceptualization, Funding acquisition, Methodology, Project administration, Writing – original draft. **Monika Engelhardt:** Funding acquisition, Investigation, Methodology, Writing – review & editing. **Mara Natasha Zeissig:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft. **Krzysztof M**

Mrozik: Investigation, Methodology, Writing – review & editing. **Duncan R Hewett:** Investigation, Methodology, Writing – review & editing. **Sandra M Dold:** Investigation, Methodology, Writing – review & editing. **Andrew Spencer:** Funding acquisition, Writing – review & editing. **Craig T Wallington-Gates:** Conceptualization, Methodology, Resources, Writing – review & editing. **Vasilios Panagopoulos:** Methodology, Resources.

Declaration of Competing Interest

None

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.leukres.2024.107469.

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