CCR1 as a therapeutic target in multiple myeloma: preventing tumour dissemination and therapeutic resistance

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Abstract

Multiple myeloma (MM) is a haematological malignancy characterised by the clonal proliferation of plasma cells (PC) within the bone marrow (BM). While the introduction of novel therapies, such as the proteasome inhibitor bortezomib, has greatly improved patient outcomes, a subset of patients still does very poorly, due to intrinsic resistance to therapy, rapid disease relapse and increased tumour dissemination. However, the mechanisms which underpin MM PC dissemination and intrinsic resistance remain poorly understood. Our laboratory has previously shown that MM PC expression of the chemokine receptor CCR1 is associated with poor prognosis in newly diagnosed patients. In this thesis, the mechanisms underlying the prognostic disadvantage of elevated CCR1 expression are explored. In addition, the prognostic significance of MM PC expression of other chemokine receptors is also investigated.

MM disease development, progression and relapse is dependent on MM PC haematogenous dissemination from one bone site to another. Currently, there is little understanding of which factors regulate spontaneous dissemination. Our previous studies suggested that elevated MM PC CCR1 expression is associated with increased numbers of circulating MM PC in newly diagnosed patients. Here, is was found that knockout of CCR1 in the MM cell line OPM2 resulted in almost no circulating tumour cells and prevented the formation of disseminated tumours following intratibial injection, suggesting that CCR1 is critical in egress from the BM to the circulation. In support of this, constitutive expression of CCR1 in 5TGM1 MM cells increased the incidence of disseminated tumours in C57BL/KaLwRij mice. Furthermore, pharmacological inhibition of CCR1 slowed the formation of disseminated tumours in intratibial RPMI-8226 or OPM2 tumour models. These studies support the further investigation of CCR1 inhibition as a therapeutic modality to inhibit MM tumour dissemination.

The poor prognosis associated with CCR1 expression in MM patients suggests that CCR1 may also play a role in therapeutic resistance. In order to investigate this, the effect of *CCR1* overexpression and knockdown in MM cell lines on response to bortezomib treatment was assessed. CCR1 knockout increased bortezomib sensitivity in vitro and in vivo, while constitutive expression of CCR1 decreased bortezomib sensitivity *in vitro*.

Furthermore, RNA sequencing analysis revealed that elevated MM PC CCR1 expression at diagnosis or at relapse was associated with poorer overall survival in patients receiving bortezomib treatment. Future studies are warranted to assess the ability of CCR1 inhibitors to increase response to bortezomib.

In order to investigate the potential role of other chemokine receptors in MM pathogenesis, we conducted an *in silico* analysis to determine the association between chemokine receptor expression on MM PCs and overall survival in MM patients. These studies identified, for the first time, that elevated CCR10 expression is associated with poor prognosis. Notably, elevated MM PC CCR10 mRNA or protein expression was not associated with other known prognostic factors in MM patients.

Taken together, these studies suggest that CCR1 is a potential target to enhance response to therapy and prevent dissemination of MM PCs. In addition, they suggest that future studies are warranted to investigate the role of CCR10 in MM pathogenesis.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Abbreviations

AF647	alexa-Fluor 647
AKT	protein Kinase B
ANOVA	analysis of variance
APEX	Assessment of Proteasome Inhibition for Extending Remissions
ATF4	activating transcription factor-4
ATF6	activating transcription factor-6
ATP	adenosine triphosphate
BAFF	B-cell activating factor
Bak	Bcl-2-antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
BIM	Bcl-2 interacting mediator of cell death
BiP	binding immunoglobulin protein
BM	bone marrow
BMEC	bone marrow endothelial cell
BMMNC	bone marrow mononuclear cell
BMSC	bone marrow stromal cell
BV421	brilliant violet 421
CAMDR	cell-adhesion mediated drug resistance
CCL2	C-C motif chemokine ligand 2
CCL27	C-C motif chemokine ligand 27
CCL28	C-C motif chemokine ligand 28
CCL3	C-C motif chemokine ligand 3
CCL5	C-C motif chemokine ligand 5
CCL7	C-C motif chemokine ligand 7
CCL8	C-C motif chemokine ligand 8
CCR1	C-C motif chemokine receptor 1
CCR10	C-C motif chemokine receptor 10
CCR2	C-C motif chemokine receptor 2
CCR3	C-C motif chemokine receptor 3

CCR4	C-C motif chemokine receptor 4
CCR5	C-C motif chemokine receptor 5
CCR6	C-C motif chemokine receptor 6
CCR7	C-C motif chemokine receptor 7
CCR8	C-C motif chemokine receptor 8
CCR9	C-C motif chemokine receptor 9
CD40L	CD40 ligand
cDNA	complementary DNA
CD1	cylin D1
CD2	cyclin D2
CHOP	CCAAT/enhancer binding protein homologous protein
CI	confidence interval
CRAB	Hypercalcemia, Renal Insufficiency, Anaemia and Bone lesions
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Ct	cycle threshold
CX3CL1	C-X_3-C motif chemokine ligand 1
CX3CR1	C-X_3-C motif chemokine receptor 1
CXCL10	C-X-C motif chemokine ligand 10
CXCL11	C-X-C motif chemokine ligand 11
CXCL12	C-X-C motif chemokine ligand 12
CXCL6	C-X-C motif chemokine ligand 6
CXCL8	C-X-C motif chemokine ligand 8
CXCL9	C-X-C motif chemokine ligand 9
CXCR1	C-X-C motif chemokine receptor 1
CXCR2	C-X-C motif chemokine receptor 2
CXCR3	C-X-C motif chemokine receptor 3
CXCR4	C-X-C motif chemokine receptor 4
CXCR5	C-X-C motif chemokine receptor 5
CXCR6	C-X-C motif chemokine receptor 6
CXCR7	C-X-C motif chemokine receptor 7
DAAD	German Academic Exchange Service

DAB	3,3′-Diaminobenzidine
DMSO	dimethyl sulphoxide
DNAJC3	DnaJ Heat Shock Protein Family Member C3
EC	endothelial cell
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
eIF-2α	eukaryotic initiation factor 2 alpha
ELISA	enzyme-linked immunosorbent assay
EMD	extramedullary disease
EMT	epithelial-to-mesenchymal transition
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation
ERK	extracellular signal-related kinase
ERN1	endoplasmic reticulum to nucleus signaling 1
EV	empty vector
FCS	foetal calf serum
FICOLL	density gradient centrifugation
FISH	fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
FMO	fluorescence minus one
FSC-A	Forward Scatter Area
G-CSF	granulocyte-colony stimulating factor
GFP	green fluorescent protein
gRNA	guide RNA
GSEA	gene set enrichment analysis
HA	human influenza hemagglutinin
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	hepatocyte growth factor
HIF-2α	hypoxia-inducible transcription factor-2α

HMCL	human multiple myeloma cell line
HR	hazard ratio
HSPA5	Heat Shock Protein Family A (Hsp70) Member 5
HY	hyperdiploidy
ICAM-1	intracellular adhesion molecule 1
IC_{50}	inhibitory concentration, 50%
IgA	immunoglobulin A
IGF-1	insulin-like growth factor 1
IGF1R	insulin-like growth factor 1 receptor
IgG HC	immunoglobulin G heavy chain
IgG LC	immunoglobulin G light chain
IL-6	interleukin 6
IL-8	interleukin 8
IMDM	Iscove's Modified Dulbecco's Medium
IMWG	International Myeloma Working Group
i.p.	intraperitoneal
IP	immunoprecipitation
IP10	IFN-γ-inducible 10 kDa protein
IRE1	inositol-requiring kinase 1
ISS	international staging system
i.t.	intratibial
I-TAC	interferon-inducible T-cell alpha chemoattractant
ITGB7	integrin subunit β7
i.v.	intravenous
JNK	c-Jun N-terminal kinase
kDa	kilodalton
KO	knockout
LB	low bone disease
LIMMA	Linear Models for Microarray Data
Mcl-1	induced myeloid leukaemia cell differentiation protein Mcl-1

MCP	monocyte chemotactic protein
MEM	minimal essential medium
MF	patient subgroup with activation of the *c-MAF* or *MAFB* proto-oncogenes
MFI	mean fluorescence intensity
$MgCl_2$	magnesium chloride
MGUS	monoclonal gammopathy of undetermined significance
Mig	monocyte/macrophage-activating IFN-γ-inducible protein
MM	multiple myeloma
MMP	matrix metalloproteinase
MMRF	Multiple Myeloma Research Foundation
MMSET	multiple myeloma SET domain
MRI	Magnetic Resonance Imaging
mRNA	messenger RNA
MS	multiple sclerosis
MS	patient subgroup with hyperactivation of *FGFR3* and *MMSET* genes
MSC	mesenchymal stem cell
NC	negative control
NK	natural killer
NOXA	phorbol-12-myristate-13-acetate-induced protein 1
NSD2	nuclear receptor binding SET domain protein 2
NSG	NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ
PAGE	polyacrylamide gel electrophoresis
p/sec/cm^2/sr	photons per second per centimetre squared per steradian
PB	peripheral blood
PBS	phosphate-buffered saline
PC	plasma cell
PCL	plasma cell leukaemia

PCR	polymerase chain reaction
PE	phycoerythrin
PE-Cy7	Phycoerythrin-cyanine dye C7
PEG	polyethylene glycol
PERK	protein kinase R-like ER kinase
PET	positron emission tomography
PFE	Phosphate-buffered saline containing foetal calf serum and EDTA
PR	proliferation
PSGL-1	P-selectin glycoprotein ligand-1
rhCCL3	recombinant human C-C motif chemokine ligand 3
RA	rheumatoid arthritis
RPMI-1640	Roswell Park Memorial Institute 1640
RT	reverse transcriptase
qPCR	real-time quantitative polymerase chain reaction
SC	stromal cell
SDS	sodium dodecyl sulphate
SDF-1	stromal cell-derived factor-1
SEM	standard error of the mean
7-AAD	7-aminoactinomycin D
sgRNA	single-stranded guide RNA
shRNA	short hairpin RNA
siRNA	small interfering RNA
SMM	smouldering multiple myeloma
SNVs	single nucleotide variants
SSC-A	Side Scatter- Area
TNF-α	tumour necrosis factor-α
TT2	Total Therapy 2
TT3	Total Therapy 3
UAMS	the University of Arkansas for Medical Science
UPR	unfolded protein response

USA	United States of America
UTR	untranslated region
VCAM-1	vascular cell–adhesion molecule-1
VLA-4	very-late antigen-4 (integrin α4β1)
WB	Western blot
WST-1	4-[3-(4-iodophenyl)-2-(4-nitophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate
XBP1	x-box protein 1
XBP1s	x-box protein 1 spliced
XCR1	X-C Motif chemokine receptor 1

Publications

Scientific Manuscripts

1. **Zeissig M.N.**, Hewett D.R., Panagopoulos V., Mrozik K.M., To L.B., Croucher P., Zannettino A.C.W., Vandyke K. Expression of the chemokine receptor CCR1 promotes the dissemination of multiple myeloma plasma cells in vivo. *Submitted to Haematologica.*

2. **Zeissig M.N.**, Zannettino A.C.W., Vandyke K. Tumour dissemination in multiple myeloma disease progression and relapse: a new therapeutic target in high-risk myeloma. *Submitted to Blood Cancer Journal.*

3. **Zeissig M.N.**, Hewett D.R., Mrozik K.M., Engelhardt M., Panagopoulos V., Zannettino A.C.W., Vandyke K. Expression of the chemokine receptor CCR1 confers resistance to bortezomib in multiple myeloma cell lines. *Manuscript in preparation.*

4. **Zeissig M.N.**, Dold S., Engelhardt M., Zannettino A.C.W., Vandyke K. Chemokine receptor profile in multiple myeloma plasma cells: expression of CCR10 is associated with poor prognosis in newly diagnosed patients. *Manuscript in preparation.*

5. Graziani G.E, Herget G.W., Ihorst G., **Zeissig M.N**., Chaidos A., Auner H., Duyster J., Wäsch R., Engelhardt M. (2019). Time from first symptom onset to the final diagnosis of Multiple Myeloma (MM) - possible risks and future solutions: retrospective and prospective 'Deutsche Studiengruppe MM' (DSMM) and 'European Myeloma Network' (EMN) analysis. *Leukemia and Lymphoma*, DOI: 10.1080/10428194.2019.1695051

6. Vandyke K., **Zeissig, M.N.**, Hewett D.R., Martin S.K., Mrozik K.M., Cheong C.M., Diamond P., To L.B., Gronthos S., Peet D.J., Croucher P.I., Zannettino A.C.W. (2017). HIF-2α promotes dissemination of plasma cells in multiple myeloma by regulating CXCL12/CXCR4 and CCR1. *Cancer Research*, 77(20):5452-5463.

Published abstracts

1. **Zeissig M.N.**, Hewett D.R., Mrozik K.M., Panagopoulos V., Engelhardt M., To L.B., Croucher P., Zannettino A.C.W., Vandyke K (2019). Therapeutic Targeting of CCR1 to Prevent Dissemination of Multiple Myeloma Plasma Cells. *Blood*, 134(Supplement_1): 3099

2. **Zeissig, M.N.**, Hewett D.R., Martin S.K., Mrozik K.M., Cheong C.M., Diamond P., To L.B., Gronthos S., Peet D.J., Croucher P.I., Vandyke K., Zannettino A.C.W. (2017). HIF-2α Upregulates CCR1 to Promote Dissemination of Plasma Cells in Multiple Myeloma. *Blood*, 130(Supplement_1): 4425

Conference Proceedings

1. **Zeissig M.N.**, Hewett D.R., Mrozik K.M., Panagopoulos V., Engelhardt M., To L.B., Croucher P.I., Zannettino A.C.W., Vandyke K. (2019). Therapeutic Targeting of CCR1 to Prevent Dissemination of Multiple Myeloma Plasma Cells. *American Society of Haematology Annual Meeting.* Orlando, Florida, December 2019. Poster presentation.

2. **Zeissig M.N.**, Vandyke K., Hewett D.R., Panagopoulos V., Engelhardt M., Croucher P.I., Zannettino A.C.W. (2019). Therapeutic Targeting of CCR1 to Prevent Dissemination of Multiple Myeloma Plasma Cells. *SAHMRI Scientific Meeting.* Adelaide, Australia, October 2019. Poster presentation.

3. **Zeissig M.N.**, Vandyke K., Hewett D.R., Panagopoulos V., Engelhardt M., Croucher P.I., Zannettino A.C.W. (2019). Therapeutic Targeting of CCR1 to Prevent Dissemination of Multiple Myeloma Plasma Cells. *Florey Postgraduate Symposium.* Adelaide, Australia, September 2019. Poster presentation.

4. **Zeissig M.N.**, Hewett D.R., Panagopoulos V., To L.B., Croucher P.I., Zannettino A.C.W., Vandyke K. (2019). The chemokine receptor CCR1 promotes dissemination of plasma cells in multiple myeloma**.** *ASMR SA Scientific Meeting.* Adelaide, Australia, June 2019. Oral presentation.

5. **Zeissig M.N.**, Vandyke K., Engelhardt M., Zannettino A.C.W. (2019). Chemokine receptor CCR1 expression promotes dissemination and reduces therapeutic sensitivity of MM cells: subsequent UKF project. *Freiburg University Clinic Medicine Annual Saig Workshop*. Freiburg, Germany, April 2019. Invited Speaker.

6. **Zeissig M.N.**, Hewett D.R., Panagopoulos V., To L.B., Croucher P.I., Zannettino A.C.W., Vandyke K. (2018). Chemokine receptor CCR1 expression promotes dissemination of plasma cells in multiple myeloma. *ANZSCDB Adelaide Meeting.* Adelaide, Australia, November 2018. Poster Presentation.

7. **Zeissig M.N.**, Hewett D.R., Panagopoulos V., To L.B., Croucher P.I., Zannettino A.C.W., Vandyke K. (2018). Chemokine receptor CCR1 expression promotes dissemination of plasma cells in multiple myeloma. *SAHMRI Scientific Meeting.* Adelaide, Australia, October 2018. 3-Minute Thesis.

8. **Zeissig M.N.**, Vandyke K., Hewett D.R., Panagopoulos V., Croucher P.I., Zannettino A.C.W. (2018). Chemokine receptor CCR1 drives dissemination of multiple myeloma plasma cells. *Florey Postgraduate Symposium.* Adelaide, Australia, September 2018. Poster presentation.

9. **Zeissig M.N.**, Hewett D.R., Panagopoulos V., To L.B., Croucher P.I., Zannettino A.C.W., Vandyke K. (2018). Chemokine receptor CCR1 expression promotes dissemination of plasma cells in multiple myeloma. *ASMR SA Scientific Meeting.* Adelaide, Australia, June 2018. Oral presentation.

10. **Zeissig, M.N.**, Hewett D.R., Martin S.K., Mrozik K.M., Cheong C.M., Diamond P., To L.B., Gronthos S., Peet D.J., Croucher P.I., Vandyke K., Zannettino A.C.W. (2017). HIF-2α Upregulates CCR1 to Promote Dissemination of Plasma Cells in Multiple Myeloma. *Cancer in SA Translational Meeting.* Adelaide, Australia, May 2018. Oral presentation.

11. **Zeissig, M.N.**, Hewett D.R., Martin S.K., Mrozik K.M., Cheong C.M., Diamond P., To L.B., Gronthos S., Peet D.J., Croucher P.I., Vandyke K., Zannettino A.C.W. (2017). HIF-2α Upregulates CCR1 to Promote Dissemination of Plasma Cells in Multiple Myeloma.

American Society of Haematology Annual Meeting. Atlanta, Georgia, December 2017. Poster presentation.

12. **Zeissig, M.N.**, Hewett D.R., Martin S.K., Mrozik K.M., Cheong C.M., Diamond P., To L.B., Gronthos S., Peet D.J., Croucher P.I., Vandyke K., Zannettino A.C.W. (2017). HIF-2α Upregulates CCR1 to Promote Dissemination of Plasma Cells in Multiple Myeloma. *ANZSCDB Adelaide Meeting.* Adelaide, Australia, November 2017. Poster Presentation.

13. **Zeissig, M.N.**, Hewett D.R., Martin S.K., Mrozik K.M., Cheong C.M., Diamond P., To L.B., Gronthos S., Peet D.J., Croucher P.I., Vandyke K., Zannettino A.C.W. (2017). HIF-2α Upregulates CCR1 to Promote Dissemination of Plasma Cells in Multiple Myeloma. *SAHMRI Scientific Meeting.* Adelaide, Australia, October 2017. 3-Minute Thesis.

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17. **Zeissig, M.N.**, Vandyke K., Hewett D.R., Zannettino A.C.W. (2017). The role of hypoxia, CXCR4 and CCR1 in the dissemination of multiple myeloma plasma cells. *ANZSCDB Adelaide Meeting.* Adelaide, Australia, November 2016. Poster Presentation.

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Chapter 1:

Introduction

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Tumour dissemination in multiple myeloma disease progression and relapse: a new therapeutic target in high-risk myeloma

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Running title:

Tumour dissemination as a therapeutic target in multiple myeloma

1.1. Abstract

Multiple myeloma (MM) is a plasma cell (PC) malignancy characterised by the presence of MM PCs at multiple sites throughout the bone marrow. Increased numbers of peripheral blood MM PCs are associated with rapid disease progression, shorter time to relapse and are a feature of advanced disease. This poses the opportunity to slow disease progression and prevent overt relapse through therapeutic targeting of dissemination. However, the mechanisms underlying MM PC dissemination are still incompletely understood, highlighting the need for further research to identify the key processes and how to target these therapeutically. In this review, the current understanding of the process of MM PC dissemination and the extrinsic and intrinsic factors potentially driving it are addressed through analysis of patient-derived MM PCs and MM cell lines as well as mouse models of homing and dissemination. In addition, we discuss how patient cytogenetic subgroups that present with highly disseminated disease such as t(4;14), t(14;16) and t(16;20) suggest that intrinsic properties of MM PC influence their ability to disseminate. Preventing dissemination may be a beneficial novel therapeutic avenue for patients with high-risk cytogenetics and highly disseminated disease.

1.2. The role of dissemination in the progression of multiple myeloma

Multiple myeloma (MM) is an incurable haematological malignancy characterised by the uncontrolled proliferation of clonal malignant plasma cells (PCs) in the bone marrow (BM).[1] Worldwide, MM accounts for 1% of all cancers,[2] with approximately 140,000 people diagnosed with myeloma every year.[3] A characteristic feature of MM is that at the time of diagnosis, MM PC are present at multiple sites throughout the skeleton.[4]

During initial disease development, malignant PCs establish themselves in BM niches that support their growth.[5] Migration and population of these transformed clonal PCs throughout the BM leads to the development of the pre-myeloma disease stages monoclonal gammopathy of undetermined significance (MGUS) or smouldering MM (SMM) and, ultimately, MM. Notably, the detection of multiple tumour lesions (>5mm) within the BM is a defining feature of MM, with elevated numbers of MRI-detectable lesions being an independent predictor of poor prognosis in newly diagnosed patients, and shorter time to progression.[6] Furthermore, over two-thirds of newly diagnosed MM patients have circulating PC, as detected by flow cytometry,[7] cytology[8] or slide-based immunofluorescence,[9] with higher numbers being an independent predictor of shorter progression-free survival[10,11] and overall survival.[7-10] Notably, the association between poor outcomes and elevated clonal PC in the PB is independent of BM tumour burden,[10] suggesting an active role for circulating PC in MM disease progression and relapse following therapy. Furthermore, the dissemination of MM PCs is a key feature of aggressive, advanced forms of MM, including development of extramedullary disease (EMD) and plasma cell leukaemia (PCL). PCL is characterised by the presence of very high (>20%) numbers of circulating PC.[15] Extra-medullary disease (EMD), characterised by the dissemination and growth of clonal PCs to soft-tissue sites, occurs in 3-5% of MM cases at diagnosis and increases to up to 20% at relapse.[16] Importantly, patients with either PCL or EMD have a poorer prognosis compared with other MM patients,[15] highlighting the association between dissemination and aggressive disease.

MGUS and smouldering MM are largely asymptomatic precursor diseases characterised by elevated PC numbers in the BM, an absence of evidence of end organ damage and, notably in a lack of multiple MRI-detectable lesions, all of which are characteristic of active MM.[4] Studies using next generation sequencing examining clonal evolution during disease progression from MGUS or SMM to MM suggest that the genetic abnormalities

leading to MM are already present at the pre-malignant stages.[14,15] These studies suggest that the outgrowth of dominant PC clones, and their subsequent population of sites throughout the BM, is a key feature of progression to MM. In support of this, increased incidence of PB circulating PCs in MGUS and SMM patients is associated with progression.[18,19] Taken together, these studies suggest that dissemination of clonal PCs is a key step for the progression to symptomatic disease.

In this review, we will discuss the current understanding of the mechanisms that underpin MM PC dissemination. Furthermore, we will discuss how this knowledge could be used to develop novel therapeutic strategies to delay disease progression and treat high-risk MM patients.

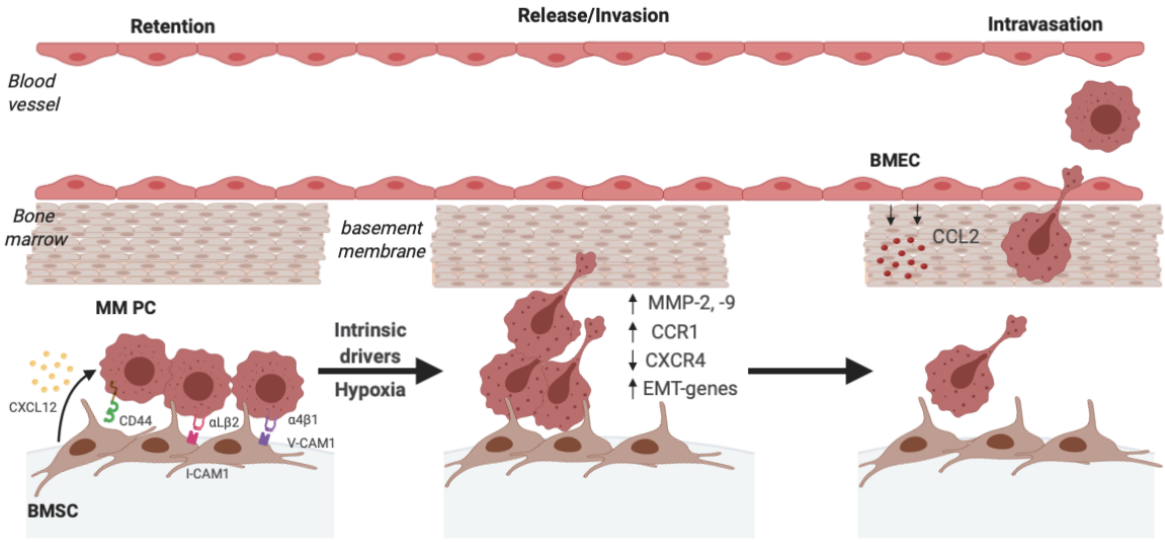
1.3. The process of dissemination in MM

Similar to the process of solid tumour metastasis, the dissemination of MM PCs is associated with a loss of their adherence to cells of the BM microenvironment that favours MM PC retention, allowing the cells to exit the niche. The tumour cells must then undergo trans-endothelial migration, mediated by chemoattractants and adhesive interactions, and intravasate (move in the blood from the tissue) where they are carried to a secondary site. The tumour cells must then arrest in the BM endothelium and extravasate (move out of the blood and into tissues) following chemotactic factors produced by BM cells. The final stage of MM PC dissemination is associated with the colonisation of new BM sites which supports tumour cell growth.[17] The following sections will describe the molecular mechanisms that are involved in MM PC dissemination, focusing on both the intrinsic properties of the MM PC that support their dissemination, as well as the extrinsic stimuli that drive this process.

1.3.1. Retention within the BM stromal niche

Bone marrow stromal cells (BMSCs) play a critical part in the BM niche that supports the growth of MM PC. Adhesion of MM PCs to BMSCs, and extracellular matrix (ECM) components that are secreted by these cells, is a crucial mechanism by which MM PCs are maintained within the niche (Figure 1.1).[17] Binding of the integrin α4β1 (also known as very late antigen 4, VLA-4), expressed by MM PCs, to vascular cell–adhesion molecule 1 (VCAM-1) and fibronectin, expressed by BMSCs, is one of the key factors mediating the strong adhesion of MM PCs to BMSCs.[18] Notably, integrin α4β1-mediated

Figure 1.1. **Multiple myeloma plasma cell intravasation is dependent on overcoming adhesive interactions within the BM and invading the vasculature basement membrane**. Release from the stromal niche is likely mediated by both external drivers such as hypoxia, and internal drivers in subclones with increased propensity to disseminate. This leads to a decrease in multiple myeloma plasma cell (MM PC) expression of key adhesion molecules such as α4β1, allowing the MM PC to overcome the retention signal mediated by CXCL12 produced by bone marrow stromal cells (BMSCs). Hypoxic MM PC upregulate epethelial to mesenchymal transition (EMT)-like genes, decrease their expression of CXCR4 and increase their expression of CCR1 to mediate mobilisation within the BM niche. Upregulation of matrix metalloproteins-2 and -9 then allows invasion into the vasculature basement membranes and intravasation into the peripheral circulation during dissemination


Retention
Release/Invasion
Intravasation
Blood vessel
BMEC
Bone marrow
basement membrane
CCL2
MM PC
Intrinsic drivers
Hypoxia
MMP-2, -9
CCR1
CXCR4
EMT-genes
CXCL12
CD44
αLβ2
α4β1
V-CAM1
I-CAM1
BMSC

binding to fibronectin decreases the response of MM cell lines to chemotactic factors *in vitro*, supporting its role in MM PC retention.[19] Additionally, the integrin αLβ2 complex (also known as lymphocyte function-associated antigen 1, LFA-1) on MM PCs mediates binding to intracellular adhesion molecule 1 (ICAM-1) on BMSCs.[20] Other adhesive factors expressed by MM PCs include CD44 variants CD44v6 and CD44v9, which mediate adhesion to BMSCs,[21] and syndecan-1 (also known as CD138), which is involved in adhesion to type 1 collagen.[22]

The adhesion of MM PCs to BMSCs and ECM can be enhanced by exogenous secreted factors which induce rapid cytoskeletal remodelling and conformational changes in integrins, thereby increasing adhesion.[18,23] The C-X-C chemokine ligand 12 (CXCL12; also known as stromal-derived factor-1 [SDF-1]), abundantly produced by BMSCs,[24] is the ligand for the C-X-C chemokine receptor 4 (CXCR4), expressed universally on MM PCs from MM patients.[25,26] Treatment of MM PCs with CXCL12 rapidly increases MM PC adhesion to fibronectin and VCAM-1 on the surface of BMSCs, through induction of conformational changes in integrin α4β1.[18] Furthermore, treatment with the CXCR4 antagonist Plerixafor (also known as AMD3100) rapidly mobilises PCs and stem cells into the blood.[23,27] Additionally, other factors may increase the adhesion of MM PCs to BMSCs by increasing expression of integrins. For example, the cytokine tumour necrosis factor alpha (TNF-α), secreted by MM PCs, acts in an autocrine fashion to increase expression of the integrin α4β1 and integrin αLβ2 complexes on MM PCs, thereby increasing adhesion of MM PC cell lines to BMSCs *in vitro*.[20] In addition, the CD40 ligand (CD40L), expressed on haematopoietic cells binds to the CD40 receptor on MM PCs and stimulates the adhesion of MM cell lines to BMSCs and fibronectin *in vitro,*[28,29] via a mechanism which may involve upregulation of integrin expression.[28] Furthermore, B-cell activating factor (BAFF), which is secreted by and expressed on the surface of BMSCs, has also been shown to increase the adhesion of MM PCs to BMSCs, although the mechanism involved is unclear.[30]

1.3.2. Release from the BM niche

To be released from the BM and intravasate into the PB, MM PCs must overcome the aforementioned adhesive interactions that act as a strong retention signal (Figure 1.1). While the microenvironmental stimuli that regulate the release from the BM niche are unclear, decreased expression of key factors involved in adhesion in the stromal niche may

play a role. Studies analysing the expression of cell surface adhesion factors in PB PCs compared with BM PCs from MM patients have shown that circulating MM PCs express less integrin α4β1 compared with BM-resident MM PCs.[31,32] In addition, studies show that there is a decrease in the activated form of β1 integrin in MM PCs in the PB compared with the BM in MM patients,[33] suggesting that downregulation of its active form may in part facilitate release from the BM. Syndecan-1 expression has also been shown to be decreased in PB MM PCs compared with their BM counterparts.[31] Notably, MM cell line expression of the enzyme heparanase-1, which is responsible for cleaving proteoglycans including syndecan-1 from the cell surface, significantly increased the spontaneous dissemination of MM cells *in vivo*, suggesting that shedding of syndecan-1 may promote dissemination.[34] Finally, PCs from PCL patients have been reported to have decreased CD40 expression compared with MGUS PCs, supporting a potential role for loss of CD40 expression in release of MM PC from the BM niche.[35]

1.3.3. Microenvironmental control of release from the BM niche

Release from the BM niche may also be facilitated by signals from the micro-environment (Figure 1.1). The BM becomes increasingly hypoxic during MM tumour growth, with highly hypoxic regions arising within the tumour mass due to rapid tumour cell growth and abnormal blood vessel formation.[36] Moreover, the role of hypoxia in tumour progression, dissemination and angiogenesis has been demonstrated in mouse models of MM.[37-39] In particular, Azab and colleagues demonstrated in a mouse model of MM that MM PC hypoxia, as assessed by pimonidazole staining, strongly correlated with both BM tumour burden and numbers of circulating MM PCs.[37] In addition, culturing MM cell lines in hypoxic conditions significantly reduced their adherence to BMSCs or to collagen *in vitro*,[40] suggesting that hypoxia may play a role in release of MM PCs from the BM niche.[37] Furthermore, hypoxia has been shown to activate the transcription factors Snail and Twist1 that are master regulators of the epithelial to mesenchymal transition (EMT), suggesting that, like in epithelial cancers, an EMT-like process may also be occurring in MM to allow release from the niche.[37] Studies by our group have demonstrated that the induction of hypoxia inducible factor HIF-2α in MM cell lines can lead to decreased response to stromal cell-derived CXCL12, which may facilitate release from the niche. HIF-2α increases CXCL12 expression by MM cell lines[41] which, in turn, reduces CXCR4 cell-surface levels on MM cells,[25] forming a feedback loop that leads to a decrease in CXCR4 signalling and a desensitisation to exogenous CXCL12.[25] In addition, our group

has demonstrated that hypoxic activation of HIF-2α leads to upregulation of the C-C chemokine receptor 1 (CCR1) in MM PCs, which may also contribute to their preferential mobilisation.[25] Notably, treatment of CCR1-positive MM cell lines with the CCR1 ligand C-C chemokine ligand 3 (CCL3; also known as macrophage inflammatory protein 1 alpha [MIP-1 α]) abrogates migration towards CXCL12 *in vitro*, suggesting that CCL3/CCR1 signalling can desensitise cells to exogenous CXCL12.[25] Taken together, these studies suggest that hypoxia may mediate a disruption to the CXCR4/CXCL12 retention signal which may allow MM PC release from the BM.

1.3.4. Intravasation

In order to undergo haematogenous dissemination, following release from the BM stromal niche, the MM PCs need to migrate towards the vasculature, and then invade and traverse through the basement membranes of the endothelium to intravasate (exit the tissue and enter the PB) (Figure 1.1).

BM endothelial cells (BMECs) are sources of secreted factors that are known chemoattractants for MM PCs, which may encourage dissemination from the primary site into the vasculature. Conditioned media from BMECs has been shown to contain chemotactic factors for MM cell lines, including CXCL12 (ligand for CXCR4) and CCL2 (also known as monocyte chemotactic protein-1 [MCP-1]; ligand for CCR2), which induce chemotaxis in MM cell lines and primary MM PCs *in vitro*.[26,42,43] In support of this, migration of mouse 5T MM cells towards BMEC conditioned media can be blocked using an antibody against CCL2, further suggesting that BMEC-derived CCL2 may promote MM PC migration towards the vasculature.[42] Collectively, these studies suggest that BMECs produce and regulate factors which may dictate the migration of MM PCs towards the endothelium and allow intravasation.

In order to intravasate, MM PCs must migrate through and invade the basement membranes of the vasculature, which are comprised of type IV collagen, laminin, and other ECM components.[44] Upregulation of MM PC secretion of specific matrix metalloproteases (MMPs), enzymes that degrade ECM components, enable invasion into the vasculature (Figure 1.1). MMP-2 and -9, which degrade ECM components including collagen IV and laminin, have been implicated in the invasion of MM PC.[45,46] Furthermore, osteopontin, produced in the BM by fibroblasts, BMECs, osteoblasts, osteoclasts,[47] is an inducer of the

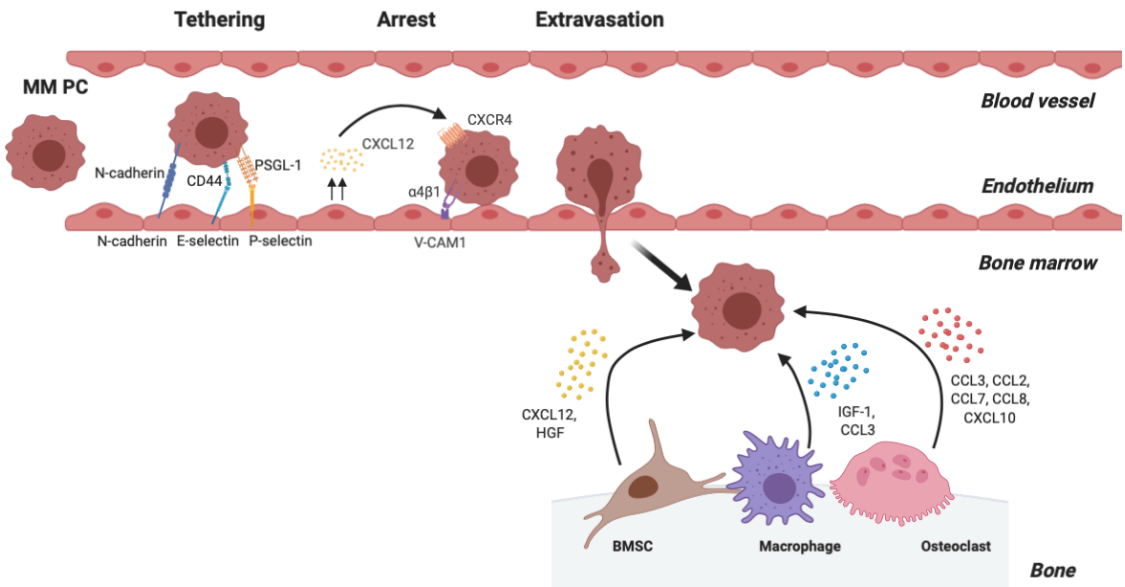
MM cell line 5T33MM transendothelial migration via upregulation of MMP-9.[48] Taken together, these studies suggest that increased MMP production in the MM microenvironment facilitates invasion of MM PC.

1.3.5. Extravasation and homing to the BM

In order to exit the the PB and establish within a new site, MM PC need to adhere to the endothelial layer and extravasate, in response to local chemoattractant factors, (Figure 1.2). MM PC adhesion to BMECs is a critical process during extravasation from the vasculature. In the trafficking of lymphocytes, primary, reversible adhesive interactions lead to "tethering", following which the lymphocyte loosely rolls along the endothelium under flow conditions, allowing it to sample the vessel for chemokines which drive extravasation and homing into the BM (Figure 1.2).[49] Tethering of MM PCs and rolling is mediated by the binding of P-selectin on the endothelium to the ligands CD44 and P-selectin glycoprotein ligand-1 (PSGL-1) on MM PCs.[50] Indeed, inhibitors which prevent PSGL-1 interacting with P-selectin resulted in a reduction of *in vivo* homing following intravenous injection of MM.1S cells.[37,51] Furthermore, using co-injection of 5T cells with an anti-CD44 antibody resulted in a decrease in 5T cell *in vivo* homing.[51] Studies by our group and those of others have also implicated the homophilic adhesion molecule N-cadherin in the adhesion of MM PCs to the endothelium. shRNA knockdown of N-cadherin in human and murine MM cell lines resulted in a reduction of the adhesion of MM cell lines to BMECs[52] without affecting transendothelial migration *in vitro*,[52,53] suggesting a role for N-cadherin in the tethering process, but not in subsequent extravasation. In support of this, N-cadherin knockdown or an N-cadherin blocking peptide decreased the homing of MM cells to the BM following intravenous injection in mice.[52,53]

Rolling along the endothelium allows the MM PC to respond to locally produced chemokines, in particular CXCL12, which induce strong adhesive interactions through stabilising integrin-mediated adhesion to the endothelium and allowing extravasation and homing into the BM (Figure 1.2). CXCL12 produced by BMECs has been shown to induce rapid activation of integrin α4β1 in primary MM PCs and MM cell lines, enabling arrest of the MM PCs through adhesion to BMEC VCAM-1, and subsequently enabling transendothelial migration during extravasation.[54] Transendothelial migration of human myeloma cell lines *in vitro*[54] and *in vivo*[55] and primary MM PC *in vitro*[54] could be completely blocked using antibodies against VCAM-1, integrin α4 or integrin β1,

Figure 1.2. **Multiple myeloma plasma cell extravasation is dependent on expression of key adhesion and chemoattractant molecules**. Tethering and rolling are mediated by E- and P-selectin expressed on endothelial cells (EC) binding to CD44 and P-selectin glycoprotein ligand-1 (PSGL-1) on multiple myeloma plasma cells (MM PCs), respectively and homodimerization of N-cadherin. Subsequently, CXCL12 is produced by the endothelium, activating MM PC-expressed integrin complex α4β1 allowing arrest through firm adhesive interactions with VCAM-1 ligand expressed on EC. Following arrest, MM PCs will undergo trans-endothelial migration following chemoattractant factors expressed within the bone marrow (BM) by BM stromal cells (BMSCs), macrophages and osteoclasts, such as IGF-1, CXCL12 and CCL3.


Tethering
Arrest
Extravasation
MM PC
Blood vessel
Endothelium
Bone marrow
N-cadherin
CD44
PSGL-1
CXCL12
CXCR4
α4β1
N-cadherin
E-selectin
P-selectin
V-CAM1
CXCL12,
HGF
IGF-1,
CCL3
CCL3, CCL2,
CCL7, CCL8,
CXCL10
BMSC
Macrophage
Osteoclast
Bone

suggesting a role for integrin α4β1-VCAM-1 interaction in adhesion to BMECs and subsequent extravasation.[54,55] In support of this, blocking of integrin α4β1 binding with an antibody delayed the *in vivo* homing of the human MM cell line MM.1S to the BM following intravenous injection.[56] Adhesion to the endothelium[57] and exposure to CXCL12[58] also triggers upregulation of MM PC production of MMP9 which facilitates proteolytic degradation of the basement membrane of the endothelium[59] to further facilitate homing to the BM.

Notably, studies using primary patient samples have demonstrated that CXCR4 is upregulated in MM PCs the in the PB compared with the BM,[25,60] potentially enabling enhanced response to local BMEC-derived CXCL12. The preferential arrest of circulating MM PCs in the BM vasculature may, at least in part, be due to the abundant expression of CXCL12 by BMECs (Figure 1.2). A murine MM cell line has been shown to preferentially adhere to endothelial cells from the BM, compared with those of other organs, suggesting a preference for the BM as a site of establishment.[61] [60,62]Notably, Sipkins and colleagues[63] have demonstrated that the MM cell line U266, and other B-cell lines, preferentially extravasates in the BM at specific vascular regions which express high levels of CXCL12 on the surface of the endothelium.

1.3.6. Establishment and colonisation of MM PC in a new BM niche

Following extravasation, it is thought that MM PC respond to locally produced chemokines and growth factors to direct their movement to their ultimate location in the BM (Figure 1.2). Recent animal studies from our group[71] and those of others,[72] have shed light on the fate of disseminating clones, and shown that the dissemination process is extremely inefficient: of the hundreds of MM cells that may reach the BM following intravenous injection, fewer than ten ultimately proliferate to form macroscopic tumours while the remaining cells are maintained in a non-proliferative state.[64,65] This suggests that the BM microenvironment in which the MM PC ultimately resides, may determine whether an individual tumour cell is destined for dormancy or proliferation.[66,67] At this time, it remains unclear whether MM PC are driven by specific microenvironment-derived factors to home to specific niches that support their growth, rather than dormancy, or whether the colonisation process is a stochastic one.

The migration of MM PCs towards specific niches in the BM may be driven by a range of chemoattractant molecules that are produced by BM cells, including BMSC, osteoclasts and macrophages, which play an important role in the MM BM niche (Table 1). For both normal PCs and MM PCs, CXCL12 represents the predominant signal that is thought to drive homing from the PB into the BM[68] and subsequently also leads to CXCL12-mediated MM PC retention (described in section 1.3.1).[60,62] CXCL12 expression is higher in the BM than in the PB,[25,60] consistent with its abundant expression by BMSCs,[69] establishing a gradient that enables homing to the BM. MM PCs have a strong migratory response towards CXCL12,[70] with CXCL12 inducing cytoskeletal remodelling that enables MM PC migration.[25,60] Blockade of CXCL12-CXCR4 binding slows homing of human MM cell lines from the PB and accumulation in the BM *in vivo*.[60,71] BMSCs also produce the hepatocyte growth factor (HGF), which further enhances the chemotactic effect of CXCL12 on MM PC.[72] Additionally, insulin-like growth factor 1 (IGF-1) has been shown to be a promigratory (chemokinetic) and chemoattractant factor for murine[73] and human[72] MM cell lines *in vitro* which synergises with CXCL12 to enhance the response to CXCL12 *in vitro*.[72] In the mouse MM 5T models, IGF-1 is a critical promoter of homing of MM PCs to the BM.[73,74] Notably, ablation of macrophages, an abundant source of IGF-1 in the BM, is sufficient to inhibit the homing of 5TGM1 cells to the BM.[74] Monocytes and macrophages also highly express the CCR1 ligand CCL3,[74] which is a promigratory factor for primary MM PCs and MM cell lines *in vitro*.[70,75] Osteoclasts are also a predominant source of a number of promigratory stimuli for MM PC, including CCL3,[75] the CCR2 ligands CCL2, CCL7 (also known as monocyte chemotactic protein-3 [MCP-3]) and CCL8 (also known as monocyte chemotactic protein-2 [MCP-2])[70,75,76] and the CXCR3 ligand CXCL10 (also known as interferon gamma-induced protein 10 [IP-10]).[70]

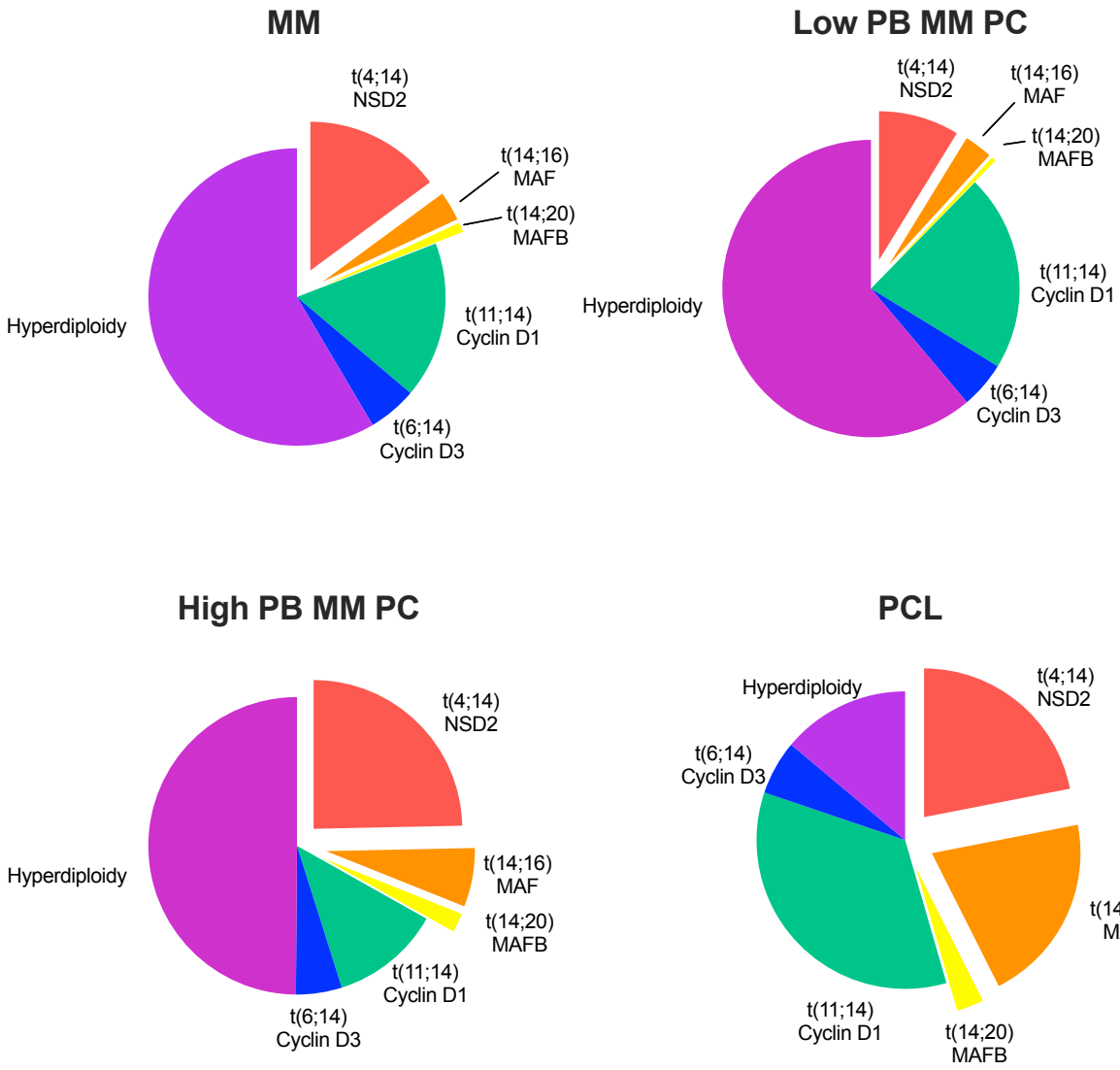
1.4. Intrinsic MM characteristics that may facilitate dissemination

It is well-established that there an association between certain chromosomal translocations that are frequently observed in MM patients including t(14;16), t(16;20) and t(4;14) and the incidence of elevated circulating PC or PCL, suggesting that these chromosomal translocations may result in an increased propensity for MM PC dissemination (Figure 1.3). While t(11;14) is also highly represented in PCL,[77] there is no clear gene expression signature that could mechanistically explain the increased propensity for dissemination in these patients, and as such was not discussed further. As

Table 1.1. Main sources of promigratory ligands in the MM bone marrow

Promigratory ligand	**Receptor**	**Predominant source in the MM bone marrow**
CXCL10[65, 94]	CXCR3	Osteoclasts and Osteoclast Precursors[95]
CXCL12[25, 26, 65]	CXCR4	Endothelial cells, BMSC, Osteoclasts[26, 96]
CCL3[25, 65, 70]	CCR1, CCR5	Osteoclasts, monocytes, macrophages[69, 70]
CCL2[42, 65, 71]	CCR2	Endothelial cells, BMSC, Osteoclasts[26, 42, 70, 71, 97]
CCL7[71]	CCR2	Osteoclasts[70]
CCL8[71]	CCR2	Osteoclasts[70]
IGF1[67, 69, 98]	IGF1R	BMSC, Osteoclasts[69, 70, 97]
HGF[67, 99]	c-Met	BM mesenchymal stem cells[97]

Figure 1.3. High-risk cytogenetics are associated with higher numbers of circulating multiple myeloma plasma cells in patients. Higher incidences of high-risk cytogenetics t(4;14), t(14;16) and t(14;20) occur in patients with high numbers of multiple myeloma plasma cells (MM PCs) in the peripheral blood (PB) (33.2%) than in patients with low PB MM PCs (12.1%). Patients with plasma cell leukemia (PCL) have the highest incidence rates of t(4;14), t(14;16) and t(14;20) (38.6%).


MM
t(4;14)
NSD2
t(14;16)
MAF
t(14;20)
MAFB
t(11;14)
Cyclin D1
t(6;14)
Cyclin D3
Hyperdiploidy
Low PB MM PC
t(4;14)
NSD2
t(14;16)
MAF
t(14;20)
MAFB
t(11;14)
Cyclin D1
t(6;14)
Cyclin D3
Hyperdiploidy
High PB MM PC
t(4;14)
NSD2
t(14;16)
MAF
t(14;20)
MAFB
t(11;14)
Cyclin D1
t(6;14)
Cyclin D3
Hyperdiploidy
PCL
Hyperdiploidy
t(4;14)
NSD2
t(6;14)
Cyclin D3
t(14;16)
MAF
t(11;14)
Cyclin D1
t(14;20)
MAFB

discussed below, there is increasing evidence for functional differences arising from t(14;16), t(16;20), and t(4;14) translocations which may underlie the propensity for dissemination observed in different genetic subtypes of MM.

1.4.1. t(14;16) and t(14;20)

The chromosomal translocations t(14;16) and t(14;20) lead to constitutive overexpression of the transcription factors MAF and MAFB, respectively. Notably, 15% of t(14;16) MM patients present with PCL, compared with 1.5% of non-t(14;16) MM patients,[78] suggesting an association with increased dissemination. The relative infrequency of t(14;20) translocations have precluded statistical analysis of the association between MM PC dissemination and t(14;20); however, t(14;20) has been reported in 1.3% to 3% of patients with elevated PB PC[79] and 2.5% of PCL patients,[80] compared with 0.8% to 1.0% of MM patients,[79] suggesting a potentially increased incidence of dissemination in these patients.

Transcriptome profiling has revealed that elevation of expression of two genes, the integrin *ITGB7* and the chemokine receptor *CX3CR1*, are highly characteristic of patients and human MM cell lines with MAF or MAFB translocations.[81,82] Overexpression and siRNA knockdown studies have demonstrated that MAF regulates integrin β7 expression in human MM cell lines.[81] Integrin β7 can form a heterodimer with integrin αE, with the αEβ7 complex regulating adhesion to E-cadherin on the surface BMSC.[81,83] Blockade of E-cadherin on BMSC[37,81] or of integrin β7 on human MM cell lines[81,83] decreases the adhesion of MM PC to BMSC *in vitro*, suggesting that this interaction may play a role in adhesion in the niche. Furthermore, shRNA knockdown of integrin β7 decreases the migration of human MM cell lines towards CXCL12 *in vitro* and furthermore, delays BM homing following intravenous injection *in vivo*.[83] Elevated integrin β7 expression in MM PC may, therefore, contribute to the increased dissemination seen in patients with aberrant MAF expression. Microarray data analyses have also shown that expression of the chemokine receptor CX3CR1 is a recurrent feature of patients with t(14;16).[82] The ligand for CX3CR1, CX3CL1, can be released in a soluble form where it can act as a chemoattractant, or alternatively, can be presented in a membrane-bound form, where it facilitates cell-cell adhesion.[84] Notably, CX3CL1/CX3CR1 binding has been implicated in playing a key role in the transendothelial migration of lymphocytes by increasing endothelial cell adhesion and migration towards chemoattractants.[85] While the functional role for CX3CR1 in MM PC is unclear, the human MM cell line RPMI-8226 has been

shown to bind to CX3CL1 *in vitro* under shear flow, suggesting that it may play a role in adhesion to endothelial cells in MM.[86] Gene expression profiling studies have also identified elevated expression of *IGF1R* and *CCR1*, the receptors for the MM PC chemoattractants IGF-1 and CCL3, respectively, are also elevated in t(14;16) human MM cell lines and primary MM PCs.[81] The expression of these key promigratory receptors may also, therefore, play a role in the MM PC dissemination in t(14;16) patients.

1.4.2. t(4;14)

The chromosomal translocation t(4;14), which leads to constitutive overexpression of the histone methyltransferase NSD2 (also known as WHSC1 and MMSET), is also associated with high risk disease and increased dissemination in MM patients. The incidence of patients with t(4;14) is 2- to 4-fold higher in patients with elevated circulating PCs, compared with those with low circulating PCs.[79] Additionally, there is some evidence for an increase in the incidence of t(4;14) in PCL patients,[87] and t(4;14) myeloma is also associated with an increased incidence of extramedullary PC tumours.[88]

Notably, the t(4;14) translocation is associated with an epithelial-to-mesenchymal transition (EMT)-like gene expression signature in MM PCs, characterised by upregulation of mesenchymal genes, including those for N-cadherin and vimentin, and the transcription factor Twist-1.[53,89] N-cadherin knockdown studies in MM cell lines have shown that N-cadherin is important in the homing of MM PCs from the vasculature to the BM.[52,53] In addition, studies from our group have shown that overexpression of Twist-1 has been shown to increase MM PCs dissemination in an intratibial model of MM *in vivo*.[89]

Like t(14;16) patients, tumour cells from t(4;14) patients and t(4;14)+ MM cell lines have been shown to have increased expression of *IGF1R* and *CCR1* receptors[81,90] which may also contribute to the increased propensity for dissemination of t(4;14) MM PC.

1.4.3. Subclonal heterogeneity and dissemination

There is evidence to suggest that, in addition to these chromosomal translocations, other secondary chromosomal abnormalities and mutations may increase the ability of individual subclones to disseminate.[31,91-93] Using FISH or whole exome sequencing on paired BM and PB PC samples from MM patients, several studies have found that in some instances subclones present in the BM were not present in the PB, and vice versa.[31,92,93] However, it

remains to be seen whether the subclonal differences observed between the BM and PB in MM patients are due to selective microenvironmental pressures, such as hypoxia or other as yet uncharacterised stimuli, that drive the efflux of a subclone in a particular microenvironment, or if certain secondary copy number changes or SNVs may increase the propensity for dissemination in MM, leading to an increased likelihood for dissemination of particular subclones. Furthermore, it is also likely that the subclonal differences may be due to the inability of single-site BM biopsy to thoroughly capture the heterogeneity of the disease.[94]

There is some evidence that points to mechanisms whereby certain subclones could have an increased propensity for dissemination. Deletion of chromosome 17p13 [del(17p)] occurs in approximately 10% of newly diagnosed MM patients.[95] Missense mutations in *TP53*, on 17p, are seen in approximately 19% of patients with del(17p), leading to a complete inactivation of functional p53.[96] There is some evidence for an increased incidence of del(17p) in PCL patients,[87] although this has not been reported in MM patients with elevated PB PC.[10] Additionally, Manier et al. identified one patient with 17p loss across the BM and PB sample with a *TP53* missense mutation which was only detectable in the PB sample, suggesting that *TP53* mutations may increase the propensity for dissemination.[93] In support of this, siRNA-mediated knockdown of p53 in NCI-H929 cells increased their invasion through Matrigel and decreased their adhesion to BMSC *in vitro*.[97] Taken together, these studies suggest a potential role for loss of p53 in migration and dissemination of MM PC.

1.5. Concluding remarks and future perspectives

One of the defining features of symptomatic MM is the presence of MM tumours at multiple sites in the BM.[4] In over two-thirds of newly diagnosed MM patients, circulating MM PC are detectable in the PB,[9-11] with higher numbers being associated with poorer overall survival.[9-12] Furthermore, highly disseminated disease, as characterised by elevated circulating MM PC and EMD, is a feature of advanced and high-risk disease and is associated with poorer overall survival.[13,15,78,98] There is also evidence to suggest that dissemination of therapy-resistant clones leads to shorter time to relapse, with higher numbers of circulating tumour cells at baseline being indicative of shorter progression-free survival, regardless of the therapy used.[12,13,99,100] These studies suggest that therapies which target key processes involved in MM PC dissemination may be useful to delay

disease progression or as a maintenance therapy to extend progression-free survival following frontline therapy. However, a greater understanding of the mechanisms that underpin the dissemination process is needed to reveal potential targets for anti-dissemination therapy. No anti-dissemination agents have as of yet made it to clinical trials, and with promising *in vivo* results this could present a novel area for the development of therapeutics. It remains to be seen, however, whether therapeutic targeting of dissemination will translate to a beneficial impact on preventing further MM dissemination and controlling disease progression. Some key outstanding questions include: What regulates spontaneous dissemination in MM? Which factors promote MM PC dissemination to soft tissue sites in advanced disease? Can anti-dissemination therapy be clinically useful to slow disease progression and/or prevent the development of overt relapse?

In conclusion, this review has highlighted the current understanding of the process of MM dissemination. Dissemination is mediated by extrinsic microenvironmental stimuli, the loss of adhesive interactions and the increase in chemotactic factors. Furthermore, intrinsic changes in MM PCs may also contribute to the propensity to disseminate. Understanding the processes of MM PC dissemination may allow the identification of novel therapeutic targets widely applicable to MM patients, especially for the prevention of overt relapse in high-risk patients.

1.6. Acknowledgements

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1.7. References

1. Dutta AK, Hewett DR, Fink JL, Grady JP, Zannettino ACW. Cutting edge genomics reveal new insights into tumour development, disease progression and therapeutic impacts in multiple myeloma. *British Journal of Haematology* **2017**; 2: 196-208
2. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. *International Journal of Cancer* **2015**; 5: 359-386
3. Cowan AJ, Allen C, Barac A, Basaleem H, Bensenor I, Curado MP, et al. Global Burden of Multiple Myeloma: A Systematic Analysis for the Global Burden of Disease Study 2016. *JAMA Oncol* **2018**;
4. Rajkumar SV. Updated Diagnostic Criteria and Staging System for Multiple Myeloma. *American Society of Clinical Oncology Educational Book* **2016**; 36: e418-e423
5. Dutta AK, Fink JL, Grady JP, Morgan GJ, Mullighan CG, To LB, et al. Subclonal evolution in disease progression from MGUS/SMM to multiple myeloma is characterised by clonal stability. *Leukemia* **2018**;
6. Walker BA, Wardell CP, Melchor L, Brioli A, Johnson DC, Kaiser MF, et al. Intraclonal heterogeneity is a critical early event in the development of myeloma and precedes the development of clinical symptoms. *Leukemia* **2013**; 384
7. Bianchi G, Kyle RA, Larson DR, Witzig TE, Kumar S, Dispenzieri A, et al. High levels of peripheral blood circulating plasma cells as a specific risk factor for progression of smoldering multiple myeloma. *Leukemia* **2013**; 3: 680-685
8. Gonsalves WI, Rajkumar SV, Dispenzieri A, Dingli D, Timm MM, Morice WG, et al. Quantification of circulating clonal plasma cells via multiparametric flow cytometry identifies patients with smoldering multiple myeloma at high risk of progression. *Leukemia* **2017**; 1: 130-135
9. Nowakowski GS, Witzig TE, Dingli D, Tracz MJ, Gertz MA, Lacy MQ, et al. Circulating plasma cells detected by flow cytometry as a predictor of survival in 302 patients with newly diagnosed multiple myeloma. *Blood* **2005**; 7: 2276-2279
10. Granell M, Calvo X, Garcia-Guiñón A, Escoda L, Abella E, Martínez CM, et al. Prognostic impact of circulating plasma cells in patients with multiple myeloma: implications for plasma cell leukemia definition. *Haematologica* **2017**; 6: 1099

11. Witzig TE, Gertz MA, Lust JA, Kyle RA, O'Fallon WM, Greipp PR. Peripheral blood monoclonal plasma cells as a predictor of survival in patients with multiple myeloma. *Blood* **1996**; 5: 1780-1787
12. Dingli D, Nowakowski GS, Dispenzieri A, Lacy MQ, Hayman SR, Rajkumar SV, et al. Flow cytometric detection of circulating myeloma cells before transplantation in patients with multiple myeloma: a simple risk stratification system. *Blood* **2006**; 8: 3384-3388
13. Gonsalves WI, Rajkumar SV, Gupta V, Morice WG, Timm MM, Singh PP, et al. Quantification of clonal circulating plasma cells in newly diagnosed multiple myeloma: implications for redefining high-risk myeloma. *Leukemia* **2014**; 10: 2060-2065
14. Moulopoulos LA, Dimopoulos MA, Smith TL, Weber DM, Delasalle KB, Libshitz HI, et al. Prognostic significance of magnetic resonance imaging in patients with asymptomatic multiple myeloma. *Journal of Clinical Oncology* **1995**; 1: 251-256
15. Sher T, Miller KC, Deeb G, Lee K, Chanan-Khan A. Plasma cell leukaemia and other aggressive plasma cell malignancies. *British Journal of Haematology* **2010**; 4: 418-427
16. Jagosky MH, Usmani SZ. Extramedullary Disease in Multiple Myeloma. *Current Hematologic Malignancy Reports* **2020**;
17. Noll JE, Williams SA, Purton LE, Zannettino ACW. Tug of war in the haematopoietic stem cell niche: do myeloma plasma cells compete for the HSC niche? *Blood Cancer Journal* **2012**; 9: e91
18. Sanz-Rodríguez F, Ruiz-Velasco N, Pascual-Salcedo D, Teixidó J. Characterization of VLA-4-dependent myeloma cell adhesion to fibronectin and VCAM-1. *British Journal of Haematology* **1999**; 4: 825-834
19. Shibayama H, Tagawa S, Hattori H, Inoue R, Katagiri S, Kitani T. Laminin and fibronectin promote the chemotaxis of human malignant plasma cell lines. *Blood* **1995**; 2: 719
20. Hideshima T, Chauhan D, Schlossman R, Richardson P, Anderson KC. The role of tumor necrosis factor alpha in the pathophysiology of human multiple myeloma: therapeutic applications. *Oncogene* **2001**; 33: 4519-4527
21. Van Driel M, Günthert U, van Kessel AC, Joling P, Stauder R, Lokhorst HM, et al. CD44 variant isoforms are involved in plasma cell adhesion to bone marrow stromal cells. *Leukemia* **2002**; 135

22. Ridley RC, Xiao H, Hata H, Woodliff J, Epstein J, Sanderson RD. Expression of syndecan regulates human myeloma plasma cell adhesion to type I collagen. *Blood* **1993**; 3: 767
23. Azab AK, Runnels JM, Pitsillides C, Moreau A-S, Azab F, Leleu X, et al. CXCR4 inhibitor AMD3100 disrupts the interaction of multiple myeloma cells with the bone marrow microenvironment and enhances their sensitivity to therapy. *Blood* **2009**; 18: 4341-4351
24. Sanz-Rodriguez F, Hidalgo A, Teixido J. Chemokine stromal cell-derived factor-1alpha modulates VLA-4 integrin-mediated multiple myeloma cell adhesion to CS-1/fibronectin and VCAM-1. *Blood* **2001**; 2: 346-351
25. Vandyke K, Zeissig MN, Hewett DR, Martin SK, Mrozik KM, Cheong CM, et al. HIF-2a Promotes Dissemination of Plasma Cells in Multiple Myeloma by Regulating CXCL12/CXCR4 and CCR1. *Cancer Research* **2017**; 20: 5452-5463
26. Pellegrino A, Ria R, Di Pietro G, Cirulli T, Surico G, Pennisi A, et al. Bone marrow endothelial cells in multiple myeloma secrete CXC-chemokines that mediate interactions with plasma cells. *Br J Haematol* **2005**; 2: 248-256
27. Broxmeyer HE, Hangoc G, Cooper S, Campbell T, Ito S, Mantel C. AMD3100 and CD26 modulate mobilization, engraftment, and survival of hematopoietic stem and progenitor cells mediated by the SDF-1/CXCL12-CXCR4 axis. *Annals of the New York Academy of Sciences* **2007**; 1-19
28. Urashima M, Chauhan D, Uchiyama H, Freeman GJ, Anderson KC. CD40 ligand triggered interleukin-6 secretion in multiple myeloma. *Blood* **1995**; 7: 1903
29. Tai YT, Podar K, Mitsiades N, Lin B, Mitsiades C, Gupta D, et al. CD40 induces human multiple myeloma cell migration via phosphatidylinositol 3-kinase/AKT/NF-kappa B signaling. *Blood* **2003**; 7: 2762-2769
30. Tai YT, Li XF, Breitkreutz I, Song W, Neri P, Catley L, et al. Role of B-cell-activating factor in adhesion and growth of human multiple myeloma cells in the bone marrow microenvironment. *Cancer Res* **2006**; 13: 6675-6682
31. Paiva B, Paino T, Sayagues JM, Garayoa M, San-Segundo L, Martin M, et al. Detailed characterization of multiple myeloma circulating tumor cells shows unique phenotypic, cytogenetic, functional, and circadian distribution profile. *Blood* **2013**; 22: 3591-3598
32. Paiva B, Perez-Andres M, Vidriales MB, Almeida J, de las Heras N, Mateos MV, et al. Competition between clonal plasma cells and normal cells for potentially

overlapping bone marrow niches is associated with a progressively altered cellular distribution in MGUS vs myeloma. *Leukemia* **2011**; 4: 697-706

33. Luque R, Garcia-Trujillo JA, Camara C, Moreno A, Eiras P, Roy G, et al. CD106 and activated-CD29 are expressed on myelomatous bone marrow plasma cells and their downregulation is associated with tumour progression. *Br J Haematol* **2002**; 1: 70-78
34. Yang Y, Macleod V, Bendre M, Huang Y, Theus AM, Miao HQ, et al. Heparanase promotes the spontaneous metastasis of myeloma cells to bone. *Blood* **2005**; 3: 1303-1309
35. Pérez-Andrés M, Almeida J, Martín-Ayuso M, Moro MJ, Martín-Nuñez G, Galende J, et al. Clonal plasma cells from monoclonal gammopathy of undetermined significance, multiple myeloma and plasma cell leukemia show different expression profiles of molecules involved in the interaction with the immunological bone marrow microenvironment. *Leukemia* **2005**; 449
36. Keith B, Simon MC. Hypoxia-inducible factors, stem cells, and cancer. *Cell* **2007**; 3: 465-472
37. Azab AK, Hu J, Quang P, Azab F, Pitsillides CM, Awwad R, et al. Hypoxia promotes dissemination of multiple myeloma through acquisition of epithelial to mesenchymal transition-like features. *Blood* **2012**; 24: 5782-5794
38. Storti P, Bolzoni M, Donofrio G, Airoldi I, Guasco D, Toscani D, et al. Hypoxia-inducible factor (HIF)-1alpha suppression in myeloma cells blocks tumoral growth in vivo inhibiting angiogenesis and bone destruction. *Leukemia* **2013**; 8: 1697-1706
39. Hu J, Handisides DR, Van Valckenborgh E, De Raeve H, Menu E, Vande Broek I, et al. Targeting the multiple myeloma hypoxic niche with TH-302, a hypoxia-activated prodrug. *Blood* **2010**; 9: 1524-1527
40. Kawano Y, Kikukawa Y, Fujiwara S, Wada N, Okuno Y, Mitsuya H, et al. Hypoxia reduces CD138 expression and induces an immature and stem cell-like transcriptional program in myeloma cells. *Int J Oncol* **2013**; 6: 1809-1816
41. Martin SK, Diamond P, Williams SA, To LB, Peet DJ, Fujii N, et al. Hypoxia-inducible factor-2 is a novel regulator of aberrant CXCL12 expression in multiple myeloma plasma cells. *Haematologica* **2010**; 5: 776-784
42. Vanderkerken K, Vande Broek I, Eizirik DL, Van Valckenborgh E, Asosingh K, Van Riet I, et al. Monocyte chemoattractant protein-1 (MCP-1), secreted by bone

marrow endothelial cells, induces chemoattraction of 5T multiple myeloma cells. *Clinical & Experimental Metastasis* **2002**; 1: 87-90

43. Johrer K, Janke K, Krugmann J, Fiegl M, Greil R. Transendothelial migration of myeloma cells is increased by tumor necrosis factor (TNF)-alpha via TNF receptor 2 and autocrine up-regulation of MCP-1. *Clin Cancer Res* **2004**; 6: 1901-1910
44. Inoue S, Osmond DG. Basement membrane of mouse bone marrow sinusoids shows distinctive structure and proteoglycan composition: A high resolution ultrastructural study. *The Anatomical Record* **2001**; 3: 294-304
45. Barille S, Akhoundi C, Collette M, Mellerin MP, Rapp MJ, Harousseau JL, et al. Metalloproteinases in multiple myeloma: production of matrix metalloproteinase-9 (MMP-9), activation of proMMP-2, and induction of MMP-1 by myeloma cells. *Blood* **1997**; 4: 1649-1655
46. Asosingh K, Menu E, Van Valckenborgh E, Vande Broek I, Van Riet I, Van Camp B, et al. Mechanisms involved in the differential bone marrow homing of CD45 subsets in 5T murine models of myeloma. *Clinical & Experimental Metastasis* **2002**; 7: 583
47. Standal T, Hjorth-Hansen H, Rasmussen T, Dahl IM, Lenhoff S, Brenne AT, et al. Osteopontin is an adhesive factor for myeloma cells and is found in increased levels in plasma from patients with multiple myeloma. *Haematologica* **2004**; 2: 174-182
48. Caers J, Gunthert U, De Raeve H, Van Valckenborgh E, Menu E, Van Riet I, et al. The involvement of osteopontin and its receptors in multiple myeloma cell survival, migration and invasion in the murine 5T33MM model. *Br J Haematol* **2006**; 4: 469-477
49. Kunkel EJ, Butcher EC. Plasma-cell homing. *Nature Reviews Immunology* **2003**; 10: 822-829
50. Yago T, Shao B, Miner JJ, Yao L, Klopocki AG, Maeda K, et al. E-selectin engages PSGL-1 and CD44 through a common signaling pathway to induce integrin α_Lβ₂-mediated slow leukocyte rolling. *Blood* **2010**; 3: 485-494
51. Asosingh K, Günthert U, De Raeve H, Van Riet I, Van Camp B, Vanderkerken K. A unique pathway in the homing of murine multiple myeloma cells: CD44v10 mediates binding to bone marrow endothelium. *Cancer research* **2001**; 7: 2862

52. Mrozik KM, Cheong CM, Hewett D, Chow AW, Blaschuk OW, Zannettino AC, et al. Therapeutic targeting of N-cadherin is an effective treatment for multiple myeloma. *Br J Haematol* **2015**; 3: 387-399

53. Groen RWJ, de Rooij MFM, Kocemba KA, Reijmers RM, de Haan-Kramer A, Overdijk MB, et al. N-cadherin-mediated interaction with multiple myeloma cells inhibits osteoblast differentiation. *Haematologica* **2011**; 11: 1653

54. Parmo-Cabañas M, Bartolomé RA, Wright N, Hidalgo A, Drager AM, Teixidó J. Integrin α4β1 involvement in stromal cell-derived factor-1α-promoted myeloma cell transendothelial migration and adhesion: role of cAMP and the actin cytoskeleton in adhesion. *Experimental Cell Research* **2004**; 2: 571-580

55. Martínez-Moreno M, Leiva M, Aguilera-Montilla N, Sevilla-Movilla S, Isern de Val S, Arellano-Sánchez N, et al. In vivo adhesion of malignant B cells to bone marrow microvasculature is regulated by α4β1 cytoplasmic-binding proteins. *Leukemia* **2016**; 4: 861-872

56. Runnels JM, Carlson AL, Pitsillides C, Thompson B, Wu J, Spencer JA, et al. Optical techniques for tracking multiple myeloma engraftment, growth, and response to therapy. *J Biomed Opt* **2011**; 1: 011006

57. Vande Broek I, Asosingh K, Allegaert V, Leleu X, Facon T, Vanderkerken K, et al. Bone marrow endothelial cells increase the invasiveness of human multiple myeloma cells through upregulation of MMP-9: evidence for a role of hepatocyte growth factor. *Leukemia* **2004**; 976

58. Menu E, Asosingh K, Indraccolo S, De Raeve H, Van Riet I, Van Valckenborgh E, et al. The involvement of stromal derived factor 1alpha in homing and progression of multiple myeloma in the 5TMM model. *Haematologica* **2006**; 5: 605-612

59. Vande Broek I, Vanderkerken K, Van Camp B, Van Riet I. Extravasation and homing mechanisms in multiple myeloma. *Clinical & Experimental Metastasis* **2008**; 4: 325-334

60. Alsayed Y, Ngo H, Runnels J, Leleu X, Singha UK, Pitsillides CM, et al. Mechanisms of regulation of CXCR4/SDF-1 (CXCL12)-dependent migration and homing in multiple myeloma. *Blood* **2007**; 7: 2708-2717

61. Vanderkerken K, Greef CD, Asosingh K, Arteta B, Veerman MD, Broek IV, et al. Selective initial in vivo homing pattern of 5T2 multiple myeloma cells in the C57BL/KalwRij mouse. *British Journal of Cancer* **2000**; 4: 953-959

62. Roccaro AM, Mishima Y, Sacco A, Moschetta M, Tai YT, Shi J, et al. CXCR4 Regulates Extra-Medullary Myeloma through Epithelial-Mesenchymal-Transition-like Transcriptional Activation. *Cell Reports* **2015**; 4: 622-635
63. Sipkins DA, Wei X, Wu JW, Runnels JM, Cote D, Means TK, et al. In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. *Nature* **2005**; 7044: 969-973
64. Hewett DR, Vandyke K, Lawrence DM, Friend N, Noll JE, Geoghegan JM, et al. DNA Barcoding Reveals Habitual Clonal Dominance of Myeloma Plasma Cells in the Bone Marrow Microenvironment. *Neoplasia* **2017**; 12: 972-981
65. Mishima Y, Moschetta M, Shi J, Mercier F, Manier S, Glavey S, et al. Clonal-Heterogeneity and Propensity for Bone Metastasis in Multiple Myeloma. *Blood* **2014**; 21: 3370-3370
66. Lawson MA, McDonald MM, Kovacic N, Hua Khoo W, Terry RL, Down J, et al. Osteoclasts control reactivation of dormant myeloma cells by remodelling the endosteal niche. *Nature Communications* **2015**; 8983
67. Khoo WH, Ledergor G, Weiner A, Roden DL, Terry RL, McDonald MM, et al. A niche-dependent myeloid transcriptome signature defines dormant myeloma cells. *Blood* **2019**; 1: 30-43
68. Tokoyoda K, Egawa T, Sugiyama T, Choi BI, Nagasawa T. Cellular niches controlling B lymphocyte behavior within bone marrow during development. *Immunity* **2004**; 6: 707-718
69. Nie Y, Waite J, Brewer F, Sunshine MJ, Littman DR, Zou YR. The role of CXCR4 in maintaining peripheral B cell compartments and humoral immunity. *The Journal of Experimental Medicine* **2004**; 9: 1145-1156
70. Moller C, Stromberg T, Juremalm M, Nilsson K, Nilsson G. Expression and function of chemokine receptors in human multiple myeloma. *Leukemia* **2003**; 1: 203-210
71. Roccaro AM, Sacco A, Purschke WG, Moschetta M, Buchner K, Maasch C, et al. SDF-1 inhibition targets the bone marrow niche for cancer therapy. *Cell Rep* **2014**; 1: 118-128
72. Ro TB, Holien T, Fagerli UM, Hov H, Misund K, Waage A, et al. HGF and IGF-1 synergize with SDF-1alpha in promoting migration of myeloma cells by cooperative activation of p21-activated kinase. *Exp Hematol* **2013**; 7: 646-655

73. Asosingh K, Gunthert U, Bakkus MH, De Raeve H, Goes E, Van Riet I, et al. In vivo induction of insulin-like growth factor-I receptor and CD44v6 confers homing and adhesion to murine multiple myeloma cells. *Cancer Res* **2000**; 11: 3096-3104
74. Opperman KS, Vandyke K, Clark KC, Coulter EA, Hewett DR, Mrozik KM, et al. Clodronate-Liposome Mediated Macrophage Depletion Abrogates Multiple Myeloma Tumor Establishment In Vivo. *Neoplasia* **2019**; 8: 777-787
75. Moreaux J, Hose D, Kassambara A, Reme T, Moine P, Requirand G, et al. Osteoclast-gene expression profiling reveals osteoclast-derived CCR2 chemokines promoting myeloma cell migration. *Blood* **2011**; 4: 1280-1290
76. Vande Broek I, Asosingh K, Vanderkerken K, Straetmans N, Van Camp B, Van Riet I. Chemokine receptor CCR2 is expressed by human multiple myeloma cells and mediates migration to bone marrow stromal cell-produced monocyte chemotactic proteins MCP-1, -2 and -3. *Br J Cancer* **2003**; 6: 855-862
77. An G, Xu Y, Shi L, Zou D, Deng S, Sui W, et al. t(11;14) multiple myeloma: a subtype associated with distinct immunological features, immunophenotypic characteristics but divergent outcome. *Leuk Res* **2013**; 10: 1251-1257
78. Avet-Loiseau H, Malard F, Campion L, Magrangeas F, Sebban C, Lioure B, et al. Translocation t(14;16) and multiple myeloma: is it really an independent prognostic factor? *Blood* **2011**; 6: 2009-2011
79. Chakraborty R, Muchtar E, Kumar SK, Jevremovic D, Buadi FK, Dingli D, et al. Risk stratification in myeloma by detection of circulating plasma cells prior to autologous stem cell transplantation in the novel agent era. *Blood Cancer Journal* **2016**; 12: e512
80. Avet-Loiseau H, Gerson F, Magrangeas F, Minvielle S, Harousseau JL, Bataille R. Rearrangements of the c-myc oncogene are present in 15% of primary human multiple myeloma tumors. *Blood* **2001**; 10: 3082-3086
81. Hurt EM, Wiestner A, Rosenwald A, Shaffer AL, Campo E, Grogan T, et al. Overexpression of c-maf is a frequent oncogenic event in multiple myeloma that promotes proliferation and pathological interactions with bone marrow stroma. *Cancer Cell* **2004**; 2: 191-199
82. Zhan F, Huang Y, Colla S, Stewart JP, Hanamura I, Gupta S, et al. The molecular classification of multiple myeloma. *Blood* **2006**; 6: 2020-2028

83. Neri P, Ren L, Azab AK, Brentnall M, Gratton K, Klimowicz AC, et al. Integrin β7-mediated regulation of multiple myeloma cell adhesion, migration, and invasion. *Blood* **2011**; 23: 6202

84. Bazan JF, Bacon KB, Hardiman G, Wang W, Soo K, Rossi D, et al. A new class of membrane-bound chemokine with a CX3C motif. *Nature* **1997**; 6617: 640-644

85. Nishimura M, Umehara H, Nakayama T, Yoneda O, Hieshima K, Kakizaki M, et al. Dual Functions of Fractalkine/CX3C Ligand 1 in Trafficking of Perforin⁺/Granzyme B⁺ Cytotoxic Effector Lymphocytes That Are Defined by CX3CR1 Expression. *The Journal of Immunology* **2002**; 12: 6173

86. Cederblad L, Rosengren B, Ryberg E, Hermansson N-O. AZD8797 is an allosteric non-competitive modulator of the human CX3CR1 receptor. *Biochemical Journal* **2016**; 5: 641

87. Chang H, Qi X, Yeung J, Reece D, Xu W, Patterson B. Genetic aberrations including chromosome 1 abnormalities and clinical features of plasma cell leukemia. *Leuk Res* **2009**; 2: 259-262

88. Katodritou E, Gastari V, Verrou E, Hadjiaggelidou C, Varthaliti M, Georgiadou S, et al. Extramedullary (EMP) relapse in unusual locations in multiple myeloma: Is there an association with precedent thalidomide administration and a correlation of special biological features with treatment and outcome? *Leukemia Research* **2009**; 8: 1137-1140

89. Cheong CM, Mrozik KM, Hewett DR, Bell E, Panagopoulos V, Noll JE, et al. Twist-1 is upregulated by NSD2 and contributes to tumour dissemination and an epithelial-mesenchymal transition-like gene expression signature in t(4;14)-positive multiple myeloma. *Cancer Letters* **2020**;

90. Bataille R, Robillard N, Avet-Loiseau H, Harousseau JL, Moreau P. CD221 (IGF-1R) is aberrantly expressed in multiple myeloma, in relation to disease severity. *Haematologica* **2005**; 5: 706-707

91. Lohr JG, Kim S, Gould J, Knoechel B, Drier Y, Cotton MJ, et al. Genetic interrogation of circulating multiple myeloma cells at single-cell resolution. *Science Translational Medicine* **2016**; 363: 363ra147

92. Mishima Y, Paiva B, Shi J, Park J, Manier S, Takagi S, et al. The Mutational Landscape of Circulating Tumor Cells in Multiple Myeloma. *Cell Rep* **2017**; 1: 218-224

93. Manier S, Park J, Capelletti M, Bustoros M, Freeman SS, Ha G, et al. Whole-exome sequencing of cell-free DNA and circulating tumor cells in multiple myeloma. *Nature Communications* **2018**; 1: 1691

94. Rasche L, Chavan SS, Stephens OW, Patel PH, Tytarenko R, Ashby C, et al. Spatial genomic heterogeneity in multiple myeloma revealed by multi-region sequencing. *Nat Commun* **2017**; 1: 268

95. Avet-Loiseau H, Attal M, Moreau P, Charbonnel C, Garban F, Hulin C, et al. Genetic abnormalities and survival in multiple myeloma: the experience of the Intergroupe Francophone du Myelome. *Blood* **2007**; 8: 3489-3495

96. Lode L, Eveillard M, Trichet V, Soussi T, Wuilleme S, Richebourg S, et al. Mutations in TP53 are exclusively associated with del(17p) in multiple myeloma. *Haematologica* **2010**; 11: 1973-1976

97. Yue Z, Zhou Y, Zhao P, Chen Y, Yuan Y, Jing Y, et al. p53 Deletion promotes myeloma cells invasion by upregulating miR19a/CXCR5. *Leukemia Research* **2017**; 115-122

98. Palumbo A, Avet-Loiseau H, Oliva S, Lokhorst HM, Goldschmidt H, Rosinol L, et al. Revised International Staging System for Multiple Myeloma: A Report From International Myeloma Working Group. *J Clin Oncol* **2015**; 26: 2863-2869

99. Peceliunas V, Janiulioniene A, Matuzeviciene R, Zvirblis T, Griskevicius L. Circulating plasma cells predict the outcome of relapsed or refractory multiple myeloma. *Leukemia & Lymphoma* **2012**; 4: 641-647

100. Keats JJ, Chesi M, Egan JB, Garbitt VM, Palmer SE, Braggio E, et al. Clonal competition with alternating dominance in multiple myeloma. *Blood* **2012**; blood-2012-2001-405985

Chapter 2:

Expression of the chemokine receptor CCR1 promotes dissemination of multiple myeloma plasma cells *in vivo*

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Expression of the chemokine receptor CCR1 promotes the dissemination of multiple myeloma plasma cells *in vivo*

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Running title:

CCR1 drives dissemination of multiple myeloma plasma cells

2.1. Abstract

Multiple myeloma (MM) disease progression is dependent on the ability of MM plasma cells (PCs) to egress from the bone marrow (BM), enter the peripheral blood and disseminate to distal BM sites. Expression of the chemokine CXCL12 by BM stromal cells is crucial for MM PC retention within the BM. However, the mechanisms which overcome CXCL12-mediated retention to enable dissemination are poorly understood. We have previously identified that treatment with the CCR1 ligand CCL3 inhibits the response to CXCL12 in MM cell lines, suggesting that CCL3/CCR1 signalling may enable egress of MM PC from the BM. Here, we determined whether CCR1 is a crucial driver of MM PC dissemination *in vivo.* Initially, we demonstrated that expression of CCR1 in the murine MM cell line 5TGM1 led to an increased incidence bone and splenic disseminated tumours following intratibial 5TGM1 cell injection in C57BL/KaLwRij mice. Furthermore, we demonstrated that CCR1 knockout in the human myeloma cell line OPM2 resulted in a >95% reduction in circulating MM PC numbers and BM and splenic tumour dissemination following intratibial injection in NSG mice. Therapeutic targeting of CCR1 with the inhibitor CCX9588 significantly reduced OPM2 or RPMI-8226 MM cell dissemination in intratibial xenograft models. Collectively, our findings suggest a novel role for CCR1 as a critical driver of BM egress of MM PCs during tumour dissemination. Furthermore, these data suggest that CCR1 may represent a potential therapeutic target for the prevention of MM tumour dissemination.

2.2. Introduction

Multiple myeloma (MM) is an incurable haematological cancer characterised by the uncontrolled proliferation of clonal plasma cells (PCs) within the bone marrow (BM).[1] One of the key features of MM is the presence of MM PCs at multiple sites throughout the BM, highlighting that dissemination of the transformed PC is a critical process during disease development.[1,2] In support of this, circulating MM PCs are detectable by flow cytometry in approximately 75% of newly diagnosed MM patients.[3] Importantly, the presence of elevated circulating MM PCs predicting faster time to progression and poorer overall survival, independent of BM tumour burden.[4-12]

The dissemination of MM PCs is a multi-step process requiring release from the supportive niche in the BM, intravasation into nearby blood vessels and subsequent extravasation and homing to a distal BM site. Integrin mediated adhesion of MM PCs to BM stromal cells (BMSCs), and extracellular matrix (ECM) components synthesised by BMSCs, is well-established to mediate retention of MM PCs within the niche.[13] For example, MM PCs express the integrin α4β1 (also known as very late antigen 4, VLA-4) that mediates adhesion to vascular cell–adhesion molecule 1 (VCAM-1) on BMSCs and to the ECM component fibronectin.[13] Importantly, the C-X-C chemokine ligand CXCL12 (also known as stromal cell-derived factor-1; SDF-1), abundantly produced by BMSCs,[14] enhances adhesion to fibronectin and VCAM-1 through binding to its receptor CXCR4 on the surface of MM PCs and inducing rapid conformational changes of the integrin α4β1 complex on MM PCs.[15] Notably, plerixafor-mediated inhibition of the CXCL12 receptor CXCR4 on MM PCs results in mobilization of MM cells to the peripheral blood (PB) in a preclinical model of MM.[15] These data suggest that CXCL12 is a critical BM retention signal for MM PCs and that overcoming the CXCL12/CXCR4 signal may be required for release from the niche during dissemination.

In a previous study by Azab and colleagues, increased hypoxia in the BM was shown to be associated with an increase in circulating MM PCs in a preclinical model.[16] Additionally, we have previously identified that overexpression of the hypoxia-inducible factor 2 alpha (HIF-2α) in MM cell lines reduces their response to exogenous CXCL12 *in vitro*, suggesting that hypoxia may overcome CXCL12-mediated retention. Furthermore, we identified that hypoxia and HIF-2α increased expression of the C-C chemokine receptor CCR1 in human MM cell lines (HMCLs).[17] CCR1 is a seven-transmembrane G-protein

coupled receptor and its most potent activator is CCL3 (also known as macrophage inflammatory protein 1 alpha; MIP-1α). Previous literature suggests that MM PCs abundantly produce CCL3.[18-21] which activates CCR1, expressed on osteoclasts, leading to increased osteolysis,[19] with inhibition of CCR1 antagonists reducing osteolysis in a murine model of MM.[22,23] In addition, CCL3 has been shown to be a potent inducer of migration of patient-derived MM PCs and MM cell lines *in vitro*.[17,19,20,24] Furthermore, in haematopoietic progenitors and natural killer (NK) cells, CCL3/CCR1 signalling drives mobilisation from the BM, in part by inactivation of CXCL12/CXCR4.[25,26] Similarly, our previous studies showed that either pre-treatment of MM cell lines with CCL3 or elevated CCR1 expression decreased tumour cell migration towards CXCL12 *in vitro.*[17] Taken together, these data suggest that hypoxia-mediated increases in CCR1 expression may desensitise cells to CXCL12-mediated BM retention and thereby facilitate dissemination. In support of this, we have previously shown that expression of CCR1 in MM PCs is associated with poorer prognosis and an increase in the number of circulating MM PCs in newly diagnosed MM patients.[17] However, a role for CCR1 in the dissemination of MM PCs *in vivo* is yet to be elucidated. Here we determined whether CCR1 overexpression can promote tumour dissemination in the syngeneic 5TGM1/KaLwRij murine model of MM. Furthermore, using xenograft models of MM, we assessed whether CCR1 knockout limits the dissemination of MM PCs *in vivo*. Lastly, we determined using MM xenograft models whether pharmacological inhibition of CCR1 can be used as a viable therapeutic strategy to limit MM PC dissemination.

2.3. Methods

2.3.1. Reagents

All reagents were sourced from Sigma-Aldrich (St Louis, MO) unless otherwise stated. Recombinant human (rh)CCL3 was sourced from R&D Systems (Minneapolis, MN). The small molecule CCR1 inhibitor CCX9588 was provided by ChemoCentryx (Mountain View, CA). For *in vitro* assays, CCX9588 was prepared at 2mM stock concentrations in DMSO and was stored at room temperature until use. Final DMSO concentration in all treatment media was 0.01%. For *in vivo* experiments, CCX9588 was prepared at 7.5mg/mL stock concentration in polyethylene glycol (PEG) vehicle and stored at room temperature until use.

2.3.2. Cell culture

All media were supplemented with 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin, 1mM sodium pyruvate and 10mM HEPES buffer, unless otherwise specified. The mouse MM cell line 5TGM1-luc (expressing a dual GFP and luciferase reporter construct)[27] was maintained in Iscove's modified Dulbecco's medium (IMDM) with 20% foetal calf serum (FCS; HyClone, QLD, Australia) and supplements. HMCLs RPMI-8226-luc (expressing GFP/luciferase)[28] and OPM2 were maintained in Roswell Park Memorial Institute Medium 1640 (RPMI-1640) with 10% FCS and supplements. All cell lines were cultured in a humidified environment with 5% carbon dioxide at 37°C.

2.3.3. Generation of 5TGM1 CCR1-expressing cell line

5TGM1-luc genomic DNA was isolated using a DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) and used for amplification of the murine *Ccr1* gene by a nested polymerase chain reaction (PCR) using Phusion high-fidelity polymerase (New England Biolabs, Ipswich, MA). Primers in the first reaction were designed to flank the *Ccr1* coding sequence with a 5' BamHI restriction site and the beginning of the human influenza hemagglutinin (HA)-tag on the 3' end (nucleotide sequence of HA-tag: TATCCTTATGATGTTCCTGATTATGCT) (Fwd 5'-GACCGGATCCTCAGCCCACCATGGAGATTTCAGAT-3'; Rev 5'-TCAGGAACATCATAAGGATAGAAGCCAGCAGAGAGCTCAT-3'). In the second reaction, the same forward primer was used, with the reverse primer designed to overlap the first reverse primer to complete the HA-tag and add a 3' NotI restriction site (Rev 5'-TTGTGCGGCCGCCTAAGCATAATCAGGAACATCATAAGGATA-3'). The HA-tag

was used for protein expression confirmation by immunoprecipitation with an anti-HA-tag antibody (Catalogue number 05-904, Sigma Aldrich), as previously described,[27] and the restriction sites enabled cloning into pLeGOiCer2 lentiviral vector[29] (gift from Boris Fehse; Addgene #27346). 5TGM1-luc cells were infected with lentivirus as previously described.[30] Briefly, for lentivirus production, HEK293T cells were transfected with pLeGOiCer2 or pLeGOiCer2-CCR1 (4μg), and packaging plasmids psPAX2 (4μg; gift from Didier Trono (Swiss Federal Institute of Technology Lausanne, unpublished); Addgene #12260) and pHCMV-EcoEnV[31] (4μg; gift from Miguel Sena-Esteves; Addgene #15802) using Lipofectamine-2000 (ThermoFisher). Lentiviral-supernatant was collected after 48 hours and 5TGM1-luc cells were infected with supernatant supplemented with 8μg/mL polybrene (Millipore, Burlington, MA). $GFP^{+}Cerulean^{+}$ cells were sorted by FACS using a FACSAria™ Fusion flow cytometer (BD Biosciences, San Jose, CA) to generate a CCR1-expressing (5TGM1-CCR1) or EV control (5TGM1-EV) cell line.

2.3.4. Generation of OPM2 CCR1-knockout cell lines

CCR1 knockout (KO) cell lines were generated using a lentiviral two-vector CRISPR-Cas9 system consisting of a Cas9 constitutive expression vector with an mCherry reporter (FuCas9Cherry; gift from Marco Herold, Addgene plasmid #7018)[32] and a doxycycline-inducible sgRNA expression vector with an eGFP reporter (FgHtUTG; gift from Marco Herold, Addgene plasmid #70183) [32] An mPlum FgH1tUTP vector was generated by digestion of the FgH1tUTG vector with BlpI and ClaI to excise the egfp gene, which was replaced with a synthesised mplum gBlock gene fragment (Integrated DNA Technologies, Newark, NJ) using isothermal assembly. Two CRISPR strategies were used (Supplementary Figure 2.1). The MIT CRISPR design software was used for the design of the sgRNAs (http://crispr.mit.edu). sgRNAs were designed to either flank the human CCR1 coding exon to delete the exon (sgRNA-A and sgRNA-B), or to target key tyrosine residues located in the ligand-binding domain (sgRNA-C). For cloning of individual sgRNAs, 24-bp oligonucleotides were synthesised (Sigma-Aldrich) including the sgRNAs sequences (gRNA-A 5'-GTTAGACTAAGATTCCTAGA-3'; gRNA-B 5'-GAGGGAATGTAATGGTGGCC-3'; gRNA-C 5'-GCCATGTGTAAGATCCTCTC-3') and 4-bp overhang for the forward (TCCC) and reverse (AAAC) oligonucleotides to enable cloning into the BsmbI site of the FgHtUT vectors.

OPM2 cells were first infected with FuCas9mCherry and FACS sorted for mCherry$^+$ cells (OPM2-Cas9) using a FACSAria™ Fusion flow cytometer. To generate empty vector control lines, OPM2-Cas9 cells were infected with the FgH1tUTG or FgH1tUTP vectors, alone, were sorted for mCherry$^+$GFP$^+$ (OPM2-EV-1) or mCherry$^+$mPlum+ (OPM2-EV-2) cells, respectively. To generate gRNA-expressing cells, OPM2-Cas9 cells were either co-infected with the FgH1TUTG-sgRNA-A and FgH1TUTG-sgRNA-B vectors or were infected with the FgH1TUTP-gRNA-C vector alone.

For isolation of the clonal OPM2-CCR1-KO-1 cell line, OPM2-Cas9 cells transfected with FgH1TUTG-sgRNA-A and FgH1TUTG-sgRNA-B vectors were sorted into 96-well plates using a FACSAria™ Fusion flow cytometer for single cell analysis. Clones were subsequently screened to identify CCR1-negative clones. Briefly, 1x105 cells per test were stained with an anti-CCR1 mouse monoclonal antibody (clone 53504; R&D Systems) or an in-house IgG2B isotype control antibody (1A6.11), followed by a goat anti-mouse biotinylated secondary antibody (Southern Biotech, Birmingham, AL), followed by a BV421-conjugated streptavidin tertiary antibody (BD Biosciences, North Ryde, Australia). Cells were analysed using a LSRFortessa flow cytometer (BD Biosciences). Genomic DNA from the CCR1-negative clones was isolated using a DNeasy kit and 35 cycles of PCR was conducted using primers designed to flank the first coding exon of human CCR1 (Fwd 5'-TGGGGTTGACCTACTAGGATT-3'; Rev 5'-TCGCTGCAATAAAGCCATTAG-3') and the products subjected to agarose gel electrophoresis to identify whole exon deletions (Supp. Figure 2.1B). Gel purified PCR products were subjected to Sanger sequencing (AGRF). This identified a clonal homozygous KO CCR1 cell line (OPM2-CCR1-KO-1) (Supp. Figure 2.1C).

For isolation of the non-clonal OPM2-KO-2 cell line, OPM2-Cas9 cells transfected with FgH1tUTP-gRNA-C vector were stained with antibodies against CCR1, as described above, and sorted on the basis of mPlum$^+$mCherry$^+$BV421$^-$ to isolate a CCR1-knockout population (OPM2-CCR1-KO-2). Mutagenesis was confirmed in the OPM2-CCR1-KO-2 line by conducting 35 cycles of PCR using primers flanking the CCR1 ligand binding domain (Fwd 5'-GCCTTTAGTAGCAGAGTAAAGACA-3'; Rev 5'-CCAGCCCAAAGAGGTTCAGTT-3'). Reannealed PCR products were subjected to heteroduplex analysis by resolution on a 1xTBE polyacrylamide gel (Supp. Figure 2.1E).

2.3.5. Quantitative real time PCR (qPCR)

Total RNA was isolated using TRIzol reagent (Thermofisher, Waltham, MA) and DNase treated using RQ1 DNase as per the manufacturer's instructions (Promega, Madison, WI). cDNA was synthesized using Superscript IV First-Strand Synthesis System (Thermofisher). Real-time qPCR was performed using a CFX Connect 9000 Real-Time PCR machine (BioRad, Hercules, CA) using primers for murine *Ccr1* (Fwd 5'-GTGGTGGGCAATGTCCTAGT-3'; Rev 5'-AGAAGCTTGCACATGGCATC-3') and *Actb* (Fwd 5' -GATCATTGCTCCTCCTGAGC-3'; Rev 5'-GTCATAGTCCG CCTAGAAGCAT-3'). Changes in gene expression were calculated relative to *Actb* using the $2^{-\Delta\Delta Ct}$ method.[33]

2.3.6. Transwell migration assays

For HMCLs, cells (1×10^5) were washed once in RPMI-1640 with 1% FCS and were seeded in 8μm transwells (Costar) in triplicate and cell migration towards rhCCL3 (100ng/mL) in RPMI-1640 with 1% FCS, or RPMI-1640 with 1% FCS alone (untreated controls) was assessed after 18 hours as previously described.[17] Where indicated, cells were treated with CCX9588 (100nM-1μM) or vehicle control for 24 hours, then washed once in RPMI-1640 with 1% FCS and resuspended in RPMI-1640 with 1% FCS containing CCX9588 prior to seeding into transwells. For the murine MM PC cell line 5TGM1, cells ($5x10^5$) were washed once in IMDM with 1% FCS and were seeded in transwells in IMDM with 1% FCS in triplicate and cell migration towards IMDM with 20% FCS and rhCCL3 (100ng/mL) or IMDM and 20% FCS alone (untreated controls) was assessed after 24 hours using a luciferase assay, as previously described.[27] Percentage cell migration is represented as normalized to the untreated controls.

2.3.7. Proliferation assays

Cells were plated at $1x10^5$ cells/mL in triplicate in phenol red-free IMDM containing 20% FCS and supplements (5TGM1) or phenol red-free RPMI-1640 containing 10% FCS and supplements (OPM2-EV-1 and RPMI-8226-luc) with or without addition of rhCCL3 (100ng/mL) or CCX9588 (0.1nM-1μM) or vehicle, in a 96-well plate. Cell numbers were assessed over 72 hours using WST-1 reagent (Roche, Basel, Switzerland) as previously described.[34]

2.3.8. Western blotting

For CCL3 stimulation experiments, OPM2-EV-1 or RPMI-8226-luc cells were treated with CCX9588 (10nM-10μM) or vehicle (0.01% DMSO) for 24 hours in serum-free RPMI-1640 containing supplements. Cells were stimulated with rhCCL3 (100ng/mL) for 5 min and cell lysates were prepared as previously described.[35] Proteins (50μg) were resolved under reducing conditions on 10% SDS-PAGE gels and transferred to 0.45μm nitrocellulose membranes. Immunoblotting was performed with the following antibodies: phosphorylated Akt (Ser473), total Akt, phosphorylated Erk1/2, total Erk1/2 (Cell Signaling Technologies, Danvers, MA, USA; all at 1:1000) and Hsc70 as a loading control (Enzo, Farmingdale, NY, USA, 1:1000). Membranes were developed using Dylight™-680 or 800 conjugated secondary antibodies (1:20,000; Invitrogen, Carlsbad, CA) and visualised using the Odyssey Infrared Imaging system (LI-COR Bioscience, Lincoln, NE, USA).

2.3.9. C57BL/KaLwRij murine model of MM

C57BL.KaLwRijHsd (KaLwRij) mice were bred and housed at the South Australian Health and Medical Research Institute (SAHMRI) Bioresources facility. All animal studies were approved by and performed in accordance with the SAHMRI Animal Ethics Committee (ID #356). Five- to six-week-old female KaLwRij mice were inoculated into the left tibia with $1x10^5$ 5TGM1-CCR1 or control 5TGM1-EV cells in 10μL phosphate-buffered saline (PBS). After 3.5 weeks, mice were injected with firefly D-luciferin (150mg/kg, diluted in PBS, 100μL i.p.), anaesthetised and after 15 min PB was collected by cardiac puncture. Mice were then humanely killed, and spleens were dissected and immediately imaged using bioluminescent imaging (Xenogen IVIS 100; Perkin Elmer). Bioluminescence signal below background (2000 p/sec/cm^2/sr) was classified as not detectable. PB was subjected to red cell lysis as described previously[36] and resuspended in PFE prior to analysis of GFP$^+$ cells on a FACSCanto II flow cytometer (BD Biosciences). Injected tibiae, and tibiae and femora from the contralateral leg, were excised and BM was flushed with 10mL PBS + 2% FCS + 2mM EDTA (PFE) and resuspended in 1mL PFE for analysis of GFP$^+$ cells on a LSRFortessa flow cytometer. GFP$^+$ tumour cells of total viable mononuclear (parent) cells below 0.01% was defined as not detectable.

2.3.10. NSG murine model of MM

NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG)[37] mice were bred and housed at the SAHMRI Bioresources facility. All animal studies were approved by and performed in accordance

with the SAHMRI Animal Ethics Committee (ID #286). Female NSG mice (5-6 weeks old) were inoculated into the left tibia with $5x10^5$ OPM2-EV-1 or OPM2-CCR1-KO-1 cells in 10µl PBS. After 28 days, long bones and PB were isolated and analysed as described above. Spleens were excised, photographed and measured and were fixed in 10% neutral buffered formalin and paraffin embedded prior to immunohistochemistry analysis as described below.

For CCR1 inhibition studies with the CCR1 small-molecule inhibitor CCX9588, mice were treated twice daily (12-hour intervals) via oral gavage with either CCR1 antagonist CCX9588 (15mg/kg) or PEG vehicle alone commencing 3 or 14 days after tumour-cell injection until day 28 at experimental endpoint. Tumour burden, circulating tumour cells and soft tissue and bone dissemination was assessed at day 28, as described above. For mice treated from day 3 post-tumour cell injection, blood from terminal cardiac bleeds was also used for performing total blood counts using a HEMAVET 950 automated blood analyser (Drew Scientific, Miami Lakes, FL, USA), and serum analysis of trough (12 hours after final dose) CCX9588 levels by liquid chromatography/mass spectrometry at Chemocentryx (Mountain View, CA).

2.3.11. Immunohistochemistry

Tumour cells in paraffin-embedded sections (5µm) were immunostained using a goat polyclonal anti–GFP antibody (Rockland, Pottstown, PA), biotinylated anti-goat secondary antibody (Vector Laboratories, CA, USA) followed by incubation with streptavidin-HRP (Vector Laboratories) and DAB (Vector Laboratories) as previously described.[38] **2.3.12.**

2.3.12. Statistical Analyses

Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA). *In vitro* assays were analysed using unpaired t-test for comparisons between cell lines, two-way ANOVA with Sidak's multiple comparisons test for migration assays, or one-way ANOVA with Tukey's multiple comparisons test for WST-1 and viability assays. *In vivo* experiments were analysed using a Mann-Whitney U test for PB and BM tumour burden, one-way ANOVA with Tukey's multiple comparisons test for splenic size analysis, or a Fisher's exact test for incidence of dissemination.

of dissemination.

2.4. Results

2.4.1. Expression of CCR1 in the mouse MM cell line 5TGM1 does not affect proliferation *in vitro* and increases incidence of splenic and bone dissemination *in vivo*

Based on our previous study showing that hypoxic MM PCs upregulate expression of CCR1 and decrease their response to CXCL12 *in vitro*,[17] we hypothesised that hypoxia-mediated elevations in CCR1 expression drive the dissemination of MM PCs *in vivo*. Initially, we assessed whether constitutive expression of CCR1 affected the migration and dissemination of the mouse MM cell line 5TGM1 which does not express detectable CCR1 basally (Figure 2.1A), and exhibits low levels of spontaneous dissemination *in vivo*.[30] Expression of functional HA-tagged CCR1 was confirmed by qPCR and by immunoprecipitation/Western blotting (Figure 2.1A, B) and by the ability of the 5TGM1-CCR1 cells to migrate towards rhCCL3 in a transwell assay (percent increase in migration over controls: 44.2 ± 8.95% [mean ± SEM]; $p<0.01$; Figure 2.1C). Expression of CCR1 did not affect the proliferation of 5TGM1 cells, relative to EV controls, either basally ($p=0.63$; Figure 2.1D) or following addition of rhCCL3 ($p=0.99$; Figure 2.1E) over a 72-hour time course.

To investigate whether CCR1 expression increased the dissemination of 5TGM1 cells *in vivo*, 5TGM1-CCR1 or EV cells were intratibially injected into C57BL/KaLwRij mice. Primary tumour burden in the injected tibiae was not significantly different between animals injected with 5TGM1-CCR1 cells and mice injected with 5TGM1-EV cells ($p=0.82$; Figure 2.2A). Similarly, the numbers of circulating MM cells in the PB or the tumour burden in the contralateral leg were also not significantly different between groups ($p=0.62$ and $p=0.41$, respectively; Figure 2.2B, C). However, there was a significant increase in the number of mice with disseminated tumour cells in the 5TGM1-CCR1 group, with 8/11 mice (73%) in this group having detectable GFP^+ cells in the contralateral leg, compared with 4/11 mice (36%) injected with 5TGM1-EV cells ($p<0.0001$; Figure 2.2D). Furthermore, an increase in the incidence of dissemination to the spleen was also observed in the 5TGM1-CCR1 group, with 9/11 mice (82%) having tumour detectable in the spleen by bioluminescence imaging, compared with 4/8 mice (50%) in the 5TGM1-EV cohort ($p<0.0001$; Figure 2.2E, F). Collectively, these data suggest that expression of CCR1 increases dissemination of MM PC, without affecting primary tumour growth.

Figure 2.1. CCR1 expression in 5TGM1 murine MM cell line increases migration towards CCL3 but does not affect proliferation. A. Expression of murine *Ccr1* mRNA was confirmed in 5TGM1-CCR1 cells by real-time PCR. **B**. CCR1-HA protein expression in 5TGM1-CCR1 cells was confirmed by immunoprecipitation using an anti-HA antibody followed by Western blotting with anti-HA antibody. A representative of two independent experiments is shown. **C**. Migration of 5TGM1-CCR1 and empty vector control (EV) cells towards 100ng/mL rhCCL3 was assessed in a transwell assay after 24 hours. **D**. Relative number of 5TGM1-CCR1 and -EV cells was assessed over 72 hours by WST-1 assay. **E.** Relative number of 5TGM1-CCR1 and -EV cells was assessed by WST-1 assay following 72 hours of culture with or without addition of 100ng/mL rhCCL3. Graphs depict mean ± SEM of three biological replicates (**A**) or three or more independent experiments (**C**-**E**). **$p<0.01$, ****$p<0.0001$, unpaired t-test (**A**), two-way ANOVA with Sidak's multiple comparison test (**C**).

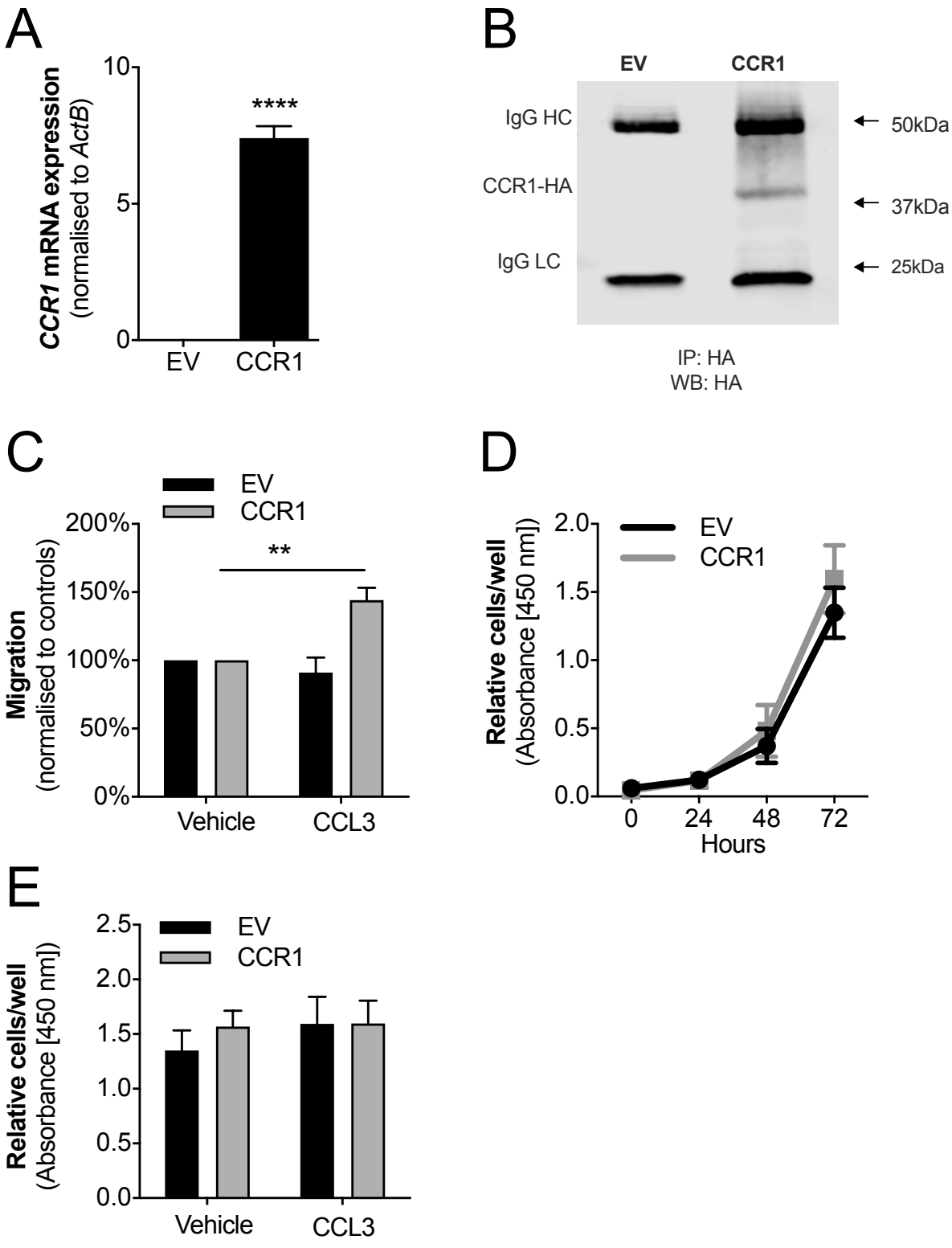
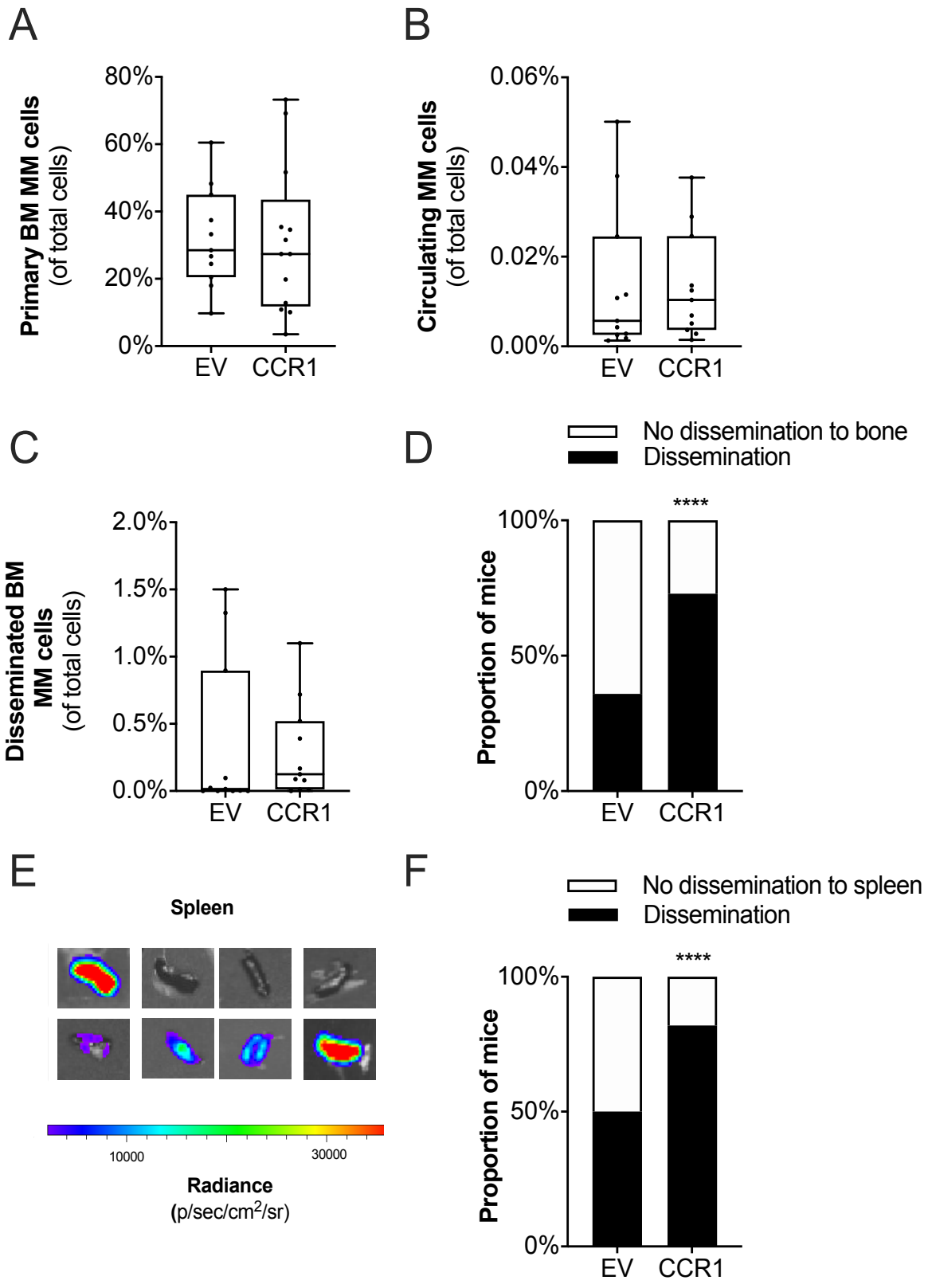

A
CCR1 mRNA expression
(normalised to ActB)
10
5
0
****
EV
CCR1
B
EV
CCR1
IgG HC
CCR1-HA
IgG LC
50kDa
37kDa
25kDa
IP: HA
WB: HA
C
EV
CCR1
Migration
(normalised to controls)
200%
150%
100%
50%
0%
**
Vehicle
CCL3
D
EV
CCR1
Relative cells/well
(Absorbance [450 nm])
2.0
1.5
1.0
0.5
0.0
0
24
48
72
Hours
E
EV
CCR1
Relative cells/well
(Absorbance [450 nm])
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
CCL3

Figure 2.2. CCR1 expression in 5TGM1 MM PCs increases incidence of bone and splenic dissemination in a C57BL/KaLwRij intratibial model of MM. A. Primary tumor burden in injected tibiae after 3.5 weeks in C57BL/KaLwRij mice injected with 5TGM1-CCR1 or control 5TGM1-EV cells. Percentage of GFP^+ MM cells of total mononuclear cells were quantitated using flow cytometry. **B**. Number of circulating 5TGM1-CCR1 or -EV cells in peripheral blood of mice, as assessed by flow cytometry. **C**. Tumor burden disseminated to the non-injected contralateral leg in mice injected with 5TGM1-CCR1 or -EV cells, as assessed by flow cytometry. **D**. Proportion of mice with detectable GFP^+ MM cells in the contralateral long bones, as assessed by flow cytometry. **E**. Spleens were collected from 8 mice (5TGM1-EV) and 11 mice (5TGM1-CCR1) and imaged using bioluminescence imaging, with representative spleens from each group shown. **F**. Proportion of mice with detectable bioluminescence signal in the spleen. Box and whisker plots depict median and interquartile range for 11 mice/group (**A**-**C**). **** $p < 0.0001$, Fisher's exact test.


A
Primary BM MM cells
(of total cells)
80%
60%
40%
20%
0%
EV
CCR1
B
Circulating MM cells
(of total cells)
0.06%
0.04%
0.02%
0.00%
EV
CCR1
C
Disseminated BM
MM cells
(of total cells)
2.0%
1.5%
1.0%
0.5%
0.0%
EV
CCR1
D
No dissemination to bone
Dissemination
****
Proportion of mice
100%
50%
0%
EV
CCR1
E
Spleen
10000
30000
Radiance
(p/sec/cm2/sr)
F
No dissemination to spleen
Dissemination
****
Proportion of mice
100%
50%
0%
EV
CCR1

2.4.2. Knockout of CCR1 in the HMCL OPM2 does not affect proliferation *in vitro* and prevents dissemination *in vivo.*

To further investigate the role of CCR1 in tumour dissemination in MM, we generated CRISPR/Cas9-mediated CCR1 knockouts in the HMCL OPM2 (Supplementary Figure 2.1). Loss of protein expression in OPM2-CCR1-KO-1 and OPM2-CCR1-KO-2 cell lines was confirmed by flow cytometry (Figure 2.3A). Furthermore, migration of OPM2-EV-1 and -2 cell lines towards rhCCL3 (OPM2-EV-1: 134.9 ± 14.5% increase in migration, $p<0.01$; OPM2-EV-2: 150.0 ± 20.6% increase in migration, $p<0.01$) was not observed in the OPM2-CCR1-KO cell lines (Figure 2.3B). Basal proliferation of OPM2 cell lines was unaffected by CCR1 knockout, as assessed over a 72-hour time-course (OPM2-KO-1: $p>0.99$, Figure 2.3C; OPM2-KO-2: $p=0.98$, Figure 2.3D). Furthermore, the addition of rhCCL3 did not affect proliferation of CCR1 KO or EV cell lines after 72 hours (OPM2-KO-1: $p=0.54$; OPM2-KO-2, $p=0.61$, Figure 2.3E).

To determine if CCR1 knockout limited MM PC dissemination *in vivo*, NSG mice were injected with either control (OPM2-EV-1) or CCR1 KO (OPM2-CCR1-KO-1) cells. Knockout of CCR1 reduced primary tumour burden by 45.5%, compared with mice inoculated with OPM2-EV-1 cells ($p<0.01$; OPM2-EV-1: 77.2 ± 7.60% of total parent population [mean ± SEM]; OPM2-KO-1: 42.1 ± 10.6%), after 4 weeks (Figure 2.4A). Circulating tumour cell numbers were significantly reduced by 97.8% in mice bearing CCR1 knockout cells compared with EV controls ($p<0.0001$; OPM2-EV-1:1.39 ± 0.305% of total parent population [mean ± SEM]; OPM2-KO-1: 0.0304 ± 0.0201%; Figure 2.4B). Additionally, dissemination of OPM2 cells from the primary tumour to the contralateral leg was observed in OPM2-EV-1 bearing mice but not in mice bearing OPM2-CCR1-KO-1 cells, with a 99.9% reduction in BM disseminated tumour cells ($p<0.0001$; OPM2-EV-1:49.5 ± 7.52% of total parent population [mean ± SEM]; OPM2-KO-1: 0.02 ± 0.005%; Figure 2.4C). Similar results were seen in the development of splenic disseminated tumours, with mice inoculated with OPM2-EV-1 cells developing splenomegaly (Figure 2.4D) from tumour cell infiltration, as confirmed by immunohistochemistry for GFP^+ cells (Figure 2.4E), which was markedly reduced in mice inoculated with OPM2-CCR1-KO-1 cells ($p<0.0001$; naïve controls: 1.07 ± 0.0413 cm in length [mean ± SEM]; OPM2-EV-1: 1.83 ± 0.0733; OPM2-KO-1: 1.01 ± 0.0410; Figure 2.4D).

Figure 2.3. Knockout of CCR1 in human OPM2 MM PCs decreases migration towards CCL3 and does not affect proliferation. A. CRISPR-Cas9-mediated knockout of CCR1 was confirmed in OPM2-CCR1-KO-1 and OPM2-CCR1-KO-2 cells by flow cytometry following staining with an anti-hCCR1 antibody or isotype control. **B**. Migration of OPM2-CCR1-KO-1 and OPM2-CCR1-KO-2 cells and EV control cells towards 100ng/mL rhCCL3, or media alone, was assessed in a transwell assay after 18 hours. Migration is expressed relative to no chemoattractant controls. **C**. Relative numbers of OPM2-CCR1-KO-1 or OPM2-EV-1 control cells, were assessed over 72 hours by WST-1 assay. **D**. Relative numbers of OPM2-CCR1-KO-2 or OPM2-EV-2 control cells, were assessed over 72 hours by WST-1 assay. **E**. The effect of 72 hours of treatment with 100ng/mL rhCCL3 on relative numbers of OPM2-CCR1-KO-1 and OPM2-CCR1-KO-2, and EV-1 and EV-2 control cells was assessed by WST-1. Graphs depict mean ± SEM of three or more independent experiments (**A**-**E**). **$p<0.001$, two-way ANOVA with Sidak's multiple comparison test.

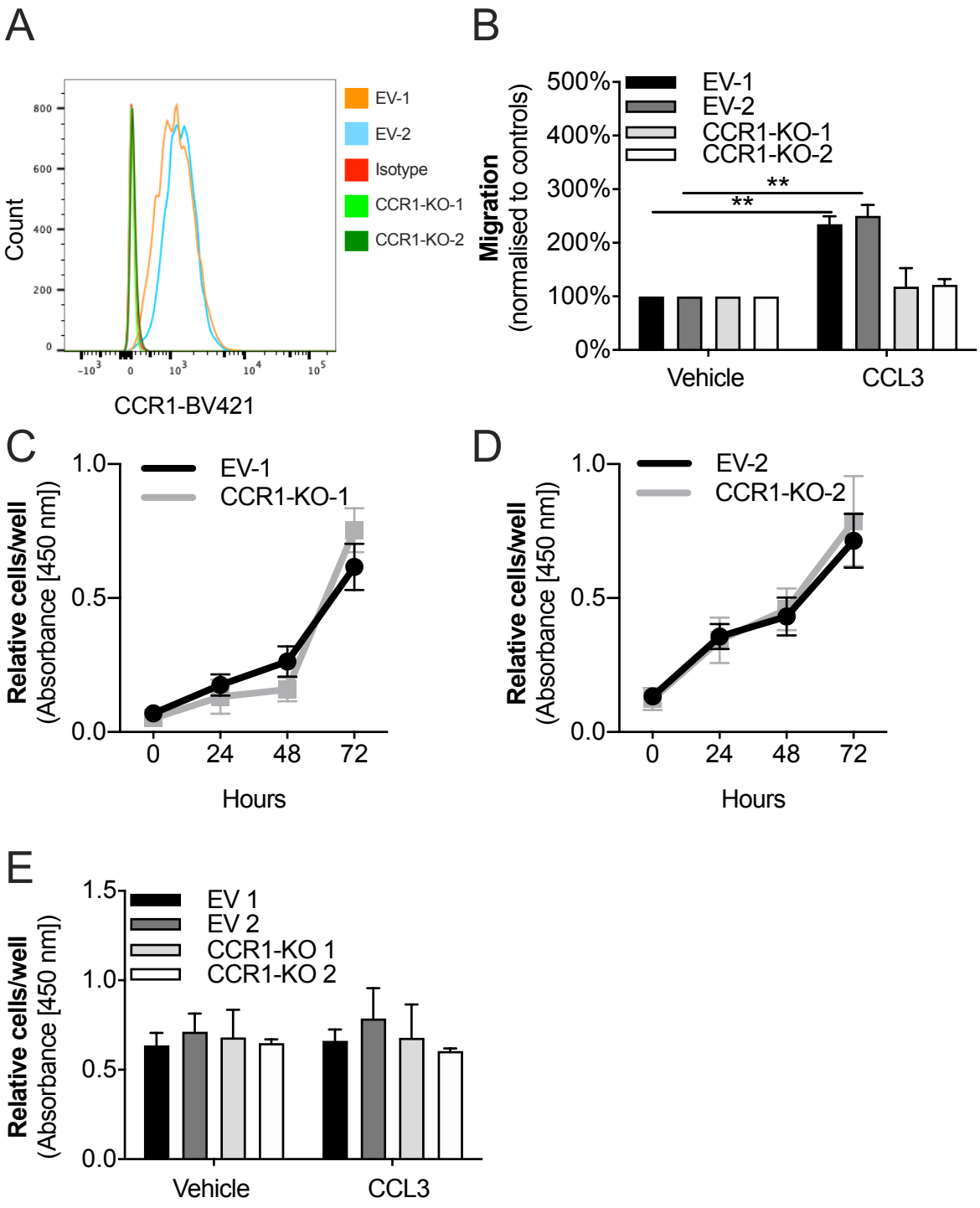
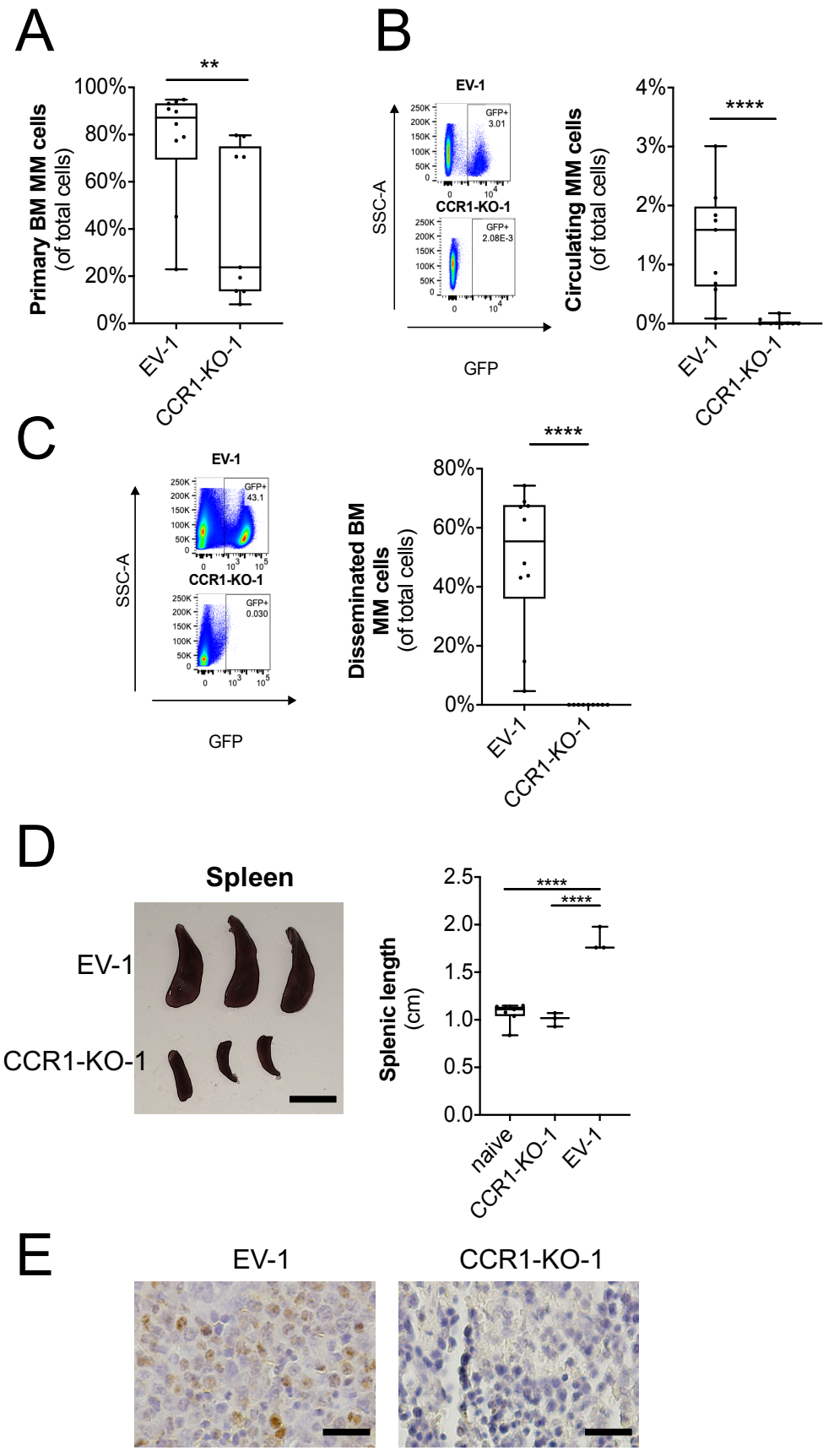

A
Count
800
600
400
200
0
EV-1
EV-2
Isotype
CCR1-KO-1
CCR1-KO-2
CCR1-BV421
B
Migration
(normalised to controls)
500%
400%
300%
200%
100%
0%
EV-1
EV-2
CCR1-KO-1
CCR1-KO-2
**
**
Vehicle
CCL3
C
Relative cells/well
(Absorbance [450 nm])
1.0
0.5
0.0
EV-1
CCR1-KO-1
0
24
48
72
Hours
D
Relative cells/well
(Absorbance [450 nm])
1.0
0.5
0.0
EV-2
CCR1-KO-2
0
24
48
72
Hours
E
Relative cells/well
(Absorbance [450 nm])
1.5
1.0
0.5
0.0
EV 1
EV 2
CCR1-KO 1
CCR1-KO 2
Vehicle
CCL3

Figure 2.4. Dissemination of human multiple myeloma cell line OPM2 in NSG mice is abrogated by knockout of CCR1. A. Primary tumor burden in injected tibiae after 4 weeks in NSG mice injected with OPM2-EV-1 or OPM2-KO-1 cells. **B**. Number of circulating OPM2-EV-1 or OPM2-KO-1 cells in peripheral blood of mice, as assessed by flow cytometry. **C**. Tumor burden disseminated to the non-injected contralateral leg in mice injected with OPM2-EV-1 or OPM2-KO-1 cells, as assessed by flow cytometry. **D**. Length of spleens collected from naïve NSG mice (n=7 mice) or mice bearing OPM2-EV-1 (n=3 mice) or OPM2-KO-1 (n=3 mice) cells were measured. Image of spleens of OPM2-EV-1- or OPM2-CCR1-KO-1-bearing mice. Scale bar: 10 mm **E**. Splenic tumor cell infiltration in mice bearing OPM2-EV-1 or OPM2-CCR1-KO-1 cells was confirmed by immunohistochemistry with an anti-GFP antibody (brown). Percentage of GFP^+ MM cells of total mononuclear cells were quantitated using flow cytometry (**A**-**C**). Representative flow plots of one mouse per group are shown (**B**-**C**). A representative of 5 mice/group is shown; scale bar: 20 μm (**E**). Box and whisker plots depict median and interquartile range, n = 9-10 mice/group. **p< 0.01, ****p< 0.0001, Mann-Whitney U test (**A**-**C**), ****p<0.0001, one-way ANOVA with Tukey's multiple comparisons test.


A
**
Primary BM MM cells
(of total cells)
100%
80%
60%
40%
20%
0%
EV-1
CCR1-KO-1
B
EV-1
GFP+
3.01
CCR1-KO-1
GFP+
2.08E-3
SSC-A
GFP
Circulating MM cells
(of total cells)
4%
3%
2%
1%
0%
****
EV-1
CCR1-KO-1
C
EV-1
GFP+
43.1
CCR1-KO-1
GFP+
0.030
SSC-A
GFP
****
Disseminated BM
MM cells
(of total cells)
80%
60%
40%
20%
0%
EV-1
CCR1-KO-1
D
Spleen
EV-1
CCR1-KO-1
****
****
Splenic length
(cm)
2.5
2.0
1.5
1.0
0.5
0.0
naive
CCR1-KO-1
EV-1
E
EV-1
CCR1-KO-1

2.4.3. The CCR1 inhibitor CCX9588 inhibits CCR1 downstream signalling and migration towards CCL3 *in vitro*

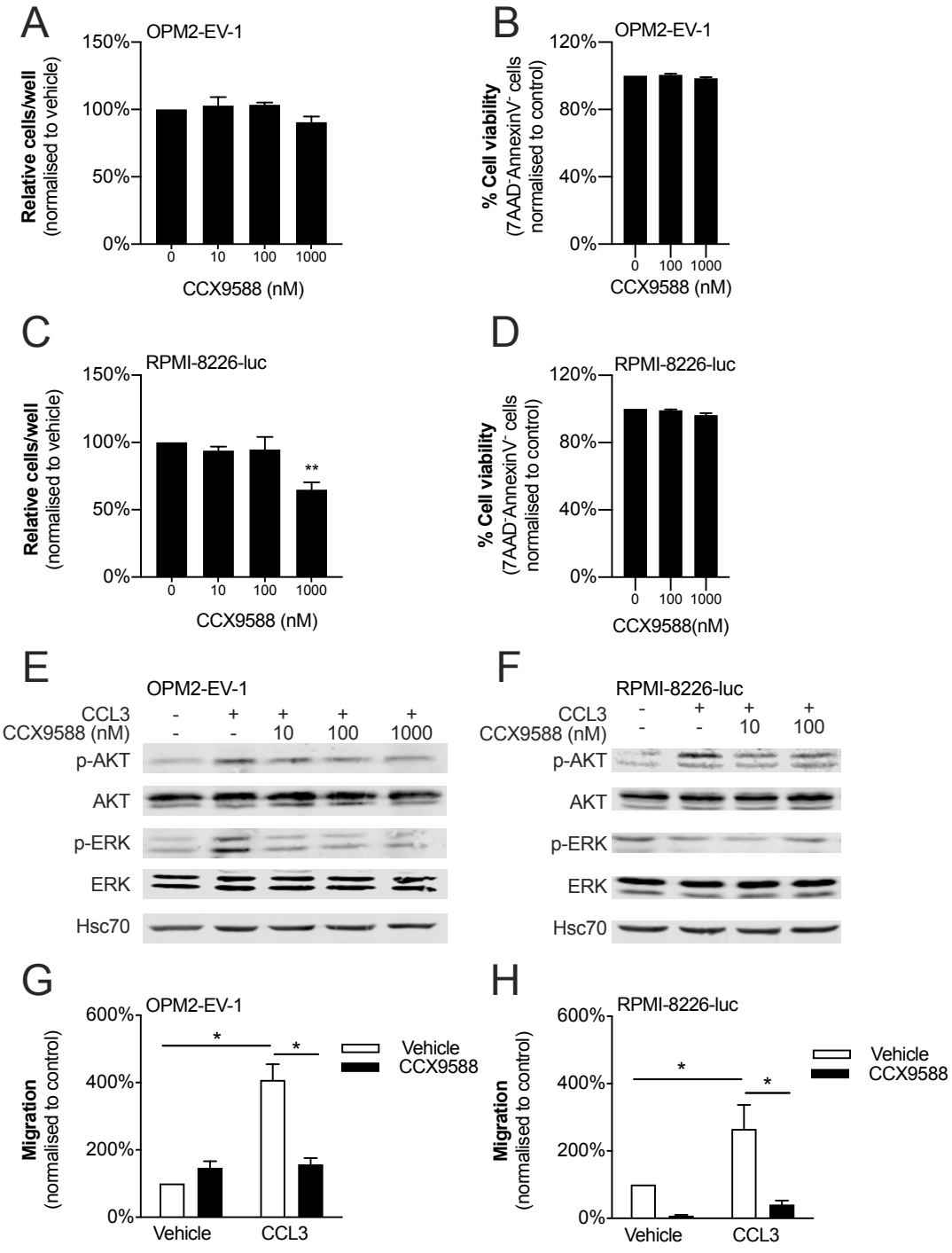
Next, the effects of a selective small molecule CCR1 inhibitor, CCX9588, on MM cells was assessed *in vitro*. To investigate whether the small molecule CCR1 inhibitor CCX9588 effects cell survival and/or proliferation, CCR1-expressing OPM2-EV-1 or RPMI-8226-luc[17] cells were cultured with increasing concentrations of CCX9588 or vehicle alone for 72 hours. Neither OPM2-EV-1 cell number ($p=0.88$, Figure 2.5A) or viability ($p=0.70$, Figure 2.5B) were affected by treatment with up to 1μM CCX9588. However, there was a 35% decrease in cell number in RPMI-8226-luc cells treated with 1μM CCX9588 ($p<0.01$, Figure 2.5C), while cell survival was unaffected by up to 1μM CCX9558 ($p=0.50$, Figure 2.5D), suggesting that this concentration may decrease cell proliferation of these cells. Based on these results, concentrations up to 100nM and 1μM were used for further characterisation in RPMI-8226-luc and OPM2 cells, respectively.

To investigate the anti-CCR1 function of CCX9588, OPM2-EV-1 or RPMI-8226-luc cells were treated with CCX9588 or vehicle alone in serum-free media for 24 hours and were then stimulated with rhCCL3 for 5 min prior to generating cell lysates. Western blot analysis showed that CCL3 treatment induced AKT and ERK1/2 phosphorylation in OPM2-EV-1 cells, which was inhibited by 10nM CCX9588 or higher (Figure 2.5E). While an increase in ERK1/2 phosphorylation was not detectable in RPMI-8226-luc cells following CCL3 stimulation, p-AKT was increased by CCL3 and this was inhibited by 10nM or higher CCX9588 pre-treatment (Figure 2.5F). In order to characterise the ability of CCX9588 to inhibit CCR1 function, OPM2-EV-1 or RPMI-8226-luc cells were pre-treated with CCX9588 (1μM or 100nM, respectively) for 24 hours prior to conducting a transwell migration assay. Pre-treatment of OPM2 or RPMI-8226 cells with CCX9588 resulted in a complete inhibition of migration towards rhCCL3 (OPM2: $p<0.001$, Figure 2.5G; RPMI-8226: $p<0.01$, Figure 2.5H).

2.4.4. CCX9588 treatment reduces dissemination of MM PCs *in vivo*

To investigate the effectiveness of CCR1 inhibition in suppressing MM PC dissemination *in vivo*, the effects of the CCR1 inhibitor CCX9588 were assessed in NSG mice bearing OPM2-EV-1 or RPMI-8226-luc cells. CCX9588 treatment did not have appreciable adverse effects on the mice, as assessed by body weight (Supplementary Figure 2.2A),

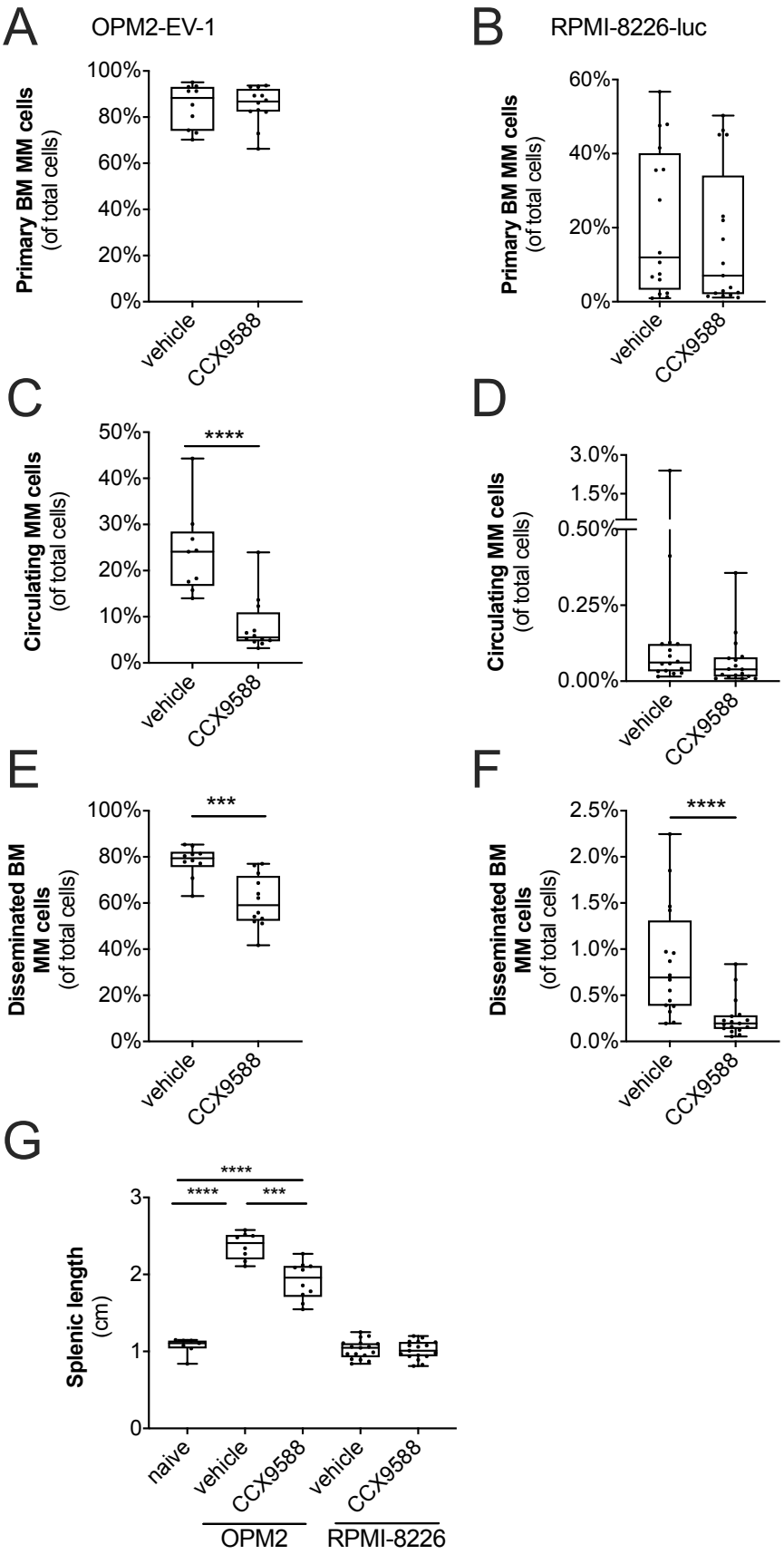
Figure 2.5. CCX9588 treatment prevents activation of CCR1 signalling in MM PCs and their migration towards CCL3 *in vitro*. A. Relative number of OPM2-EV-1 cells was assessed after 72 hours of treatment with 10nM-1μM CCX9588 (all containing 0.01% DMSO), or 0.01% DMSO vehicle alone. **B**. Viability of OPM2-EV-1 cells was assessed after 72 hours of treatment with 10nM-1μM CCX9588 (all containing 0.01% DMSO), or 0.01% DMSO vehicle alone. **C**. Relative number of RPMI-8226-luc cells was assessed after 72 hours of treatment with 10nM-1μM CCX9588 (all containing 0.01% DMSO), or 0.01% DMSO vehicle alone. **D**. Viability of RPMI-8226-luc cells was assessed after 72 hours of treatment with 10nM-1μM CCX9588, or 0.01% DMSO vehicle. **E**. OPM2-EV-1 cells were treated with CCX9588 (10nM-1μM) or media alone for 24 hours, and cells were stimulated with 100ng/mL rhCCL3 for 5 minutes. Cells were lysed and Western blotting was performed with antibodies against p-Akt, p-ERK1/2, total AKT and total ERK. Hsc70 was used as a loading control. A representative of 3 experiments is shown. **F**. RPMI-8226-luc cells were treated with CCX9588 (10nM-1μM) or media alone for 24 hours, and cells were stimulated with 100ng/mL rhCCL3 for 5 minutes. Cells were lysed and Western blotting was performed with antibodies against p-Akt, p-ERK1/2, total AKT and total ERK. Hsc70 was used as a loading control. A representative of 2 experiments is shown. **G**. OPM2-EV-1 cells were treated with 1μM CCX9588 or 0.01% DMSO vehicle control for 24 hours and migrated towards 100ng/ml rhCCL3 or media alone. **H**. RPMI-8226-luc cells were treated with 100nM CCX9588 or 0.01% DMSO vehicle control for 24 hours and migrated towards 100ng/ml rhCCL3 or media alone. Graphs depict mean ± SEM of three or more independent experiments (**A-D, G-H**). **p<0.01, *p<0.05, one-way ANOVA with Tukey's multiple comparisons test (**C**) two-way ANOVA with Sidak's multiple comparisons test (**G-H**).


A
OPM2-EV-1
Relative cells/well
(normalised to vehicle)
150%
100%
50%
0%
0
10
100
1000
CCX9588 (nM)
B
OPM2-EV-1
% Cell viability
(7AAD-AnnexinV- cells
normalised to control)
120%
80%
40%
0%
0
100
1000
CCX9588 (nM)
C
RPMI-8226-luc
Relative cells/well
(normalised to vehicle)
150%
100%
50%
0%
**
0
10
100
1000
CCX9588 (nM)
D
RPMI-8226-luc
% Cell viability
(7AAD-AnnexinV- cells
normalised to control)
120%
80%
40%
0%
0
100
1000
CCX9588(nM)
E
OPM2-EV-1
CCL3
CCX9588 (nM)
- + + + +
- - 10 100 1000
p-AKT
AKT
p-ERK
ERK
Hsc70
F
RPMI-8226-luc
CCL3
CCX9588 (nM)
- + + +
- - 10 100
p-AKT
AKT
p-ERK
ERK
Hsc70
G
OPM2-EV-1
Migration
(normalised to control)
600%
400%
200%
0%
*
*
Vehicle
CCX9588
Vehicle
CCL3
H
RPMI-8226-luc
Migration
(normalised to control)
600%
400%
200%
0%
*
*
Vehicle
CCX9588
Vehicle
CCL3

behaviour or physical appearance, or analysis of PB cell counts (Supplementary Table 2.1). Mean trough serum concentration of CCX9588 achieved in vivo was 328nM (range: 76.8-886nM; Supplementary Figure 2.2B).

In mice bearing OPM2-EV-1 or RPMI-8226-luc cells, primary tumour burden was unaffected by CCX9588 treatment compared with vehicle controls as assessed by flow cytometry (OPM2-EV-1: p=0.91, Figure 2.6A; RPMI-8226-luc: p=0.49, Figure 2.6B). Consistent with the effect of CCR1 knockout in OPM2 cells, we observed *in vivo* a 66% decrease in the mean number of circulating tumour cells in the OPM2-EV-1 model ($p<0.0001$; vehicle: 23.9 ± 3.12% of total parent population [mean ± SEM]; CCX9588: 8.05 ± 1.72%; Figure 2.6C); while the decrease in circulating tumour cells in the RPMI-8226-luc model did not reach statistical significance (p=0.09; vehicle: 0.233± 0.146% of total parent population [mean ± SEM]; CCX9588: 0.0662 ± 0.0210%; Figure 2.6D). CCX9588 treatment significantly reduced dissemination to the bone, with a 22% reduction in mean tumour burden in the contralateral limb in the OPM2-EV-1 model (vehicle: 78.1 ± 2.12% of total parent population [mean ± SEM]; CCX9588: 60.8 ± 3.24%; $p<0.001$; Figure 2.6E) and a 70% reduction in mean tumour burden in the contralateral limb in the RPMI-8226-luc model ($p<0.0001$; vehicle: 0.855 ± 0.152% of total parent population [mean ± SEM]; CCX9588: 0.259 ± 0.0510%; Figure 2.6F) compared with controls. Furthermore, the degree of splenomegaly resulting from tumour infiltration in the OPM2-EV-1 model was significantly reduced compared with vehicle controls in CCX9588-treated mice ($p<0.001$; non-tumour control: 1.10 ± 0.0413 cm in length [mean ± SEM]; vehicle: 2.37 ± 0.0618 cm; CCX9588: 1.92± 0.0767 cm; Figure 2.6G). Splenomegaly was not observed in the RPMI-8226-luc model, precluding assessment of the effect of CCX9588 on splenic dissemination (Figure 2.6G). When CCX9588 treatment was delayed until two weeks post OPM2-EV-1 tumour cell inoculation, CCX9588-treated mice showed significantly reduced numbers of circulating tumour cells ($p<0.01$; vehicle: 13.5 ± 2.50% of total parent population [mean ± SEM]; CCX9588: 4.73 ± 0.560%; Supplementary Figure 2.3B) although delayed treatment did not significantly decrease tumour burden in the contralateral leg (p=0.08; Supplementary Figure 2.3C).

Figure 2.6. CCR1 inhibition reduces circulating MM PC numbers and tumor dissemination in NSG mice bearing OPM2 or RPMI-8226 cells. A. Primary tumor burden in injected tibiae after 4 weeks in NSG mice injected with OPM2-EV-1 cells and treated Days 3-28 with CCX9588 (15mg/kg) or vehicle control at 12-hour intervals. **B**. Primary tumor burden in injected tibiae after 4 weeks in NSG mice injected with RPMI-8226-luc cells and treated Days 3-28 with CCX9588 (15mg/kg) or vehicle control at 12-hour intervals. **C**. Number of circulating OPM2-EV-1 cells in peripheral blood of mice treated Days 3-28 with CCX9588 (15mg/kg) or vehicle control at 12-hour intervals. **D**. Number of circulating RPMI-8226-luc cells in peripheral blood of mice treated Days 3-28 with CCX9588 (15mg/kg) or vehicle control at 12-hour intervals. **E**. Tumor burden disseminated to the non-injected contralateral leg in mice injected with OPM2-EV-1 cells treated Days 3-28 with CCX9588 (15mg/kg) or vehicle control at 12-hour intervals. **F**. Tumor burden disseminated to the non-injected contralateral leg in mice injected with RPMI-8226-luc cells treated Days 3-28 with CCX9588 (15mg/kg) or vehicle control at 12-hour intervals. **G**. Spleens collected from naïve NSG mice or vehicle- or CCX9558-treated mice bearing OPM2-EV-1 or RPMI-8226-luc cells were measured to assess the degree of splenomegaly. Naïve mice splenic sizes are duplicated from Fig. 4D for comparison. Box and whisker plots depict median and interquartile range, n=10-12 mice/group (**A**,**C**,**E**), n=17 mice/group (**B**,**D**,**F**), n=7-17 mice/group (**G**). **p<0.01, ***p<0.001, ****p<0.0001, Mann-Whitney test (**C**,**E-F**), ordinary one-way ANOVA with Tukey's multiple comparison test (**G**).


A
OPM2-EV-1
Primary BM MM cells
(of total cells)
100%
80%
60%
40%
20%
0%
vehicle
CCX9588
B
RPMI-8226-luc
Primary BM MM cells
(of total cells)
60%
40%
20%
0%
vehicle
CCX9588
C
Circulating MM cells
(of total cells)
****
50%
40%
30%
20%
10%
0%
vehicle
CCX9588
D
Circulating MM cells
(of total cells)
3.0%
1.5%
0.50%
0.25%
0.00%
vehicle
CCX9588
E
Disseminated BM
MM cells
(of total cells)
***
100%
80%
60%
40%
20%
0%
vehicle
CCX9588
F
Disseminated BM
MM cells
(of total cells)
****
2.5%
2.0%
1.5%
1.0%
0.5%
0.0%
vehicle
CCX9588
G
Splenic length
(cm)
****
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***
3
2
1
0
naive
vehicle
CCX9588
vehicle
CCX9588
OPM2
RPMI-8226

2.5. Discussion

Symptomatic MM is characterised by the presence of multiple tumours throughout the skeleton, and in some patients, soft tissues. The dissemination of MM PCs is central to the progression of disease and subsequent disease relapse, highlighting the therapeutic potential of targeting of key factors that regulate dissemination to delay disease progression and prevent overt relapse. While the inhibition of several factors, including selectins,[39] N-cadherin[34,40] and CXCR4[41] have been demonstrated to slow BM homing of MM cells *in vivo*, very few genes have been demonstrated to play a role in the spontaneous dissemination of MM PCs from the BM in MM. For example, overexpression of heparanase, an enzyme that cleaves heparan sulfate chains, has been reported to increase the incidence of spontaneous dissemination of MM cells in a mouse MM xenograft model.[42] Additionally, recent data suggests that the transcription factor Twist-1 increases dissemination in an intratibial 5TGM1/KaLwRij model *in vivo*.[43] Furthermore, as far as we are aware, no therapeutic interventions have been described that can inhibit spontaneous dissemination of MM PCs *in vivo*. Here, our findings suggest a novel role for the chemokine receptor CCR1 in regulating the egress of MM PCs from the BM to the circulation during dissemination. These findings are consistent with a role for CCR1 in metastasis in other cancer settings, with a study showing that shRNA-knockdown of CCR1 decreased migration of hepatocellular carcinoma cells *in vitro* and reduced the incidence of lung metastasis *in vivo*.[44]

CCL3 is known to act as a potent chemoattractant for murine and human MM cell lines and patient-derived MM PCs *in vivo.*[17,20,45] In accordance with these previous studies, we demonstrated that CCL3 acts as a chemoattractant for OPM2 and RPMI-8226 cells, which could be blocked with CCR1 KO or by treatment with the CCR1 inhibitor CCX9588. Furthermore, expression of CCR1 in the 5TGM1 murine cell line resulted in a chemotactic response to CCL3 that was not observed in EV controls, *in vitro*. While CCL3 has been shown to be produced by MSC[20] and osteoclasts[19,20] in the BM, the most abundant source of CCL3 in the BM in MM patients is suggested to be the MM PCs themselves.[18-21] CCL3 is abundantly expressed by MM cell lines and primary MM PCs[18,19,24] and is present in high levels in the BM of MM patients.[21,46] It is therefore likely that autocrine CCL3 production would interfere with any chemoattractant effect of exogenous CCL3 in the MM BM and would not contribute significantly to the decreased dissemination with CCR1 inhibition or KO observed here. Interestingly, we found that treatment of RPMI-8226-luc

CCL3. This is consistent with a previous study, whereby the CCR1 inhibitor BX471 prevented basal migration of the human acute monocytic leukaemia cell line THP-1.[47] This may suggest that autocrine CCL3 production in these cells may act to increase random non-directional migration (chemokinesis) in these cells, as has been described for chemokines CCL2 and IGF-1 in MM cell lines.[48,49] Alternatively, CCR1 has been suggested to signal without the presence of ligand and induce agonist-independent migration of THP-1 cells and the murine pre-B lymphoma cell line L1.2.[47] Decreased basal migration or chemokinesis of these cells in the presence of CCR1 inhibitor may, therefore, in part be responsible for the decrease in dissemination of RPMI-8226 cells observed *in vivo*.

We have previously demonstrated that CCL3 treatment of HMCLs reduces their capacity to migrate towards exogenous CXCL12 or undergo cytoskeletal remodelling in response to CXCL12 treatment.[17] Additionally, we demonstrated that migration towards CXCL12 of the human MM cell line U266, which produces abundant CCL3, could be restored following CCR1 knockout or treatment with a CCR1 inhibitor, strongly suggesting that CCL3/CCR1 signalling is responsible for blocking migration towards CXCL12 in these cells.[17] Here, we demonstrate that CCR1 expression increases the capacity for dissemination, while CCR1 inhibition or KO decreases the formation of disseminated tumours in mouse models. We postulate that this is due to changes in CCR1 signalling interfering with CXCL12-mediated BM retention. We have previously shown that CCL3-treatment of HMCLs results in a decreased chemotactic response to CXCL12 but no effect on CXCR4 cell-surface levels *in vitro*, suggesting that CCL3/CCR1 signalling can interfere with downstream CXCL12/CXCR4 signalling.[17] In support of this, CCL3/CCR1 signalling drives mobilisation of haematopoietic progenitors and NK cells from the BM in part through inactivation of CXCR4.[25,26,50] Our findings that CCR1 KO or inhibition significantly decreases the exit of MM PCs from the BM into the PB support our hypothesis that hypoxia-mediated upregulation of CCR1 may be critical for overcoming CXCL12-mediated BM retention and enabling mobilisation.

We observed no effect of CCR1 expression or KO, in the presence or absence of exogenous CCL3, on the proliferation of MM cell lines *in vitro*. This was despite the ability of CCL3 to induce AKT and ERK phosphorylation, which are involved in known survival/proliferation pathways in MM.[24] This contrasts with a previous study suggesting

that recombinant CCL3 increases HMCL proliferation *in vitro*.[24] As such, the possibility that the relatively high serum concentration used in the current study could be providing sufficient other growth factors to mask the effects of CCL3 *in vitro* here cannot be excluded. We also saw no effect of CCX9588 treatment on the proliferation of OPM2 cells, but a decrease in the proliferation of RPMI-8226 cells with 1μM CCX9588 treatment. In contrast, a previous study has observed that treatment with the CCR1 inhibitor CCX721 at high doses had no effect on the proliferation of RPMI-8226 cells *in vitro,*[23] suggesting that the effects observed at high concentrations of CCX9588 here may be due to off-target effects. Importantly, CCR1 expression in the 5TGM1 cell line, or treatment with a CCR1 inhibitor in OPM2 and RPMI-8226 models did not affect primary tumour growth *in vivo*. Inhibition of CCL3 or CCR1 in the murine 5T2MM and 5TGM1 models has previously been shown to decrease primary BM tumour growth, but not growth of subcutaneous tumours or cells *in vitro*.[22,23] This suggests that CCL3/CCR1 inhibitors may effect growth factor production by cells of the BM microenvironment to indirectly affect 5TMM tumour growth.[23] Similar effects were observed with osteoclast ablation using zoledronate, suggesting that these results may be secondary to decreased osteoclast activity/numbers in this model.[23] The CCR1 inhibitor MLN3897 has previously been shown to decrease the pro-proliferative effects of osteoclast coculture on a CCR1-negative HMCL, at least in part through indirectly decreasing osteoclast IL6 secretion, supporting the idea that effects of CCR1 inhibition on tumour growth in some *in vivo* models may be due to secondary effects on osteoclasts.[19] However, we found no effect of CCR1 inhibitor treatment on primary tumour growth in the RPMI-8226 or OPM2 xenograft model, suggesting that inhibition of microenvironmental CCR1 was not contributing to the effects we observed here. Notably, we have previously demonstrated that treatment with the CXCR4 inhibitor T140 had no effect on intratibial RPMI-8226 tumour growth, despite dramatic effects on osteolysis and decreased osteoclast numbers, suggesting that inhibition of osteoclasts does not affect primary tumour growth in this model.[28] We did, however, observe *in vivo* that mice injected with OPM2-CCR1-KO cells had lower primary tumour burden compared with controls. As we did not observe an impact on primary tumour growth in any of the other mouse models, this suggests a potential clonal variance effect. Alternatively, there is a potential indirect effect, whereby an inability of the cells to leave the primary tumor site may be causing environmental pressures, such as an increase in hypoxia,[51] that is slowing their proliferation.

Importantly, we are the first to assess the efficacy of the small molecule CCR1 inhibitor CCX9588 on dissemination in a pre-clinical model of MM. CCX9588 has been previously reported to decrease chemotaxis of T-cells towards liver conditioned media *in vitro*.[52] CCX9588 is an analogue of CCX354, which has previously been investigated as a therapeutic for rheumatoid arthritis in a clinical trial,[53] and CCX721, which has been shown to have anti-osteolytic activity in an *in vivo* MM model.[23] Initially we showed that CCX9588 can decrease CCL3-induced phosphorylation of AKT and ERK in a dose-dependent manner in OPM2 cells. We also saw a similar dose-dependent reduction in AKT phosphorylation in RPMI-8226 cells, however under these conditions we did not observe an induction in ERK phosphorylation with CCL3 stimulation and therefore were not able to assess the effects of the inhibitor on p-ERK levels in these cells. Our *in vivo* studies have demonstrated that CCR1 inhibition can decrease dissemination of MM PCs in OPM2 and RPMI-8226 xenograft models. The OPM2 cell line was originally established from a patient with plasma cell leukemia,[54] and produces a rapidly growing and highly disseminated disease model in NSG mice. In our study, treatment with the CCR1 inhibitor CCX9588 only had a relatively small effect on disseminated tumour burden in the contralateral leg, suggesting that residual disseminating cells during treatment are sufficient to populate the marrow at secondary sites. In contrast, RPMI-8226 cells, derived from a MM patient with no detectable circulating MM PCs on smear,[55] produces a less aggressive model, with a more dramatic reduction in the formation of disseminated BM tumours with CCR1 inhibitor treatment in our study. While we were not able to completely prevent dissemination of MM PCs using CCX9588 at this dose, these studies suggest that impeding the egress of MM PCs from the BM to the circulation could slow the development of disease. Further studies are required to determine whether combination therapy with other anti-myeloma agents, or more intensive treatment regimens, could achieve an enhanced effect on tumour dissemination and subsequent outgrowth in these models.

In summary, our studies have identified a novel role for the chemokine receptor CCR1 in the context of MM pathogenesis, demonstrating that CCR1 is a key driver of MM PCs egress from the BM to the circulation during dissemination. Furthermore, we have shown that inhibition of CCR1 via therapeutic targeting or KO can slow dissemination. Together with previous studies demonstrating that targeting of CCR1 prevents the development of severe osteolytic lesions *in vivo*, our study demonstrating that CCR1 regulates

dissemination presents CCR1 as an attractive therapeutic target for MM. Future preclinical studies are warranted to investigate whether therapeutic inhibition of CCR1 has efficacy as a maintenance therapy, extending post-therapy remission and preventing overt relapse.

2.6. Acknowledgements

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2.7. References

1. Rajkumar SV. Updated Diagnostic Criteria and Staging System for Multiple Myeloma. *American Society of Clinical Oncology Educational Book* **2016**; 36: e418-e423
2. Ghobrial IM. Myeloma as a model for the process of metastasis: implications for therapy. *Blood* **2012**; 1: 20-30
3. Gonsalves WI, Rajkumar SV, Gupta V, Morice WG, Timm MM, Singh PP, et al. Quantification of clonal circulating plasma cells in newly diagnosed multiple myeloma: implications for redefining high-risk myeloma. *Leukemia* **2014**; 10: 2060-2065
4. Chakraborty R, Muchtar E, Kumar SK, Jevremovic D, Buadi FK, Dingli D, et al. Risk stratification in myeloma by detection of circulating plasma cells prior to autologous stem cell transplantation in the novel agent era. *Blood Cancer J* **2016**; 12: e512
5. Chakraborty R, Muchtar E, Kumar SK, Jevremovic D, Buadi FK, Dingli D, et al. Serial measurements of circulating plasma cells before and after induction therapy have an independent prognostic impact in patients with multiple myeloma undergoing upfront autologous transplantation. *Haematologica* **2017**; 8: 1439-1445
6. Peceliunas V, Janiulioniene A, Matuzeviciene R, Zvirblis T, Griskevicius L. Circulating plasma cells predict the outcome of relapsed or refractory multiple myeloma. *Leukemia & Lymphoma* **2012**; 4: 641-647
7. Dingli D, Nowakowski GS, Dispenzieri A, Lacy MQ, Hayman SR, Rajkumar SV, et al. Flow cytometric detection of circulating myeloma cells before transplantation in patients with multiple myeloma: a simple risk stratification system. *Blood* **2006**; 8: 3384-3388
8. Nowakowski GS, Witzig TE, Dingli D, Tracz MJ, Gertz MA, Lacy MQ, et al. Circulating plasma cells detected by flow cytometry as a predictor of survival in 302 patients with newly diagnosed multiple myeloma. *Blood* **2005**; 7: 2276-2279
9. Witzig TE, Gertz MA, Lust JA, Kyle RA, O'Fallon WM, Greipp PR. Peripheral blood monoclonal plasma cells as a predictor of survival in patients with multiple myeloma. *Blood* **1996**; 5: 1780-1787
10. Bianchi G, Kyle RA, Larson DR, Witzig TE, Kumar S, Dispenzieri A, et al. High levels of peripheral blood circulating plasma cells as a specific risk factor for progression of smoldering multiple myeloma. *Leukemia* **2013**; 3: 680-685

11. Kumar S, Rajkumar SV, Kyle RA, Lacy MQ, Dispenzieri A, Fonseca R, et al. Prognostic value of circulating plasma cells in monoclonal gammopathy of undetermined significance. *J Clin Oncol* **2005**; 24: 5668-5674
12. Gonsalves WI, Rajkumar SV, Dispenzieri A, Dingli D, Timm MM, Morice WG, et al. Quantification of circulating clonal plasma cells via multiparametric flow cytometry identifies patients with smoldering multiple myeloma at high risk of progression. *Leukemia* **2017**; 1: 130-135
13. Sanz-Rodríguez F, Ruiz-Velasco N, Pascual-Salcedo D, Teixidó J. Characterization of VLA-4-dependent myeloma cell adhesion to fibronectin and VCAM-1. *British Journal of Haematology* **1999**; 4: 825-834
14. Nie Y, Waite J, Brewer F, Sunshine MJ, Littman DR, Zou YR. The role of CXCR4 in maintaining peripheral B cell compartments and humoral immunity. *The Journal of Experimental Medicine* **2004**; 9: 1145-1156
15. Azab AK, Runnels JM, Pitsillides C, Moreau A-S, Azab F, Leleu X, et al. CXCR4 inhibitor AMD3100 disrupts the interaction of multiple myeloma cells with the bone marrow microenvironment and enhances their sensitivity to therapy. *Blood* **2009**; 18: 4341-4351
16. Azab AK, Hu J, Quang P, Azab F, Pitsillides CM, Awwad R, et al. Hypoxia promotes dissemination of multiple myeloma through acquisition of epithelial to mesenchymal transition-like features. *Blood* **2012**; 24: 5782-5794
17. Vandyke K, Zeissig MN, Hewett DR, Martin SK, Mrozik KM, Cheong CM, et al. HIF-2a Promotes Dissemination of Plasma Cells in Multiple Myeloma by Regulating CXCL12/CXCR4 and CCR1. *Cancer Research* **2017**; 20: 5452-5463
18. Uneda S, Hata H, Matsuno F, Harada N, Mitsuya Y, Kawano F, et al. Macrophage inflammatory protein-1 alpha is produced by human multiple myeloma (MM) cells and its expression correlates with bone lesions in patients with MM. *British Journal of Haematology* **2003**; 1: 53-55
19. Vallet S, Raje N, Ishitsuka K, Hideshima T, Podar K, Chhetri S, et al. MLN3897, a novel CCR1 inhibitor, impairs osteoclastogenesis and inhibits the interaction of multiple myeloma cells and osteoclasts. *Blood* **2007**; 10: 3744-3752
20. Moreaux J, Hose D, Kassambara A, Reme T, Moine P, Requirand G, et al. Osteoclast-gene expression profiling reveals osteoclast-derived CCR2 chemokines promoting myeloma cell migration. *Blood* **2011**; 4: 1280-1290

21. Roussou M, Tasidou A, Dimopoulos MA, Kastritis E, Migkou M, Christoulas D, et al. Increased expression of macrophage inflammatory protein-1α on trephine biopsies correlates with extensive bone disease, increased angiogenesis and advanced stage in newly diagnosed patients with multiple myeloma. *Leukemia* **2009**; 11: 2177-2181
22. Menu E, De Leenheer E, De Raeve H, Coulton L, Imanishi T, Miyashita K, et al. Role of CCR1 and CCR5 in homing and growth of multiple myeloma and in the development of osteolytic lesions: a study in the 5TMM model. *Clin Exp Metastasis* **2006**; 5-6: 291-300
23. Dairaghi DJ, Oyajobi BO, Gupta A, McCluskey B, Miao S, Powers JP, et al. CCR1 blockade reduces tumor burden and osteolysis in vivo in a mouse model of myeloma bone disease. *Blood* **2012**; 7: 1449-1457
24. Lentzsch S, Gries M, Janz M, Bargou R, Dorken B, Mapara MY. Macrophage inflammatory protein 1-alpha (MIP-1 alpha) triggers migration and signaling cascades mediating survival and proliferation in multiple myeloma (MM) cells. *Blood* **2003**; 9: 3568-3573
25. Broxmeyer HE, Hangoc G, Cooper S, Campbell T, Ito S, Mantel C. AMD3100 and CD26 modulate mobilization, engraftment, and survival of hematopoietic stem and progenitor cells mediated by the SDF-1/CXCL12-CXCR4 axis. *Annals of the New York Academy of Sciences* **2007**; 1-19
26. Bernardini G, Sciume G, Bosisio D, Morrone S, Sozzani S, Santoni A. CCL3 and CXCL12 regulate trafficking of mouse bone marrow NK cell subsets. *Blood* **2008**; 7: 3626-3634
27. Cheong CM, Chow AW, Fitter S, Hewett DR, Martin SK, Williams SA, et al. Tetraspanin 7 (TSPAN7) expression is upregulated in multiple myeloma patients and inhibits myeloma tumour development in vivo. *Experimental Cell Research* **2015**; 1: 24-38
28. Diamond P, Labrinidis A, Martin SK, Farrugia AN, Gronthos S, To LB, et al. Targeted Disruption of the CXCL12/CXCR4 Axis Inhibits Osteolysis in a Murine Model of Myeloma-Associated Bone Loss. *JBMR* **2009**; 7: 1150-1161
29. Weber K, Bartsch U, Stocking C, Fehse B. A multicolor panel of novel lentiviral "gene ontology" (LeGO) vectors for functional gene analysis. *Mol Ther* **2008**; 4: 698-706

30. Hewett DR, Vandyke K, Lawrence DM, Friend N, Noll JE, Geoghegan JM, et al. DNA Barcoding Reveals Habitual Clonal Dominance of Myeloma Plasma Cells in the Bone Marrow Microenvironment. *Neoplasia* **2017**; 12: 972-981
31. Sena-Esteves M, Tebbets JC, Steffens S, Crombleholme T, Flake AW. Optimized large-scale production of high titer lentivirus vector pseudotypes. *J Virol Methods* **2004**; 2: 131-139
32. Aubrey BJ, Kelly GL, Kueh AJ, Brennan MS, O'Connor L, Milla L, et al. An inducible lentiviral guide RNA platform enables the identification of tumor-essential genes and tumor-promoting mutations in vivo. *Cell Reports* **2015**; 8: 1422-1432
33. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods* **2001**; 4: 402-408
34. Mrozik KM, Cheong CM, Hewett D, Chow AW, Blaschuk OW, Zannettino AC, et al. Therapeutic targeting of N-cadherin is an effective treatment for multiple myeloma. *Br J Haematol* **2015**; 3: 387-399
35. Fitter S, Dewar AL, Kostakis P, To LB, Hughes TP, Roberts MM, et al. Long-term imatinib therapy promotes bone formation in CML patients. *Blood* **2008**; 5: 2538-2547
36. Opperman KS, Vandyke K, Clark KC, Coulter EA, Hewett DR, Mrozik KM, et al. Clodronate-Liposome Mediated Macrophage Depletion Abrogates Multiple Myeloma Tumor Establishment In Vivo. *Neoplasia* **2019**; 8: 777-787
37. Ishikawa F, Yasukawa M, Lyons B, Yoshida S, Miyamoto T, Yoshimoto G, et al. Development of functional human blood and immune systems in NOD/SCID/IL2 receptor {gamma} chain(null) mice. *Blood* **2005**; 5: 1565-1573
38. Fitter S, Matthews MP, Martin SK, Xie J, Ooi SS, Walkley CR, et al. mTORC1 Plays an Important Role in Skeletal Development by Controlling Preosteoblast Differentiation. *Molecular and Cellular Biology* **2017**; 7: e00668-00616
39. Asosingh K, Günthert U, De Raeve H, Van Riet I, Van Camp B, Vanderkerken K. A unique pathway in the homing of murine multiple myeloma cells: CD44v10 mediates binding to bone marrow endothelium. *Cancer research* **2001**; 7: 2862
40. Groen RWJ, de Rooij MFM, Kocemba KA, Reijmers RM, de Haan-Kramer A, Overdijk MB, et al. N-cadherin-mediated interaction with multiple myeloma cells inhibits osteoblast differentiation. *Haematologica* **2011**; 11: 1653

41. Roccaro AM, Mishima Y, Sacco A, Moschetta M, Tai YT, Shi J, et al. CXCR4 Regulates Extra-Medullary Myeloma through Epithelial-Mesenchymal-Transition-like Transcriptional Activation. *Cell Reports* **2015**; 4: 622-635
42. Yang Y, Macleod V, Bendre M, Huang Y, Theus AM, Miao HQ, et al. Heparanase promotes the spontaneous metastasis of myeloma cells to bone. *Blood* **2005**; 3: 1303-1309
43. Cheong CM, Mrozik KM, Hewett DR, Bell E, Panagopoulos V, Noll JE, et al. Twist-1 is upregulated by NSD2 and contributes to tumour dissemination and an epithelial-mesenchymal transition-like gene expression signature in t(4;14)-positive multiple myeloma. *Cancer Letters* **2020**;
44. Zhu Y, Gao X-M, Yang J, Xu D, Zhang Y, Lu M, et al. C-C chemokine receptor type 1 mediates osteopontin-promoted metastasis in hepatocellular carcinoma. *Cancer Science* **2018**; 3: 710-723
45. Moller C, Stromberg T, Juremalm M, Nilsson K, Nilsson G. Expression and function of chemokine receptors in human multiple myeloma. *Leukemia* **2003**; 1: 203-210
46. Choi SJ, Cruz JC, Craig F, Chung H, Devlin RD, Roodman GD, et al. Macrophage inflammatory protein 1-alpha is a potential osteoclast stimulatory factor in multiple myeloma. *Blood* **2000**; 2: 671-675
47. Gilliland CT, Salanga CL, Kawamura T, Trejo J, Handel TM. The Chemokine Receptor CCR1 Is Constitutively Active, Which Leads to G Protein-independent, β-Arrestin-mediated Internalization. *The Journal of Biological Chemistry* **2013**; 45: 32194-32210
48. Vanderkerken K, Asosingh K, Braet F, Van Riet I, Van Camp B. Insulin-Like Growth Factor-1 Acts as a Chemoattractant Factor for 5T2 Multiple Myeloma Cells. *Blood* **1999**; 1: 235-241
49. Johrer K, Janke K, Krugmann J, Fiegl M, Greil R. Transendothelial migration of myeloma cells is increased by tumor necrosis factor (TNF)-alpha via TNF receptor 2 and autocrine up-regulation of MCP-1. *Clin Cancer Res* **2004**; 6: 1901-1910
50. Lord BI, Woolford LB, Wood LM, Czaplewski LG, McCourt M, Hunter MG, et al. Mobilization of Early Hematopoietic Progenitor Cells With BB-10010: A Genetically Engineered Variant of Human Macrophage Inflammatory Protein-1α. *Blood* **1995**; 12: 3412-3415

51. Muz B, de la Puente P, Azab F, Luderer M, Azab AK. Hypoxia promotes stem cell-like phenotype in multiple myeloma cells. *Blood Cancer Journal* **2014**; 12: e262-e262

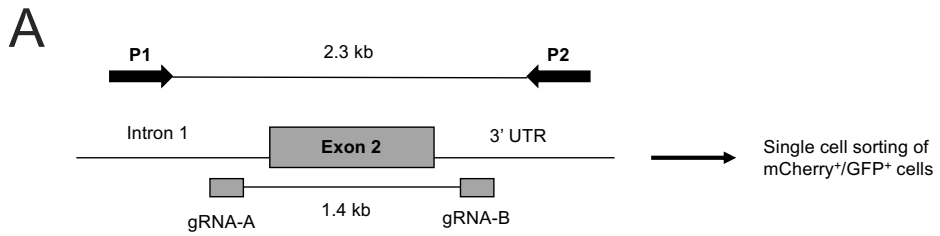
52. Conroy MJ, Galvin KC, Kavanagh ME, Mongan AM, Doyle SL, Gilmartin N, et al. CCR1 antagonism attenuates T cell trafficking to omentum and liver in obesity-associated cancer. *Immunology & Cell Biology* **2016**; 6: 531-537

53. Tak PP, Balanescu A, Tseluyko V, Bojin S, Drescher E, Dairaghi D, et al. Chemokine receptor CCR1 antagonist CCX354-C treatment for rheumatoid arthritis: CARAT-2, a randomised, placebo controlled clinical trial. *Ann Rheum Dis* **2013**; 3: 337-344

54. Katagiri S, Yonezawa T, Kuyama J, Kanayama Y, Nishida K, Abe T, et al. Two distinct human myeloma cell lines originating from one patient with myeloma. *Int J Cancer* **1985**; 2: 241-246

55. Matsuoka Y, Moore GE, Yagi Y, Pressman D. Production of free light chains of immunoglobulin by a hematopoietic cell line derived from a patient with multiple myeloma. *Proc Soc Exp Biol Med* **1967**; 4: 1246-1250

Supplementary Figure 2.1. Verification of OPM2 CCR1 knockout cell lines. **A**. Schematic diagram of CRISPR-Cas9 strategy OPM2 to generate CCR1-KO-1 cell line. P1 and P2 represent forward and reverse sequencing primers, respectively, and location of gRNA-A and -B binding sites are indicated. **B**. PCR to confirm CRISPR-Cas9 deletion of the CCR1 exon in OPM2-CCR1-KO-1 cells compared with non-deleted CCR1 sequence in OPM2-EV-1 cells. **C**. Sanger sequencing of OPM2-CCR1-KO-1 deletion band. **D**. Schematic diagram of CRISPR-Cas9 strategy OPM2 to generate CCR1-KO-2 cell line. P1 and P2 represent forward and reverse sequencing primers, respectively, and location of gRNA-C binding site is indicated. **E**. CRISPR-Cas9 mutagenesis of the CCR1 exon was confirmed by PCR and acrylamide gel electrophoresis of OPM2-CCR1-KO-2 cells compared with OPM2-EV-2 control cells.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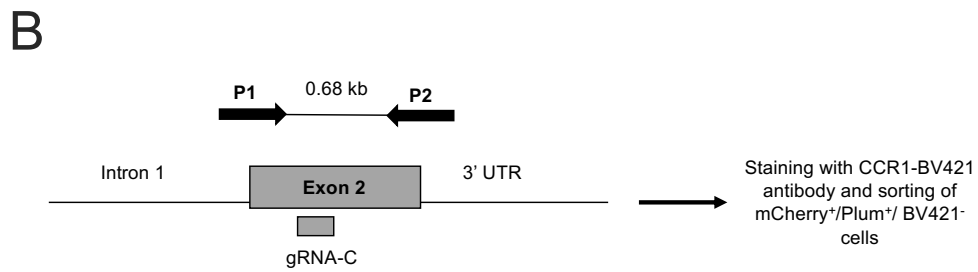
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Staining with CCR1-BV421
antibody and sorting of
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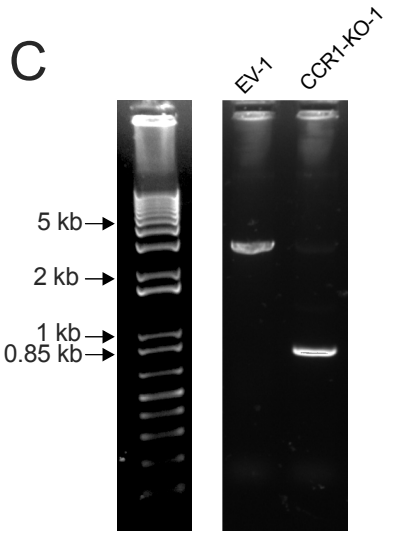
cells
gRNA-C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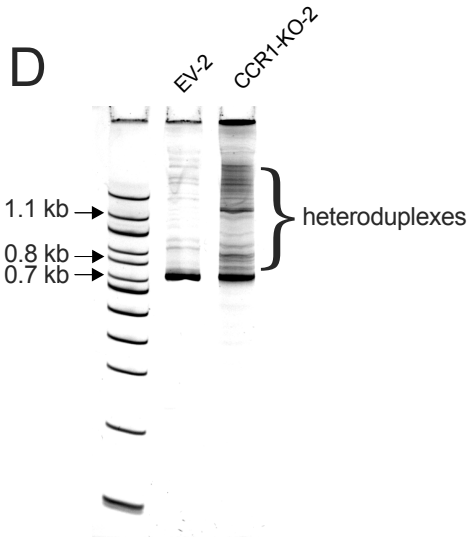
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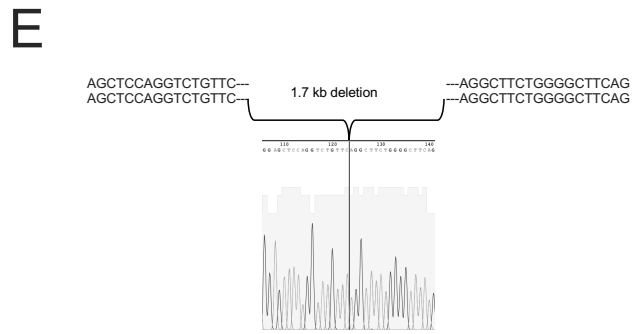
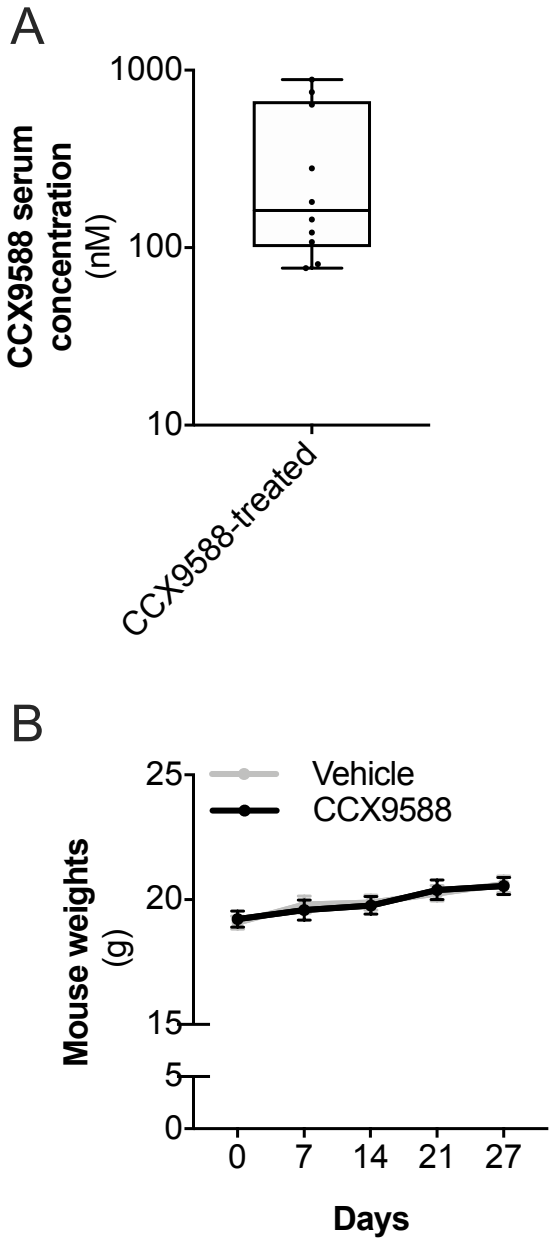

E
AGCTCCAGGTCTGTTC---
AGCTCCAGGTCTGTTC---
1.7 kb deletion
---AGGCTTCTGGGGCTTCAG
---AGGCTTCTGGGGCTTCAG

Supplementary Figure 2.2. Four-week treatment of NSG mice with twice daily treatment with the CCR1 inhibitor CCX9558 was well-tolerated *in vivo*. A. Trough serum concentrations of CCX9588 (C_{min}) were measured 12 hours after the final dose in OPM2-tumor bearing CCX9588-treated NSG mice. **B**. Weights of OPM2-tumor bearing NSG mice were treated with PEG vehicle or CCX9588 via twice daily oral gavage daily for 25 days. Graph depicts median and interquartile range, n=10 mice (**A**), mean ± SEM, n=10-12 mice/group (**B**).

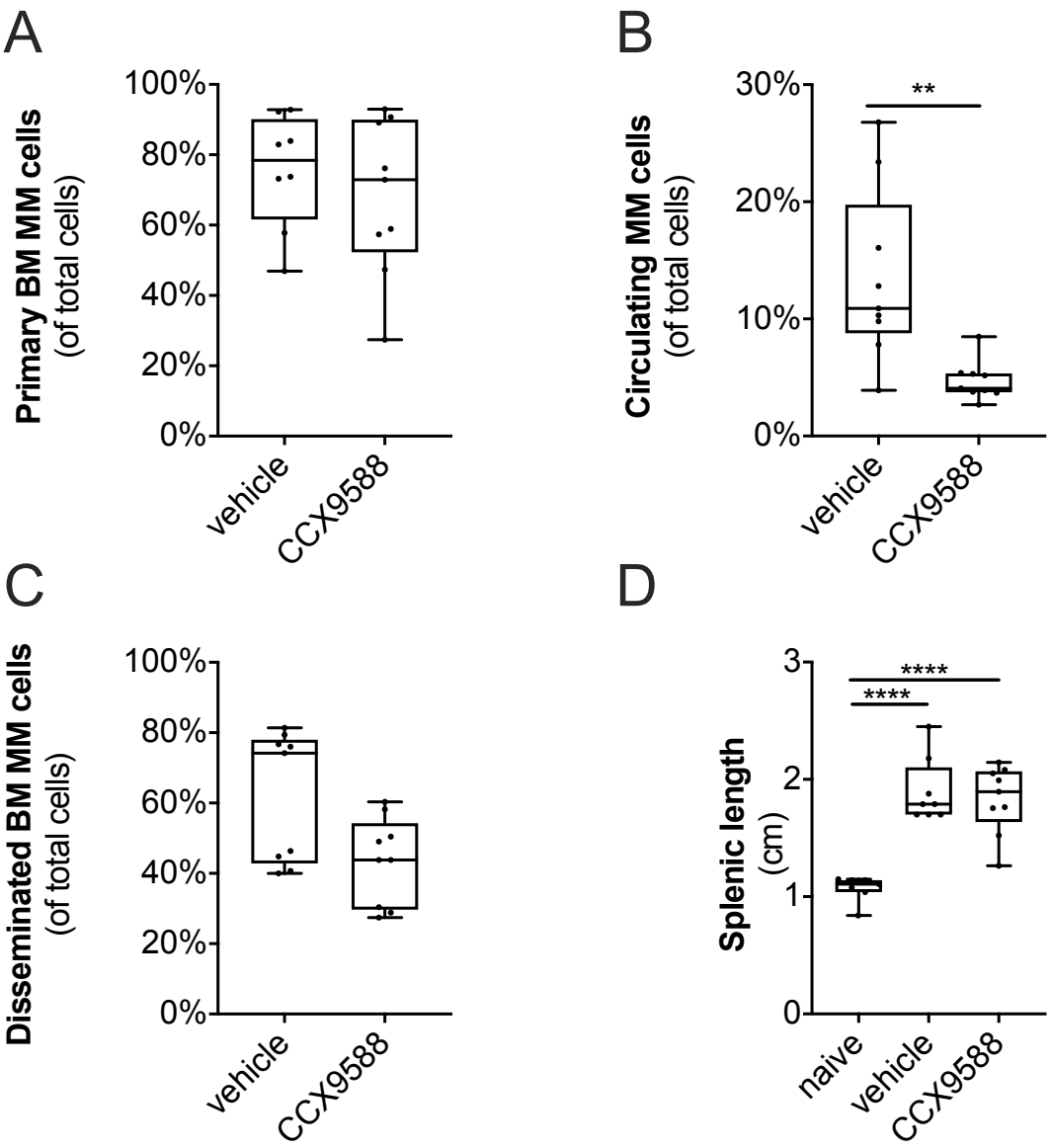

A
1000
100
10
CCX9588 serum concentration (nM)
CCX9588-treated
B
25
20
15
5
0
Vehicle
CCX9588
Mouse weights (g)
0
7
14
21
27
Days

Supplementary Table 2.1. Complete blood counts of OPM2-tumour bearing NSG[1] mice following 25-day CCX9588 treatment

	Naive[2]	Vehicle[2]	CCX9588[2]	*P*-value[3]
Parameter	n=3	n=10	n=9	
White blood cells (K/μL)	1.66 ± 0.39	1.94 ± 0.15	2.06 ± 0.23	0.65
Red blood cells (K/μL)	6.30 ± 0.87	6.76 ± 0.21	6.52 ± 0.25	0.96
Hemoglobin (g/dL)	9.48 ± 1.34	8.41 ± 0.28	8.13 ± 0.34	0.61
Hematocrit (%)	33.18 ± 4.78	34.37 ± 1.15	32.89 ± 1.27	0.81
Mean corpuscular hemoglobin (pg)	15.00 ± 0.25	12.48 ± 0.12	12.47 ± 0.12	0.014
Mean corpuscular hemoglobin concentration (g/dL)	28.62 ± 0.49	24.49 ± 0.27	24.71 ± 0.14	0.015
Red blood cell distribution width (%)	18.15 ± 0.13	17.79 ± 0.13	17.84 ± 0.17	0.45
Platelets (M/μL)	863 ± 242.3	524.3 ± 34.67	492.6 ± 64.34	0.34

[1]9-10 weeks of age at the time of cardiac bleed
[2]mean +/- SEM
[3]Kruskal-Wallis Test

Supplementary Figure 2.3. Targeting of CCR1 reduces circulating tumour cell numbers in NSG mice with established OPM2 tumour cells. Targeting of CCR1 reduces circulating tumor cell numbers in NSG mice with established OPM2 tumor cells. A. Primary tumor burden in injected tibiae after 4 weeks in NSG mice injected with OPM2-EV-1 cells and treated Days 14-28 with CCX9588 (15mg/kg) or vehicle control at 12-hour intervals. **B**. Number of circulating OPM2-EV-1 cells in peripheral blood of mice treated Days 14-28 with CCX9588 (15mg/kg) or vehicle control at 12-hour intervals, as assessed by flow cytometry. **C**. Number of circulating RPMI-8226-luc cells in peripheral blood of mice treated Days 14-28 with CCX9588 (15mg/kg) or vehicle control at 12-hour intervals, as assessed by flow cytometry. **D**. Spleens collected from naïve NSG mice or vehicle- or CCX9558-treated mice bearing OPM2-EV-1 cells were measured to assess the degree of splenomegaly. Naïve mice splenic sizes are duplicated from Fig. 4D for comparison. Box and whisker plots depict median and interquartile ranges, n=9 mice/group (**A**-**C**), n=7-9 mice per group (**D**). **p<0.01, Mann-Whitney test (**B**), ****p<0.0001, ordinary one-way ANOVA with Tukey's multiple comparison test (**D**).


A
Primary BM MM cells
(of total cells)
100%
80%
60%
40%
20%
0%
vehicle
CCX9588
B
Circulating MM cells
(of total cells)
30%
20%
10%
0%
**
vehicle
CCX9588
C
Disseminated BM MM cells
(of total cells)
100%
80%
60%
40%
20%
0%
vehicle
CCX9588
D
Splenic length
(cm)
3
2
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Chapter 3:

Expression of the chemokine receptor CCR1 confers resistance to bortezomib in multiple myeloma cell lines

Statement of Authorship

Title of Paper	Expression of the chemokine receptor CCR1 confers resistance to bortezomib in multiple myeloma cell lines
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Contribution to the Paper	Primary author of manuscript Designed and performed experiments Data analysis and interpretation		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	01/04/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Critical review of manuscript

Signature		Date	8/4/2020

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Contribution to the Paper	Assisted with experiments Critical review of manuscript		
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Name of Co-Author	Vasilios Panagopoulos		
Contribution to the Paper	Assisted with experiments		
Signature		Date	09/04/2020

Name of Co-Author	Sandra M. Dold		
Contribution to the Paper	Assisted with experiments		
Signature		Date	03.04.2020

Name of Co-Author	Monika Engelhardt		
Contribution to the Paper	Experimental design		
Signature		Date	03/04/2020

Name of Co-Author	Andrew C.W. Zannettino		
Contribution to the Paper	Conceptualisation and critical review of manuscript Experimental design		
Signature		Date	02/04/2020

Name of Co-Author	Kate Vandyke		
Contribution to the Paper	Conceptualisation and critical review of manuscript Experimental design		
Signature		Date	02/04/20

Expression of the chemokine receptor CCR1 confers resistance to bortezomib in multiple myeloma cell lines

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* These authors contributed equally to the work

Running title:

CCR1 confers multiple myeloma cell line resistance to bortezomib

3.1. Abstract

The introduction of the proteasome inhibitor, bortezomib, for the treatment of the haematological malignancy multiple myeloma (MM), has improved survival rates. However, a large proportion of patients exhibit resistance to bortezomib for which the mechanisms remain to be fully elucidated. We have previously shown that elevated CCR1 expression in MM plasma cells (PCs) is associated with poor prognosis in newly diagnosed MM patients. Here, we hypothesise that the poor prognosis conferred by CCR1 expression is, in part, due to decreased MM PC sensitivity to bortezomib. Initially, we expressed CCR1 in the murine MM cell line 5TGM1 and found that CCR1 expression conferred a decreased sensitivity to bortezomib treatment *in vitro*. In addition, CRISPR-Cas9-mediated knockout of CCR1 in human OPM2 MM PCs rendered OPM2 cells significantly more sensitive to bortezomib treatment *in vitro* and *in vivo*. In contrast, we demonstrated that addition of recombinant CCL3 or siRNA-mediated knockdown of autocrine *CCL3,* did not affect response to bortezomib *in vitro*. CCR1 expression was shown to negatively regulate the expression of the unfolded protein response receptor IRE1, which may, in part, be responsible for the decreased sensitivity to bortezomib of CCR1 expressing cell lines. In support of this, CCR1 levels inversely correlated with IRE1 levels in primary BM MM PCs. Notably, CCR1 is expressed in over 60% of newly diagnosed MM patients, with elevated CCR1 expression at diagnosis, or induction of CCR1 expression in BM MM PCs at relapse being associated with poor prognosis in patients treated with bortezomib-based regimens. Taken together, these studies suggest that CCR1 expression decreases sensitivity of MM PCs to bortezomib therapy via a mechanism that may involve downregulation of IRE1. As bortezomib is commonly used in frontline and second-line therapeutic regimens in MM, future studies should investigate whether CCR1 inhibition could improve response to bortezomib in relapsed and refractory patients.

3.2. Introduction

Multiple myeloma (MM) is an incurable haematological malignancy characterised by the uncontrolled growth of clonal plasma cells (PC) within the bone marrow (BM).[1] MM is preceded by the asymptomatic precursor disease monoclonal gammopathy of undetermined significance (MGUS), characterised by the presence of low numbers of clonal PC in the BM but no evidence of end organ damage that is typically associated with overt MM.[1] In Europe,[2] the USA[3] and Australia,[4] the proteasome inhibitor, bortezomib, is a common frontline treatment, used in approximately 50% of MM patients. Since its introduction, bortezomib has led to significant improvements in patient survival, with an increase in the relative five-year survival rate to almost 50%.[5] However, resistance to bortezomib represents a major hurdle in the successful treatment of MM patients.[6,7] For instance, approximately 20% of patients present with intrinsic therapeutic resistance and do not respond to frontline bortezomib-containing regimens.[8] In addition, acquired resistance is common in patients treated with bortezomib, with approximately 40-50% of patients relapsing or becoming unresponsive to retreatment.[9,10] Despite the clinical need, therapeutic strategies to overcome bortezomib resistance are lacking, highlighting the need to fully understand the mechanisms of bortezomib resistance in MM.

Proteasome inhibitors, like bortezomib, exert broad anti-tumour effects, including causing the accumulation of misfolded proteins leading to activation of the unfolded protein response (UPR), and stabilisation of pro-apoptotic molecules which induce apoptosis.[7,11] Activation of the UPR is thought to be the mechanism by which bortezomib exerts it major anti-tumour effect as MM PCs undergo ER expansion to accommodate the synthesis of secreted immunoglobulin.[12,13] Bortezomib binds to the active site of the 26S proteasome,[14] inducing ER stress through the accumulation of unfolded proteins and prevention of ER-associated degradation. 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 (Hsp70) member 5 [HSPA5]) from the three UPR transmembrane stress sensors: inositol-requiring kinase 1 (IRE1, also known as endoplasmic reticulum to nucleus signalling 1 [ERN1]), protein kinase R-like ER kinase (PERK) and activating transcription factor 6 (ATF6). Importantly, IRE1, PERK and ATF6 signalling induce the expression of genes responsible for adaption to stress conditions;[15,16] however, bortezomib induces

prolonged ER stress, leading to the induction of pro-apoptotic signalling pathways in, what is termed, the terminal UPR response.[13,17]

The C-C motif chemokine receptor 1 (CCR1) is a G protein-coupled receptor whose most potent ligand is the C-C motif chemokine ligand 3 (CCL3; also known as macrophage inflammatory protein 1 alpha (MIP-1α)). CCL3 activation of CCR1 results in the activation of PI3K/AKT and MEK/ERK signalling pathways associated with cell survival.[18] We have previously shown that elevated BM MM PC expression of *CCR1* is associated with a poor prognosis in newly diagnosed MM patients in the Total Therapy 2 and 3 clinical trials which included bortezomib-based regimens.[19] As shown in Chapter 2, CCR1 expression plays a pivotal role in MM PC dissemination *in vivo*, without affecting cell proliferation *in vitro* or *in vivo*. Notably, a previous study suggests that CCR1/CCL3 signalling may play a role in bortezomib and chemotherapy resistance in transformed B-cell lines,[20] suggesting a potential mechanism which could account for the association between elevated CCR1 and poor prognosis in MM. We therefore hypothesised that the prognostic disadvantage of elevated CCR1 expression is due, in part, to increased MM PC resistance to bortezomib. In the studies presented here, we investigated whether CCR1 overexpression or knockout affected survival of MM cell lines following bortezomib therapy *in vitro* and *in vivo*, and whether addition of CCL3 or endogenous CCL3/CCR1 signalling influenced this response. In addition, we assessed the expression of CCR1 on BM MM PCs isolated from newly diagnosed MM patients using flow cytometry and examined the association between CCR1 expression and survival of MM patients treated with bortezomib-based regimens.

3.3. Methods

3.3.1. Cell culture

All cell culture reagents were sourced from Sigma-Aldrich (St Louis, MO) unless otherwise stated. All media were supplemented with 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin, 1mM sodium pyruvate and 10mM HEPES. Human MM cell lines OPM2-EV-1, OPM2-CCR1-KO-1 (Chapter 2) and U266 were maintained in Roswell Park Memorial Institute 1640 (RPMI-1640) medium with 10% foetal calf serum (FCS; Thermo Fisher Scientific, Waltham, MA) and supplements. The mouse MM cell lines 5TGM1-EV and 5TGM1-CCR1 (Chapter 2) were maintained in Iscove's modified Dulbecco's medium (IMDM) with 20% FCS and supplements. All cell lines were routinely cultured in a humidified environment with 5% carbon dioxide at 37°C. Where indicated, cells were treated with recombinant human (rh) CCL3 (100 ng/mL; R&D Systems, Minneapolis, MN) and/or bortezomib (0-160nM; Selleck Chemicals, Houston, TX).

3.3.2. *In vitro* bortezomib sensitivity assays

WST-1 assays were conducted as previously described.[21] For 5TGM1 cell lines, cells were plated at $1x10^5$ cells/mL in triplicate in phenol red free IMDM medium containing 20% FCS and supplements. For OPM2 and U266 cell lines, cells were plated at $1x10^5$ cells/mL in triplicate in phenol red free RPMI-1640 medium containing 10% FCS and supplements. Where indicated, cells were seeded in media containing bortezomib (0-160nM, all containing 0.002% DMSO) with, or without, rhCCL3 (100 ng/mL). After 24 hours, WST-1 reagent (Roche, Basel, Switzerland) was added and cells incubated at 37°C as per manufacturer's instructions and absorbance was read on an iMark™ Microplate Absorbance Reader at 450nM (BioRad) to quantitate relative numbers of viable cells per well.

3.3.3. siRNA-mediated knockdown of *CCL3*

All reagents were sourced from Thermo Fisher Scientific, unless otherwise specified. Silencer Select Negative Control No. 1 siRNA (Cat. 4390843) and pre-designed *CCL3*-targeting siRNAs (Assay ID s12568, siRNA#1; Assay ID s199846, siRNA#2) were pre-incubated in Opti-MEM at 150nM with 15µl/mL Lipofectamine RNAiMAX reagent for 15 minutes at room temperature. U266 or OPM2 cells were washed and resuspended in Opti-minimal essential medium (MEM) at $2.5x10^5$ cells/mL and siRNA-lipid complexes were then added dropwise, for a final concentration of 25nM siRNA and 2.5µL/mL

Lipofectamine RNAiMAX reagent, and flasks were gently rocked to mix. After 6 hours at 37°C, cells were diluted 2-fold with antibiotic-free RPMI-1640 containing 10% FCS and supplements and cultured for 2 (OPM2) or 3 (U266) days. At day 2 or 3, cells were harvested for RNA isolation using TRIzol reagent.

3.3.4. Real time quantitative PCR (qPCR)

Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific) and DNase treated using RQ1 DNase as per manufacturer's instructions (Promega, Madison, WI). Complementary DNA (cDNA) was synthesized using Superscript IV First-Strand Synthesis System (Thermo Fisher Scientific). qPCR was performed using a CFX Connect 9000 Real-Time PCR machine (BioRad). Sequences for human and mouse primers are outlined in Supplementary Table 3.1. Changes in gene expression were calculated relative to *ACTB/ActB* using the $2^{-\Delta\Delta Ct}$ method.[22]

3.3.5. Western blotting

MM cell lines were treated with bortezomib (5-20nM) or vehicle (0.002% DMSO) for 6 hours in RPMI-1640 medium containing 10% FCS and supplements (OPM2-EV-1 and OPM2-CCR1-KO-1) or IMDM medium containing 20% FCS and supplements (5TGM1-EV and 5TGM1-CCR1). Cells were washed once in phosphate buffered saline (PBS) and cell lysates were prepared as previously described.[23] Proteins (50µg) were resolved under reducing conditions on 10% SDS-PAGE gels and transferred to 0.45µm nitrocellulose membranes. Immunoblotting was performed with the following antibodies: phosphorylated JNK and total IRE1α (Cell Signaling Technologies, Danvers, MA, USA; both at 1:1000) and α-tubulin as a loading control (Abcam, Cambridge, UK; 1:5000). Membranes were developed using Dylight™-680 or 800 conjugated secondary antibodies (Invitrogen, Carlsbad, CA; 1:20,000) and visualised using the Odyssey Infrared Imaging system (LI-COR Bioscience, Lincoln, NE, USA).

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

NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG)[24] mice were bred and housed at the SAHMRI Bioresources facility. All animal studies were approved by and performed in accordance with the SAHMRI Animal Ethics Committee (ID #286). Female NSG mice (5-6 weeks old) were inoculated with $5x10^5$ OPM2-EV-1 or OPM2-CCR1-KO-1 cells in 10µl PBS via intratibial injection. Mice were administered bortezomib (0.7mg/kg) or 0.03% DMSO

control diluted in PBS intravenously on days 7, 11, 14, 18, 21 and 25 post tumour cell injection. On day 28, mice were humanely euthanised and tumour cell-injected tibiae were isolated. Tibiae were flushed with 10mL PBS containing 2% FCS and 2mM EDTA (PFE), centrifuged for 10mins at 1400rpm and resuspended in 1mL PFE prior to analysis of GFP-positive tumour cells by flow cytometry using a LSRFortessa flow cytometer (BD Biosciences).

3.3.7. Patient samples

Ethical approval for this study was obtained from the University of Freiburg Medical Centre Ethics Review Committee and all patients provided written, informed consent, in accordance with the Declaration of Helsinki. Posterior superior iliac spine BM aspirates were collected at initial diagnosis from 28 MM patients that had not received prior therapy [median age: 68 years (range: 49–84); male:female ratio 1.15:1] and 8 patients with MGUS[1] [median age: 74 years (range: 53-88); male:female ratio 1.7:1]. All MM patients fulfilled the diagnostic criteria for active MM disease.[1] The patients presented at the Freiburg Universitätsklinikum between 15th June 2007 and 30th April 2019 (Freiburg, Germany). BM was collected in potassium EDTA tubes and mononuclear cells (BMMNC) were isolated by density gradient centrifugation (Ficoll) and were cryopreserved prior to use as described previously.[25]

3.3.8. Flow cytometry

Cell surface CCR1 expression was assessed on viable CD38^{++}/CD138$^{+/-}$/CD45^{lo}/CD19^{-} malignant PC in MM and MGUS patients by multicolour flow cytometry (FACSARIA III; BD Biosciences, San Jose, CA) as previously described.[19] Briefly, 3x10^5 mononuclear cells per test were stained with either an anti-CCR1-PE (clone 53504; R&D systems, Minneapolis, MN) or no PE-conjugated primary antibody [fluorescence minus one (FMO) control], in combination with CD38-PE-Cy7 (clone HIT2; BioLegend, San Diego, CA), CD138-AlexaFluor-647 (clone B-A38; BioRad, Hercules, CA), CD45-FITC (clone J.33; Beckman Coulter, Brea, CA), and CD19-Brilliant Violet 421 (clone HIB19; BioLegend) antibodies. Cells were stained with the viability dye hydroxystilbamidine (FluoroGold; Invitrogen Life Technologies, Carlsbad, CA) immediately before analysis. Viable, single cells were gated on the basis of FSC and SSC characteristics and FluoroGold negativity and malignant PC were identified as CD38-bright cells. As CD138 was often lost upon freezing, CD138 positivity was not required for identification of the MM PC population.

Contaminating cells were subsequently excluded by gating on the CD45-low and CD19-negative population. A minimum of 100 cells was required to fulfil these criteria to be identified as a malignant PC population. A minimum of $5x10^4$ total nucleated cells were analysed per test, with a minimum of $1x10^5$ cells analysed per patient. CCR1 expression was quantitated as the change in the median fluorescence intensity (ΔMFI), defined as the difference in MFI between the CCR1-stained sample and the FMO control. CCR1 positivity was defined, based on the lowest detectable signal, as above ΔMFI of 83.

3.3.9. Patient RNA-sequencing analysis

RNA-sequencing data was obtained from the Multiple Myeloma Research Foundation (MMRF) CoMMpass (MMRF-COMMPASS) dataset, accessed via the NIH NCI GDC Data Portal (9 August 2019). Gene expression data (FPKM) for CD138-selected BM PC was included from all MM patients (n=43) who had RNA-sequencing performed from a sample taken at diagnosis (initial) and a sample taken following at least one line of therapy which included bortezomib (subsequent). Patients were categorised as having low tumour expression of *CCR1* (*CCR1* < 10 FPKM at both initial and subsequent biopsy; n=26; median age: 70 years [37-83]; male:female ratio 1.36:1), high *CCR1* (*CCR1* ≥ 10 FPKM at initial biopsy; n=7; median age: 66 years [39-70]; male:female ratio 1.31:1) or increased *CCR1* (initial *CCR1* < 10 FPKM and subsequent *CCR1* ≥ 10 FPKM; n=10; median age: 66 years [50-82]; male:female ratio 2.33:1). Median time between initial and subsequent samples was 490.5 days [range: 59-1419 days]. Overall survival was reported as death from any cause.

3.3.10. Statistical Analyses

Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA). *In vitro* assays were analysed using an unpaired t-test for comparison between two cell lines, one-way ANOVA with Tukey's multiple comparisons test for analysis of CCL3 gene expression, two-way ANOVA with Sidak's multiple comparison's test for time-course assays or two-way ANOVA with Tukey's multiple comparisons test for bortezomib dose response assays. *In vivo* experiment was analysed using a two-way ANOVA with Sidak's multiple comparisons test. For analysis of CCR1 expression on BM MM PCs a Mann-Whitney test was used. Overall survival was assessed using Kaplan-Meier curves; comparisons between groups were made using the log-rank (Mantel-Cox) test and the Mantel-Haenszel hazard ratio. Correlation was assessed using Spearman

correlation co-efficient. Differences were considered statistically significant when the p value was less than 0.05.

3.4. Results

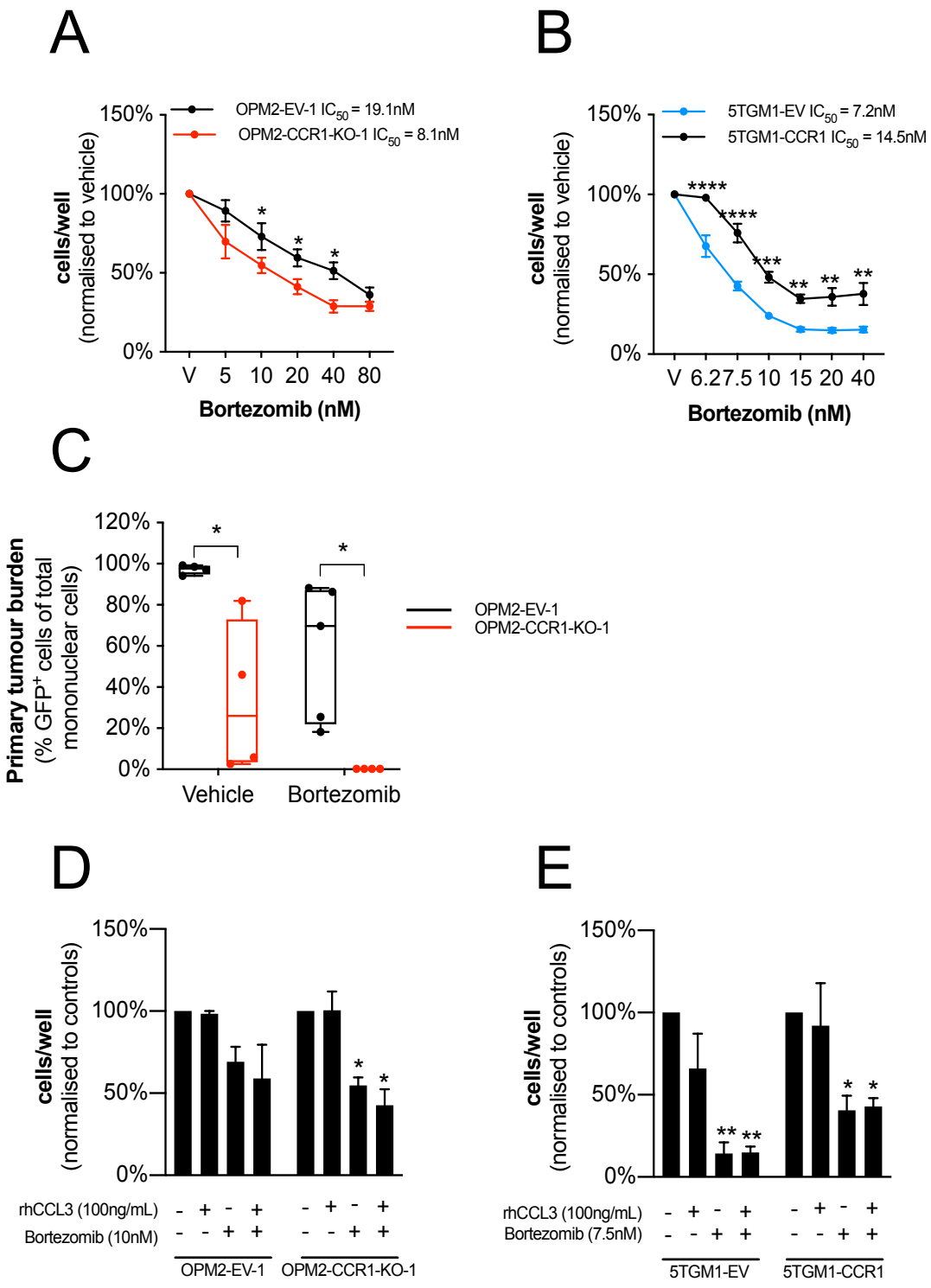
3.4.1. Expression of CCR1 decreases sensitivity of MM cell lines to bortezomib *in vitro* and *in vivo*

We have previously shown that elevated *CCR1* expression in CD138-selected BM PC is associated with poor prognosis in newly diagnosed MM patients.[19] 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 effects of bortezomib on CCR1-knockout OPM2-CCR1-KO-1 cells and control CCR1-expressing OPM2-EV-1 cells. There was a significantly higher reduction in cell number of OPM2-CCR1-KO-1 cells compared with OPM2-EV-1 cells when treated with 10nM, 20nM and 40nM bortezomib *in vitro* (OPM2-EV-1 IC_{50}: 19.1nM, CCR1-KO-1 IC_{50}: 8.1nM, $p<0.01$, Figure 3.1A). In addition, response to bortezomib treatment was investigated in CCR1-expressing 5TGM1-CCR1 cells and CCR1-negative 5TGM1-EV controls. We observed a significantly lower reduction in cell number of 5TGM1-CCR1 cells compared with 5TGM1-EV cells when treated with 6.25nM bortezomib or more *in vitro* (5TGM1-EV IC_{50}: 7.2nM, 5TGM1-CCR1 IC_{50}: 14.5nM, $p<0.0001$, Figure 3.1B).

Next, we assessed the effect of CCR1 expression on the sensitivity of OPM2 cells to bortezomib *in vivo* using an intratibial orthotopic xenograft MM model. As reported in Chapter 2, we observed a significant reduction in tumour burden in NSG mice injected with OPM2-CCR1-KO-1 cells compared with OPM2-EV-1 cells in the saline-treated control groups ($p<0.05$, Figure 3.1C). Notably, OPM2-CCR1-KO-1 tumours were significantly more sensitive to bortezomib therapy than OPM2-EV-1 tumours ($p<0.05$, Figure 3.1C). In mice inoculated with OPM2-EV-1 control cells, bortezomib treatment resulted in a 41.0% reduction in mean primary tumour burden, compared with saline-treated controls (saline: 97.3 ± 1.12 [mean ± SEM], bortezomib: 57.6 ± 15.0, Figure 3.1C). Notably, in mice injected with OPM2-CCR1-KO-1 cells, there was a 99.9% reduction in mean primary tumour burden following bortezomib treatment compared with saline controls (saline: 34.1 ± 18.8 [mean ± SEM], bortezomib: 0.250 ± 0.0290, Figure 3.1C).

3.4.2. Decreased sensitivity of MM cell lines to bortezomib conferred by CCR1 is independent of exogenous or endogenous CCL3

Figure 3.1. Expression of CCR1 confers resistance to bortezomib in MM cell lines *in vitro* and *in vivo.* **A.** Relative number of OPM2 CRISPR-Cas9 CCR1 knockout (OPM2-CCR1-KO-1) or empty vector (OPM2-EV-1) control cells treated with bortezomib (0-80nM) for 24 hours as assessed by WST-1. **B.** Relative number of 5TGM1 cells expressing CCR1 (5TGM1-CCR1) or 5TGM1-EV controls treated with bortezomib (0-40nM) for 24 hours as assessed by WST-1. **C.** Primary tumour burden of OPM2-EV-1 or OPM2-CCR1-KO-1 inoculated NSG mice treated with bortezomib (0.7mg/kg) or saline vehicle control (0.33% DMSO) intravenously on days 7, 11, 14, 18, 21 and 25 post-tumour cell injection. **D**. OPM2-CCR1-KO-1 or OPM2-EV-1 cells were treated with either bortezomib (10nM) with, or without, the addition of recombinant human rhCCL3 (100ng/mL), or with saline control (0.001% DMSO) with, or without, the addition of rhCCL3 for 24 hours and relative cell numbers were assessed by WST-1. **E**. 5TGM1-CCR1 or 5TGM1-EV cells were treated with either bortezomib (7.5nM) with, or without, the addition of recombinant human rhCCL3 (100ng/mL), or with saline control (0.001% DMSO) with, or without, the addition of rhCCL3 for 24 hours and relative cell numbers were assessed by WST-1. Graphs depict mean ± SEM for two (**D,E**) or four to six (**A**,**B**) independent experiments. n=4-5 mice/group (**C**). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, two-way ANOVA with Sidak's (**A**-**C**) or Tukey's (**D**,**E**) multiple comparisons test.


A
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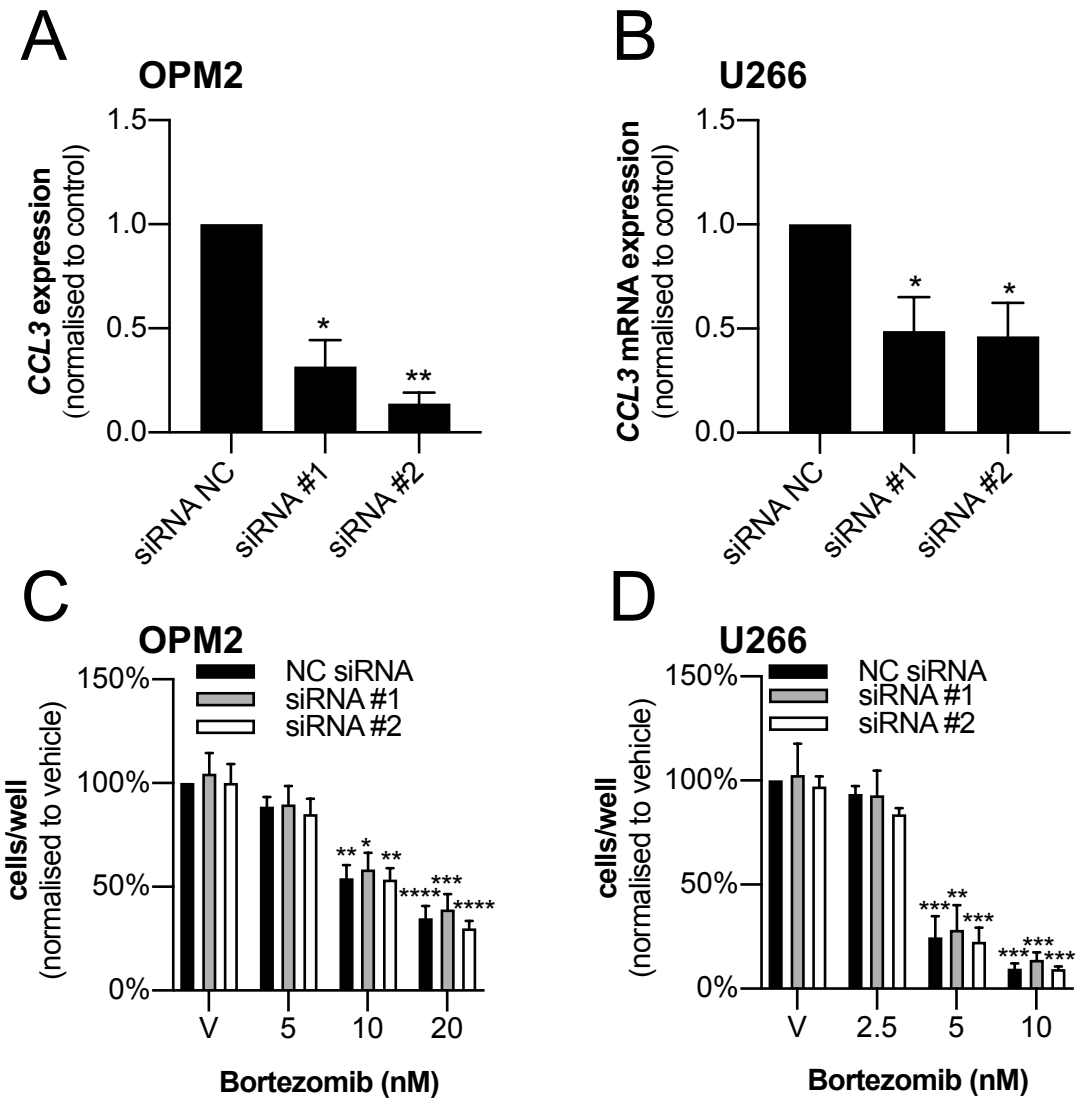
cells/well
(normalised to vehicle)
OPM2-EV-1 IC50 = 19.1nM
OPM2-CCR1-KO-1 IC50 = 8.1nM
V 5 10 20 40 80
Bortezomib (nM)
B
150%
100%
50%
0%
cells/well
(normalised to vehicle)
5TGM1-EV IC50 = 7.2nM
5TGM1-CCR1 IC50 = 14.5nM
V 6.27.5 10 15 20 40
Bortezomib (nM)
C
120%
100%
80%
60%
40%
20%
0%
Primary tumour burden
(% GFP+ cells of total
mononuclear cells)
Vehicle
Bortezomib
OPM2-EV-1
OPM2-CCR1-KO-1
D
150%
100%
50%
0%
cells/well
(normalised to controls)
rhCCL3 (100ng/mL)
Bortezomib (10nM)
OPM2-EV-1
OPM2-CCR1-KO-1
E
150%
100%
50%
0%
cells/well
(normalised to controls)
rhCCL3 (100ng/mL)
Bortezomib (7.5nM)
5TGM1-EV
5TGM1-CCR1

CCL3 is the most potent CCR1 ligand, and we have previously shown that CCL3 activates CCR1 signalling (Chapter 2). Previous studies have shown that treatment with a CCL3-neutralising antibody increased the cytotoxic effect of bortezomib in a human immortalised B-cell line *in vitro*.[20] To determine the role of CCL3 in CCR1-mediated resistance of MM PC to bortezomib, we initially assessed the effects of exogenous CCL3 on sensitivity to bortezomib *in vitro*. Consistent with our previous findings (Chapter 2), the addition of rhCCL3 had no effect on the basal proliferation of OPM2 (OPM2-EV-1: $p>0.99$, OPM2-CCR1-KO-1: $p>0.99$, Figure 3.1D) or 5TGM1 (5TGM1-EV: $p=0.31$, 5TGM1-CCR1: $p=0.97$, Figure 3.1E) cells. Notably, while we observed that bortezomib significantly reduced OPM2-CCR1-KO-1 and 5TGM1-CCR1 and 5TGM1-EV cell numbers compared with untreated controls ($p<0.05$, Figure 3.1D, E), the addition of rhCCL3 showed no effect on the sensitivity of OPM2 (OPM2-EV-1: $p=0.87$, OPM2-CCR1-KO-1: $p=0.76$, 10nM bortezomib, Figure 3.1D) or 5TGM1 (5TGM1-EV: $p>0.99$, 5TGM1-CCR1: $p>0.99$, 7.5nM bortezomib, Figure 3.1E) cells to bortezomib treatment.

As we did not observe that exogenous CCL3 influenced the sensitivity of MM cell lines to bortezomib treatment, we hypothesised that endogenous production of CCL3 could be responsible for the differential response to bortezomib treatment mediated by CCR1. To investigate this, we knocked down *CCL3* expression using siRNA in OPM2 cells (which endogenously expresses low amounts of CCL3), and in the human myeloma cell line U266 (which endogenously expresses abundant CCL3).[19] Initially, qPCR was conducted to confirm the timepoint required for optimal (>50%) knockdown for CCL3 in each cell line compared with negative control (NC) siRNA (OPM2 siRNA #1: 68% decrease, $p<0.05$; OPM2 siRNA #2: 86% decrease, $p<0.01$; U266 siRNA #1: 51% decrease, $p < 0.05$; U266 siRNA #2: 54% decrease, $p < 0.05$; Figure 3.2A,B). Based on these analyses, bortezomib treatment was conducted following 48 hours of siRNA treatment for OPM2 cells and 72 hours for U266 cells. When the cells were subsequently treated with bortezomib for 24 hours, *CCL3* knockdown in OPM2 and U266 cells was found to have no effect on their sensitivity to bortezomib treatment *in vitro* compared with their respective NC siRNA controls (OPM2: $p=0.98$, Figure 3.2C; U266: $p=0.97$, Figure 3.2D). Taken together, these studies demonstrate that neither exogenous nor endogenous CCL3 modulated the response of MM cell lines to bortezomib *in vitro*.

3.4.3. CCR1 negatively regulates IRE1 expression in MM cell lines

Figure 3.2. Increased resistance to bortezomib conferred by CCR1 expression is independent of CCL3/CCR1 signalling. A. *CCL3* expression 48 hours following transfection of OPM2 cells with *CCL3*-targeting siRNA (siRNA #1 and siRNA #2) (normalised to negative control (NC) siRNA). **B**. *CCL3* expression 48 hours following transfection of U266 cells with *CCL3*-targeting siRNA (siRNA #1 and siRNA #2) (normalised to negative control (NC) siRNA expression). **C**. OPM2 cells were treated with bortezomib (5-20nM) for 24 hours following transfection with NC or *CCL3*-targeting siRNA (siRNA #1 and #2) and relative cell numbers were assessed by WST-1. **D**. U266 cells were treated with bortezomib (2.5-10nM) for 24 hours following transfection with NC or *CCL3*-targeting siRNA (siRNA #1 and #2) and relative cell numbers were assessed by WST-1. Graphs depict mean ± SEM for two (**A**, **C**) or three (**B**, **D**) independent experiments. Data normalised to *ACTB* control (**A**,**B**). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, one-way ANOVA with Tukey's multiple comparisons test (**A**, **B**), two-way ANOVA with Tukey's multiple comparisons test (**C**, **D**).


A
OPM2
CCL3 expression
(normalised to control)
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*
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siRNA NC
siRNA #1
siRNA #2
B
U266
CCL3 mRNA expression
(normalised to control)
1.5
1.0
0.5
0.0
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siRNA NC
siRNA #1
siRNA #2
C
OPM2
NC siRNA
siRNA #1
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cells/well
(normalised to vehicle)
150%
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Previous studies have shown decreased expression of genes that trigger the ER stress response pathways to be associated with resistance to bortezomib.[12,26-28] 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 basal gene expression of downstream targets of the UPR receptors IRE1, PERK and ATF6. The downstream effector of the PERK signalling pathway, *ATF4,* was not differentially expressed in OPM2-CCR1-KO-1 (*ATF4:* p=0.446, Figure 3.3A) or 5TGM1-CCR1 (*Atf4:* p=0.573, Figure 3.3B) cells compared with respective EV controls. Similarly, the ATF6 signalling pathway target gene *HSPA5* (gene for BiP) was not differentially expressed in OPM2-CCR1-KO-1 (*HSPA5*: p=0.385, Figure 3.3C) or 5TGM1-CCR1 (*Hspa5*: p=0.966, Figure 3.3D) cells compared with respective EV controls. However, we found that the IRE1 downstream effector gene DnaJ heat shock protein family member C3 (*DNAJC3*; also known as protein kinase inhibitor p58 (p58IPK)) was significantly increased in OPM2-CCR1-KO-1 cells compared with OPM2-EV-1 cells ($p<0.05$, Figure 3.3E). However, there was no change in *Dnajc3* expression in 5TGM1-CCR1 cells compared with controls (p=0.924, Figure 3.3F).

As expression of the IRE1 target gene *DNAJC3* was increased in CCR1 knockout cells, we further investigated this UPR pathway in our CCR1-modified cell lines. ER stress is known to activate the IRE1 endonuclease which, in turn, splices the X-box binding protein 1 (*XBP1*) mRNA into its active form, *XBP1s*. XBP1s then functions as a transcription factor which activates expression of genes responsible for protein folding and homeostasis, including *DNAJC3*.[29] Basal levels of *ERN1* (the gene encoding IRE1), total (spliced and unspliced forms) *XBP1* and *XBP1s* mRNA were significantly increased in OPM2-CCR1-KO-1 cells compared with OPM2-EV-1 cells (*ERN1*: $p<0.05$, Figure 3.4A; *XBP1* total: $p<0.01$, Figure 3.4B; *XBP1s*: $p<0.05$, Figure 3.4C). Furthermore, CCR1-expressing 5TGM1 cells basally expressed lower levels of *Ern1*, total *Xbp1* and *Xbp1s* compared with 5TGM1-EV controls (*Ern1*: $p<0.05$, Figure 3.4D; *Xbp1* total: $p<0.05$, Figure 3.4E; *Xbp1s*: $p<0.05$, Figure 3.4F), further suggesting that CCR1 may negatively regulate this pathway.

We then investigated IRE1 pathway signalling in the response to bortezomib in our CCR1 modified MM cell lines (Figure 3.5). In line with the increase in basal *ERN1* expression seen in OPM2-CCR1-KO-1 cells, we found a concomitant increase in basal IRE1 protein

Figure 3.3. CCR1 expression negatively regulates a target gene downstream of the IRE1 pathway but not genes downstream of the PERK and ATF6 pathways. A. Basal PERK target gene *ATF4* expression in OPM2-CCR1-KO-1 or OPM2-EV-1 control cells as assessed by qPCR. **B.** Basal PERK target gene *Atf4* expression in 5TGM1-CCR1 or 5TGM1-EV control cells as assessed by qPCR. **C**. Basal ATF6 target gene *HSPA5* expression in OPM2-CCR1-KO-1 or OPM2-EV-1 control cells as assessed by qPCR. **D.** Basal ATF6 target gene *Hspa5* expression in 5TGM1-CCR1 or 5TGM1-EV control cells as assessed by qPCR. **E**. Basal IRE1 target gene *DNAJC3* expression in OPM2-CCR1-KO-1 or OPM2-EV-1 control cells as assessed by qPCR. **F.** Basal IRE1 target gene *Dnajc3* expression in 5TGM1-CCR1 or 5TGM1-EV control cells as assessed by qPCR. Graphs depict mean ± SEM of 4 or more independent experiments normalised to *ACTB/ActB* controls. *$p<0.05$, unpaired t-test.

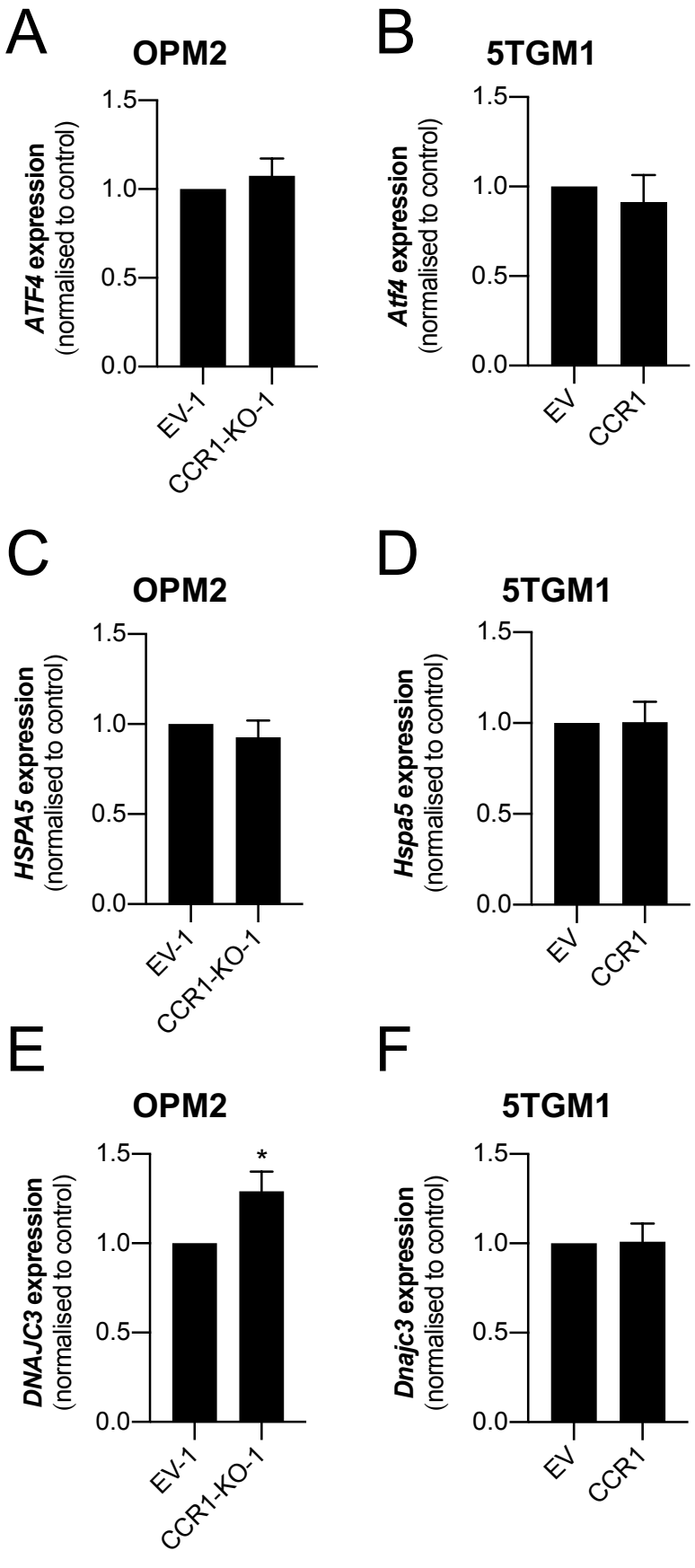

A
OPM2
ATF4 expression
(normalised to control)
1.5
1.0
0.5
0.0
EV-1
CCR1-KO-1
B
5TGM1
Atf4 expression
(normalised to control)
1.5
1.0
0.5
0.0
EV
CCR1
C
OPM2
HSPA5 expression
(normalised to control)
1.5
1.0
0.5
0.0
EV-1
CCR1-KO-1
D
5TGM1
Hspa5 expression
(normalised to control)
1.5
1.0
0.5
0.0
EV
CCR1
E
OPM2
DNAJC3 expression
(normalised to control)
*
1.5
1.0
0.5
0.0
EV-1
CCR1-KO-1
F
5TGM1
Dnajc3 expression
(normalised to control)
1.5
1.0
0.5
0.0
EV
CCR1

Figure 3.4. CCR1 expression negatively regulates expression of IRE1 and its pathway. A. Basal *ERN1* (gene for IRE1) expression, as assessed by qPCR, in OPM2-EV-1 or OPM2-CCR1-KO-1 cells. **B.** Basal *XBP1* total (spliced and unspliced forms) expression, as assessed by qPCR, in OPM2-EV-1 or OPM2-CCR1-KO-1 cells. **C**. Basal *XBP1* spliced expression, as assessed by qPCR, in OPM2-EV-1 or OPM2-CCR1-KO-1 cells. **D**. Basal *Ern1* expression, as assessed by qPCR, in 5TGM1-EV or 5TGM1-CCR1 cells. **E**. Basal *Xbp1* total (spliced and unspliced forms) expression, as assessed by qPCR, in 5TGM1-EV or 5TGM1-CCR1 cells. **F**. Basal *Xbp1* spliced expression, as assessed by qPCR, in 5TGM1-EV or 5TGM1-CCR1 cells. Graphs depict mean ± SEM of 4 or more independent experiments normalised to *ACTB/ActB controls*. *p<0.05, **p<0.01, ***p<0.001, unpaired t-test.

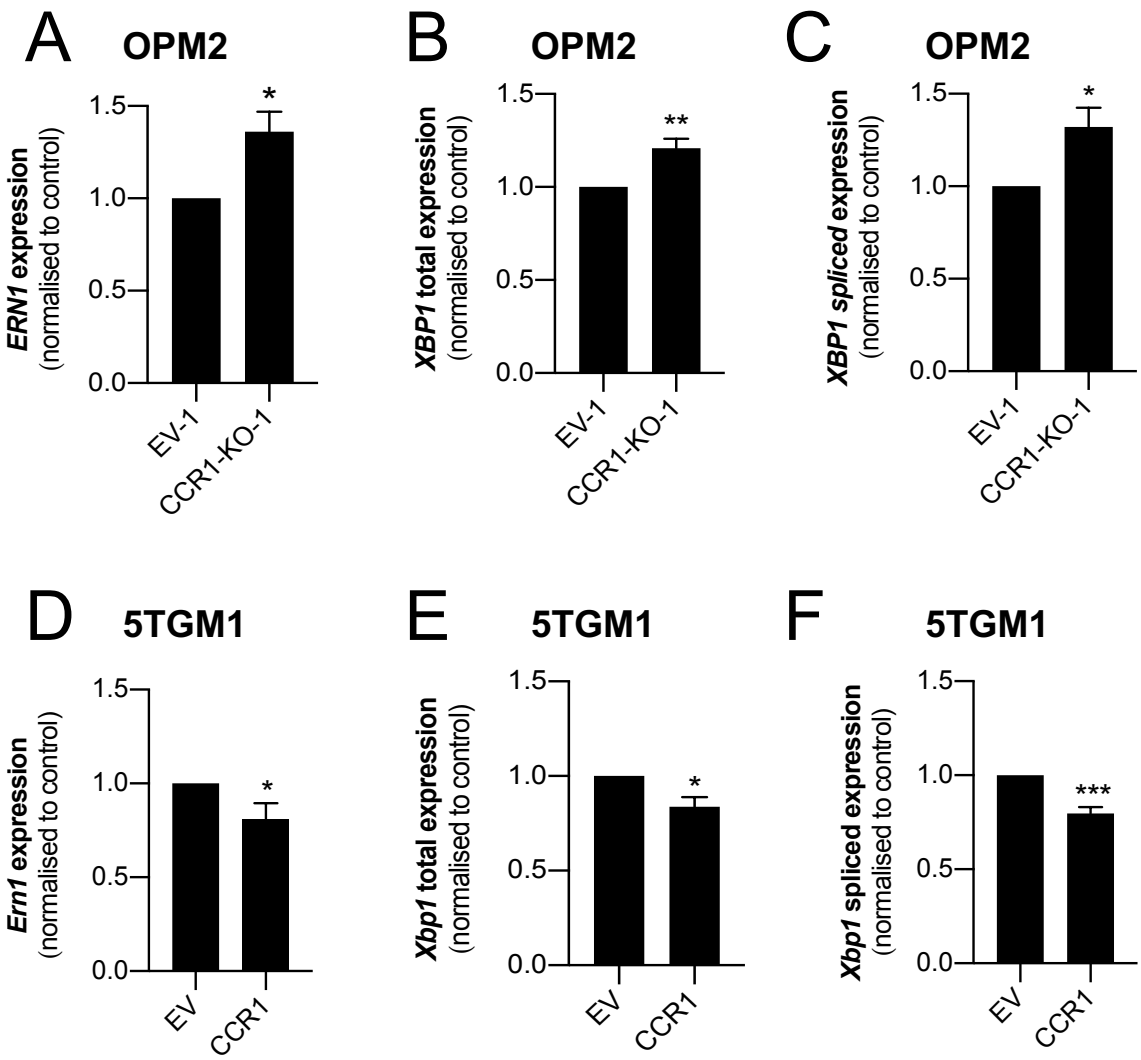
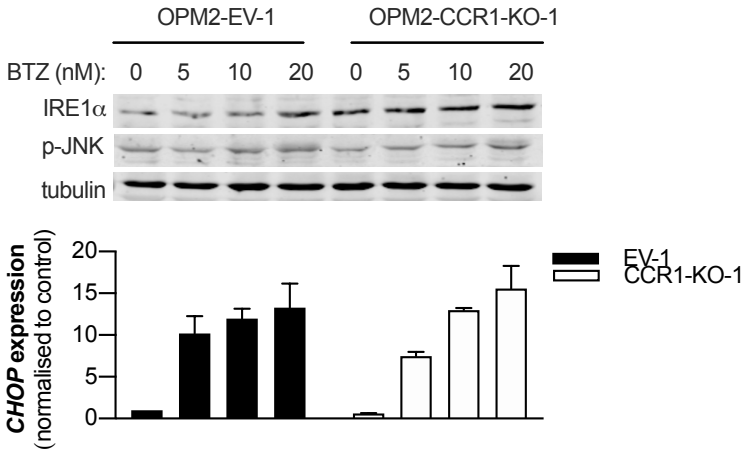
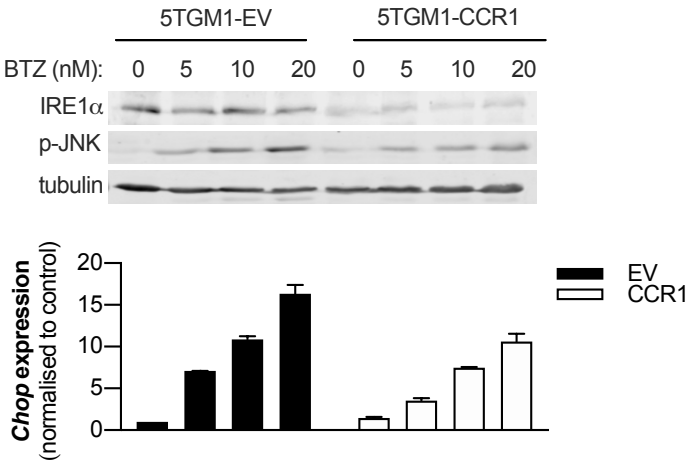

A
OPM2
ERN1 expression
(normalised to control)
1.5
1.0
0.5
0.0
*
EV-1
CCR1-KO-1
B
OPM2
XBP1 total expression
(normalised to control)
1.5
1.0
0.5
0.0
**
EV-1
CCR1-KO-1
C
OPM2
XBP1 spliced expression
(normalised to control)
1.5
1.0
0.5
0.0
*
EV-1
CCR1-KO-1
D
5TGM1
Ern1 expression
(normalised to control)
1.5
1.0
0.5
0.0
*
EV
CCR1
E
5TGM1
Xbp1 total expression
(normalised to control)
1.5
1.0
0.5
0.0
*
EV
CCR1
F
5TGM1
Xbp1 spliced expression
(normalised to control)
1.5
1.0
0.5
0.0
***
EV
CCR1

Figure 3.5. CCR1 expression decreases IRE1 protein expression both basally and following bortezomib treatment in MM cell lines. A. OPM2-EV-1 and OPM2-CCR1-KO-1 cells were exposed to increasing concentrations of bortezomib for 6 hours and examined by Western blot for IRE1α and p-JNK proteins. Tubulin was used as a loading control. qPCR analysis for *CHOP* was performed in parallel and graph depicts one representative of three independent experiments. **B.** 5TGM1-EV and 5TGM1-CCR1 cells were exposed to increasing concentrations of bortezomib for 6 hours and examined by Western blot for IRE1α and p-JNK proteins. Tubulin was used as a loading control. qPCR analysis for *CHOP* was performed in parallel and graph depicts one representative of four independent experiments normalised to *ActB* control. **C.** Schematic diagram of how CCR1 expression regulates IRE1 expression and subsequent pro-apoptotic signalling to confer changes in bortezomib sensitivity. Western blot depicts one of three independent experiments (**A**, **B**). ****$p<0.0001$, two-way ANOVA with Sidak's multiple comparisons test (**B**).

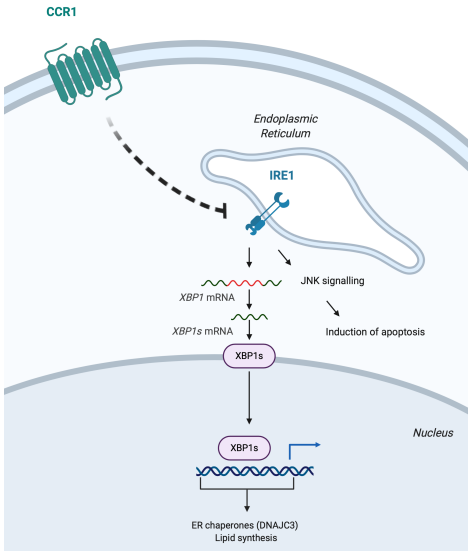
A


OPM2-EV-1
OPM2-CCR1-KO-1
BTZ (nM): 0 5 10 20 0 5 10 20
IRE1α
p-JNK
tubulin
CHOP expression
(normalised to control)
20
15
10
5
0
EV-1
CCR1-KO-1

B


5TGM1-EV
5TGM1-CCR1
BTZ (nM): 0 5 10 20 0 5 10 20
IRE1α
p-JNK
tubulin
Chop expression
(normalised to control)
20
15
10
5
0
EV
CCR1

C


CCR1
Endoplasmic
Reticulum
IRE1
XBP1 mRNA
XBP1s mRNA
JNK signalling
Induction of apoptosis
XBP1s
Nucleus
XBP1s
ER chaperones (DNAJC3)
Lipid synthesis

expression in OPM2-CCR1-KO-1 compared with EV cells, as assessed by Western blotting (Figure 3.5A). In line with this, we also found a decrease in basal Ire1 protein expression in 5TGM1-CCR1 compared with EV cells, as shown by Western blotting (Figure 3.5B). Furthermore, treatment with 20nM bortezomib for 6 hours led to an induction in IRE1 protein expression in OPM2-EV-1 cells, but no induction was observed in OPM2-CCR1-KO-1 cells due to the high basal expression levels. In addition, induction of Ire1 protein was not detectable in 5TGM1 cell lines at the bortezomib concentrations used here.

Under sustained ER stress, a terminal UPR response leads to increased expression of the pro-apoptotic transcription factor CCAAT/enhancer binding protein homologous protein (CHOP).[13] In addition, IRE1 also activates a cell death pathway via phosphorylation of the stress kinase, c-Jun N-terminal kinase (JNK), leading to activation of caspase-dependent apoptosis.[17] To this end, we assessed levels of *CHOP* and phosphorylated JNK (p-JNK) in our CCR1-modified cell lines in response to bortezomib. Bortezomib treatment led to a dose-dependent induction of p-JNK and *CHOP* mRNA in OPM2-EV-1 and OPM2-CCR1-KO-1; however, neither basal nor bortezomib-induced p-JNK or *CHOP* levels were significantly different between the cell lines (Figure 3.5A). In contrast, while bortezomib induced a dose-dependent increase in p-JNK and *CHOP* in both 5TGM1-CCR1 and 5TGM1-EV cells, the magnitude of the increase in p-JNK and *CHOP* in response to bortezomib treatment was lower in 5TGM1-CCR1 cells compared with EV controls (Figure 5B). Taken together, these data suggest that CCR1 expression negatively regulates IRE1 expression in OPM2 and 5TGM1 cell lines. Furthermore, in 5TGM1 cells, this leads to reduced induction of p-JNK and CHOP expression in response to bortezomib treatment, consistent with a decreased induction of apoptosis pathways in these cells (Figure 3.5C).

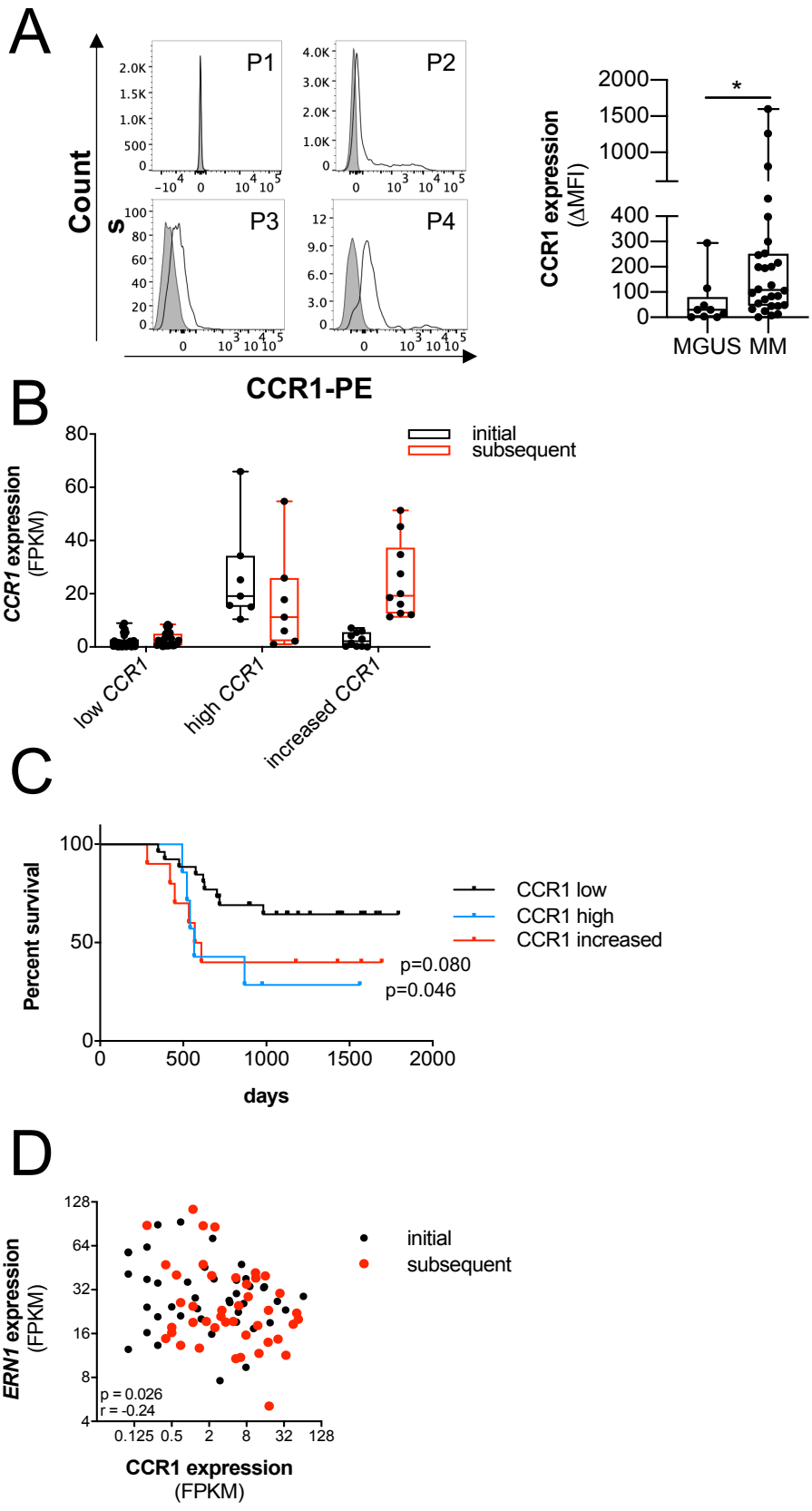
3.4.4. Elevated CCR1 expression at diagnosis, or induction of CCR1 expression at relapse, is associated with poorer prognosis in MM patients

Using *in silico* analysis, we have previously shown that *CCR1* expression in BM MM PCs is elevated in approximately 70% of newly diagnosed MM patients compared with BM PCs from healthy individuals.[19] Furthermore, we showed that 81% (13/16) of newly diagnosed MM patients express detectable CCR1 on BM MM PCs by flow cytometry.[19] Here, we used multi-colour flow cytometry to examine CCR1 expression on $CD38^{++}/CD138^{+/-}/CD45^{lo}/CD19^{-}$ BM MM PCs in a cohort of 28 newly diagnosed MM and

8 MGUS patients. In accordance with our previous study, we found that the majority, 64.3% (18/28), of MM expressed detectable CCR1 (Figure 3.6A). Furthermore, CCR1 protein expression was detectable in 25% (2/8) of MGUS patients (Figure 3.6A), and was found to be significantly higher in BM PC from MM patients (ΔMFI median: 108, range: -36-1598) than those from MGUS patients (ΔMFI median: 8.60, range: -36-295; $p<0.05$, Figure 3.6A), consistent with our previous microarray analysis.[19]

To investigate whether *CCR1* expression is associated with disease response to bortezomib therapy in MM patients, we used RNA-sequencing analysis of *CCR1* expression in CD138-selected BM MM PCs isolated from a cohort of MM patients collected as part of the CoMMpass study. In this cohort, BM samples were taken at diagnosis (initial) and following at least one line of therapy which included bortezomib at relapse (subsequent). We observed that 16.3% (n=7/43) of patients had high (≥ 10 FPKM) *CCR1* expression at baseline. In the patients with low (< 10 FPKM) *CCR1* expression at baseline, 72.2% (n=26/36) patients maintained low *CCR1* expression both at initial and subsequent samples. In contrast, in 27.8% (n=10/36) of patients which displayed low initial *CCR1* expression, showed elevated (≥ 10 FPKM) *CCR1* expression in the subsequent sample post-therapy (Figure 3.6B). Importantly, high *CCR1* BM MM PC expression at diagnosis was significantly associated with poor prognosis, compared with patients in the low *CCR1* group ($p<0.05$, hazard ratio=4.3 [95% CI: 1.03-18.1], Figure 3.6C). Furthermore, there was a trend towards induction of *CCR1* expression post-therapy also being associated with poor prognosis ($p=0.080$, hazard ratio=3.0 [95% CI: 0.88-10.44], Figure 3.6C). Notably, MM PC expression of *CCR1* was found to be inversely correlated with *ERN1* (IRE1) expression in MM patients following therapy ($r=-0.24$, $p=0.026$, Figure 3.6D), consistent with the negative regulation of IRE1 by CCR1 observed *in vitro*.

Figure 3.6. Elevated CCR1 expression at diagnosis, or induction of CCR1 expression at relapse is associated with poorer prognosis in MM patients. A. Multicolour FACS analysis was performed to assess the expression of CCR1 on $CD38^{++/}CD138^{+/-/}CD45l^{o}/CD19^{-}$ BM MM patient plasma cells. Plots of anti-CCR1-PE-stained cells (open histogram) and fluorescence minus one (FMO) controls (filled histogram) are shown for four representative patients (P1–P4). CCR1 expression (ΔMFI) is shown for $CD38^{++}/CD138^{+/-}/CD45^{lo}/CD19^{-}$ BM MM PC isolated from 8 MGUS and 28 newly diagnosed MM patients. **B**. Gene expression data (FPKM) for CD138-selected BM MM PC for patients with a sample taken at diagnosis (initial) and a sample taken following at least one line of therapy with bortezomib (subsequent) (CoMMpass dataset, n=43). Patients were categorised as having low tumour expression of *CCR1* (*CCR1* < 10 FPKM at both initial and subsequent biopsy; n=26), high *CCR1* (*CCR1* ≥ 10 FPKM at initial biopsy; n=7) or increased *CCR1* (initial *CCR1* < 10 FPKM and subsequent *CCR1* ≥ 10 FPKM; n=10). **C**. Kaplan-Meier plots of overall survival are shown for MM patients stratified based on low CD138-selected BM MM PC expression of *CCR1* (*CCR1* < 10 FPKM at both initial and subsequent biopsy; n=26), high *CCR1* (*CCR1* ≥ 10 FPKM at initial biopsy; n=7) or increased *CCR1* (initial *CCR1* < 10 FPKM and subsequent *CCR1* ≥ 10 FPKM; n=10) (CoMMpass dataset, n=43). **D**. *CCR1* expression plotted against *ERN1* (gene for IRE1) expression in CD138-selected BM MM PC isolated from MM patients at a sample taken at diagnosis (initial) or at a sample taken following at least one line of therapy with bortezomib (subsequent) (CoMMpass dataset, n=43). r and p values are shown for Spearman correlation analysis (**D**). Box plot depicts median with interquartile range (**A**). *p<0.05, Mann-Whitney test (**A**).


A
Count
P1
P2
P3
P4
CCR1-PE
CCR1 expression
(ΔMFI)
MGUS MM
*
B
CCR1 expression
(FPKM)
initial
subsequent
low CCR1
high CCR1
increased CCR1
C
Percent survival
days
CCR1 low
CCR1 high
CCR1 increased
p=0.080
p=0.046
D
ERN1 expression
(FPKM)
initial
subsequent
p = 0.026
r = -0.24
CCR1 expression
(FPKM)

3.5. Discussion

While the introduction of the proteasome inhibitor bortezomib has greatly improved the survival of MM patients, intrinsic or acquired resistance to bortezomib is commonly observed in MM patients, leading to poor initial response to therapy or relapse.[6,7] As bortezomib is commonly used as the backbone to therapeutic regimens,[2-4] resistance to bortezomib poses a challenge for the successful treatment of MM patients. However, the mechanisms of resistance remain poorly understood, and it is crucial to identify these mechanisms in order to overcome resistance.

Chemokine receptors have been shown to play a role in chemotherapeutic resistance in multiple cancer types. For example, the interleukin 8 receptor, CXCR2, has been implicated in drug resistance in prostate and breast cancer cell lines, with shRNA knockdown or pharmaceutical inhibition significantly increasing the cytotoxicity of chemotherapeutics.[30,31] Additionally, inhibition of CXCR4 increased the sensitivity of leukaemic[32] and pancreatic cancer cell lines to therapy *in vitro.*[33] Elevated expression of CXCR4 was also associated with lower chemosensitivity in epithelial ovarian cancer patients.[34] Furthermore, addition of the chemokine CCL25, the ligand for CCR9, has been shown to reduced therapy-induced cell death of ovarian cancer cell lines.[35] Using microarray analysis, we previously showed that *CCR1* is expressed in approximately 70% of newly diagnosed MM patients, and elevated BM MM PC expression of *CCR1* is associated with poor prognosis in newly diagnosed patients treated with bortezomib-based regimens.[19] Here, we report for the first time, a role for CCR1 in mediating the resistance of MM PCs to bortezomib treatment. Using CCR1-expression or CCR1-knockout in murine and human MM cell lines *in vitro,* we showed that CCR1-expression decreased sensitivity, while CCR1-knockout increased sensitivity to bortezomib. In support of this, CCR1 has been shown to play a role in the resistance of prostate cancer cells to therapeutics, with a taxane-resistant PC3 prostate cancer cell line expressing CCR1 at high levels compared with taxane-sensitive controls *in vitro.*[36] Furthermore, analysis of publicly available RNA-sequencing data from BM MM PCs isolated from MM patients prior to therapy and at relapse supported our *in vitro* findings that CCR1 increases MM PC bortezomib resistance, demonstrating that elevated CCR1 expression pre-treatment, or induction of CCR1 expression at relapse, may be associated with poor prognosis in MM patients treated with bortezomib-based therapeutic regimens. This is in accordance with our previous microarray analysis of *CCR1* expression in patients enrolled in the Total

Therapy 2 and Total Therapy 3 clinical trials which included bortezomib-based regimens, where we found that elevated *CCR1* expression at diagnosis was associated with poor prognosis.[19] Previous analysis of patients in the Total Therapy 3 trial showed that expression of the transcription factor MAF is also associated with poor prognosis in these patients.[37,38] Furthermore, in overexpression and knockout studies, MAF expression was shown to increase resistance to bortezomib in MM cell lines.[37] Interestingly, expression of CCR1 is known to be driven by MAF in human MM cell lines.[37,39] The data presented here suggest that elevated CCR1 could, in part, mediate the prognostic disadvantage of elevated MAF expression observed in these previous studies.

The primary ligands for CCR1 are CCL3 and CCL5,[40] with CCL3 being both the most potent CCR1 ligand and the most highly expressed CCR1 ligand in MM PCs.[41-44] Previous studies have shown inconsistent results with respect to the potential chemoprotective effects of CCL3. Lentzsch and colleagues demonstrated that addition of CCL3, even at very high concentrations, did not affect the response of the human MM cell line MM.1S to dexamethasone or melphalan *in vitro*.[18] In contrast, treatment with a CCL3-neutralising antibody or siRNA-mediated CCL3 knockdown has been shown to increase the cytotoxic effect of bortezomib in a human immortalised B-cell line *in vitro*.[20] Furthermore, a melphalan-resistant RPMI-8226 cell line, which expressed abundant CCL3, could be re-sensitised to melphalan therapy with the addition of the CCL3-neutralising antibody.[20] While these studies suggest that CCL3 signalling can play a role in resistance to therapeutics, here we show 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, or high levels of the other 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, a number of G-protein coupled receptors, including CCR1, have been shown to be capable of adopting an active conformation and inducing downstream signalling in the absence of ligand.[45,46] Studies in human and murine leukocytes have suggested that CCR1 can take on an active conformational state in the absence of ligand, enabling G-protein-dependent signalling that leads to increased F-actin polymerisation and migration, which can be blocked using a

CCR1 inhibitor.[46] This suggests that the CCL3-independent effects of CCR1 expression observed here may be attributable to constitutive CCR1 signalling in the absence of ligand.

Adhesion to BM stromal cells (BMSCs) and extracellular matrix, mediated by integrin α4β1 (also known as very late antigen 4, VLA-4) and CD44, has been implicated in the resistance to the anti-MM drugs vincristine,[47] dexamethasone,[47] lenolidamide,[48] doxorubicin[49] and melphalan[49] in a process known as cell adhesion-mediated drug resistance (CAMDR). However, co-culture of MM PCs with BMSCs has been shown to have minimal effect on response to bortezomib.[47] Bortezomib was shown to decrease the expression of the α4 (also known as CD49d) subunit of integrin α4β1, suggesting that bortezomib treatment may counteract the effects of CAMDR, thereby sensitising MM cells to other antimyeloma agents.[47] Indeed, addition of bortezomib decreased the effects of CAMDR for vincristine or dexamethasone and increased tumour cell death *in vitro*.[47] In the studies presented here, we found that NSG mice injected with OPM2-CCR1-KO-1 cells have significantly lower tumour burden compared with mice inoculated with OPM2-EV-1 cells when treated with bortezomib. As we also observed that OPM2-CCR1-KO-1 cells are more sensitive to bortezomib therapy compared with EV controls in cell suspension *in vitro,* this suggests that CCR1 mediates resistance to bortezomib in part through a CAMDR independent mechanism.

There is strong evidence to suggest that the response of MM PCs to bortezomib is dependent on activation of the IRE1 pathway and the splicing of XBP1 to its active form, XBP1s.[12,26,27] In support of this, decreased IRE1 or XBP1 levels have previously been suggested to be associated with increased resistance to bortezomib both in patients and in cell line models.[26,27] Leung-Hagesteijn and colleagues showed that levels of XBP1s target gene expression in BM MM PCs from MM patients pre-treatment predicted the response to single-agent bortezomib therapy, with significantly lower expression in patients who relapsed following bortezomib treatment compared with patients who had a complete response.[26] Similar results were seen in an independent study, showing that BM MM PC total (spliced and unspliced) *XBP1* mRNA prior to bortezomib treatment was significantly lower in patients that subsequently did not respond to bortezomib, compared with bortezomib-responsive patients.[27] Evidence also suggests siRNA- or shRNA-mediated knockdown of either XBP1s[12,26] or IRE1[26] in human and murine MM cell lines increases resistance to bortezomib *in vitro*. Consistent with this, we have shown an association

between CCR1 expression, decreased IRE1 expression and bortezomib resistance in MM cell lines. In MM PCs lines that express CCR1 (OPM-2-EV-1 and 5TGM1-CCR1 cells), there was considerably lower basal stress (as seen by IRE1α) than CCR1-negative cells. Thus, CCR1 expression allows the MM PCs to better tolerate subsequent stresses, hence are more resistant to bortezomib. In support of this, our data suggests that decreased IRE1 expression with CCR1 expression in 5TGM1 cells was associated with decreased activation of apoptosis-associated signalling pathways downstream of IRE1, in response to bortezomib treatment, as reflected by decreased induction of *CHOP* and p-JNK levels. However, this finding was not replicated in OPM2 cells. This may be due to the early timepoint (6 hours) used here. In addition, using RNA-sequencing data from MM patients, we found that *CCR1* expression inversely correlates with *IRE1* expression, supporting our *in vitro* findings that CCR1 downregulates IRE1 expression. CCR1 activation results in the phosphorylation and activation of the PI3K/AKT signalling pathway. Notably, AKT has been suggested to regulate the UPR.[50,51] Mimura and colleagues showed that inhibition of AKT increased the IRE1 and p-IRE1 levels in a dose-dependent manner in human MM cell lines.[52] In further support of this, inhibition of AKT has been shown to increase the sensitivity of MM cell lines to bortezomib *in vitro* and *in vivo* in multiple studies.[52-54] 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 expression.

In summary, this study identifies for the first time a novel role for CCR1 in the resistance of MM PC to bortezomib therapy, through a mechanism that may involve decreased expression of IRE1. Furthermore, our study shows that elevated CCR1 expression at diagnosis and/or following induction of CCR1 expression at relapse is associated with poor prognosis in MM patients treated with bortezomib. These results are of potential importance as bortezomib is commonly used in frontline and relapse therapeutic regimens, suggesting that CCR1 may be a useful biomarker to predict the response to bortezomib therapy. Future studies examining whether CCR1 inhibition can re-sensitise resistant MM PC to bortezomib therapy, and to investigate whether CCR1 plays a role in sensitivity to other MM drugs, are warranted.

3.6. Acknowledgements

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3.7. References

1. Rajkumar SV. Updated Diagnostic Criteria and Staging System for Multiple Myeloma. *American Society of Clinical Oncology Educational Book* **2016**; 36: e418-e423
2. Raab MS, Cavo M, Delforge M, Driessen C, Fink L, Flinois A, et al. Multiple myeloma: practice patterns across Europe. *Br J Haematol* **2016**; 1: 66-76
3. Song X, Cong Z, Wilson K. Real-world treatment patterns, comorbidities, and disease-related complications in patients with multiple myeloma in the United States. *Current Medical Research and Opinion* **2016**; 1: 95-103
4. Tomlinson R. Multiple myeloma: Updated approach to management in 2018. *Aust J Gen Pract* **2018**; 8: 526-529
5. Kumar SK, Dispenzieri A, Lacy MQ, Gertz MA, Buadi FK, Pandey S, et al. Continued improvement in survival in multiple myeloma: changes in early mortality and outcomes in older patients. *Leukemia* **2014**; 5: 1122-1128
6. Niewerth D, Jansen G, Assaraf YG, Zweegman S, Kaspers GJ, Cloos J. Molecular basis of resistance to proteasome inhibitors in hematological malignancies. *Drug Resist Updat* **2015**; 18-35
7. McConkey DJ, Zhu K. Mechanisms of proteasome inhibitor action and resistance in cancer. *Drug Resist Updat* **2008**; 4-5: 164-179
8. Palumbo A, Avet-Loiseau H, Oliva S, Lokhorst HM, Goldschmidt H, Rosinol L, et al. Revised International Staging System for Multiple Myeloma: A Report From International Myeloma Working Group. *J Clin Oncol* **2015**; 26: 2863-2869
9. Petrucci MT, Giraldo P, Corradini P, Teixeira A, Dimopoulos MA, Blau IW, et al. A prospective, international phase 2 study of bortezomib retreatment in patients with relapsed multiple myeloma. *Br J Haematol* **2013**; 5: 649-659
10. Sood R, Carloss H, Kerr R, Lopez J, Lee M, Druck M, et al. Retreatment with bortezomib alone or in combination for patients with multiple myeloma following an initial response to bortezomib. *Am J Hematol* **2009**; 10: 657-660
11. Gomez-Bougie P, Wuillème-Toumi S, Ménoret E, Trichet V, Robillard N, Philippe M, et al. Noxa Up-regulation and Mcl-1 Cleavage Are Associated to Apoptosis Induction by Bortezomib in Multiple Myeloma. *Cancer Research* **2007**; 11: 5418
12. Lee A-H, Iwakoshi NN, Anderson KC, Glimcher LH. Proteasome inhibitors disrupt the unfolded protein response in myeloma cells. *Proceedings of the National Academy of Sciences* **2003**; 17: 9946

13. Obeng EA, Carlson LM, Gutman DM, Harrington WJ, Jr., Lee KP, Boise LH. Proteasome inhibitors induce a terminal unfolded protein response in multiple myeloma cells. *Blood* **2006**; 12: 4907-4916
14. Adams J. The development of proteasome inhibitors as anticancer drugs. *Cancer Cell* **2004**; 5: 417-421
15. Blais JD, Filipenko V, Bi M, Harding HP, Ron D, Koumenis C, et al. Activating transcription factor 4 is translationally regulated by hypoxic stress. *Mol Cell Biol* **2004**; 17: 7469-7482
16. Okada T, Yoshida H, Akazawa R, Negishi M, Mori K. Distinct roles of activating transcription factor 6 (ATF6) and double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK) in transcription during the mammalian unfolded protein response. *Biochem J* **2002**; Pt 2: 585-594
17. Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, et al. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* **2000**; 5453: 664-666
18. Lentzsch S, Gries M, Janz M, Bargou R, Dorken B, Mapara MY. Macrophage inflammatory protein 1-alpha (MIP-1 alpha) triggers migration and signaling cascades mediating survival and proliferation in multiple myeloma (MM) cells. *Blood* **2003**; 9: 3568-3573
19. Vandyke K, Zeissig MN, Hewett DR, Martin SK, Mrozik KM, Cheong CM, et al. HIF-2a Promotes Dissemination of Plasma Cells in Multiple Myeloma by Regulating CXCL12/CXCR4 and CCR1. *Cancer Research* **2017**; 20: 5452-5463
20. Tsubaki M, Takeda T, Tomonari Y, Mashimo K, Koumoto YI, Hoshida S, et al. The MIP-1alpha autocrine loop contributes to decreased sensitivity to anticancer drugs. *J Cell Physiol* **2018**; 5: 4258-4271
21. Mrozik KM, Cheong CM, Hewett D, Chow AW, Blaschuk OW, Zannettino AC, et al. Therapeutic targeting of N-cadherin is an effective treatment for multiple myeloma. *Br J Haematol* **2015**; 3: 387-399
22. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods* **2001**; 4: 402-408
23. Fitter S, Dewar AL, Kostakis P, To LB, Hughes TP, Roberts MM, et al. Long-term imatinib therapy promotes bone formation in CML patients. *Blood* **2008**; 5: 2538-2547

24. Ishikawa F, Yasukawa M, Lyons B, Yoshida S, Miyamoto T, Yoshimoto G, et al. Development of functional human blood and immune systems in NOD/SCID/IL2 receptor {gamma} chain(null) mice. *Blood* **2005**; 5: 1565-1573
25. Zannettino ACW, Farrugia AN, Kortesidis A, Manavis J, To LB, Martin SK, et al. Elevated serum levels of stromal-derived factor-1α are associated with increased osteoclast activity and osteolytic bone disease in multiple myeloma patients. *Cancer Research* **2005**; 5: 1700-1709
26. Leung-Hagesteijn C, Erdmann N, Cheung G, Keats Jonathan J, Stewart AK, Reece Donna E, et al. Xbp1s-Negative Tumor B Cells and Pre-Plasmablasts Mediate Therapeutic Proteasome Inhibitor Resistance in Multiple Myeloma. *Cancer Cell* **2013**; 3: 289-304
27. Ling SCW, Lau EKK, Al-Shabeeb A, Nikolic A, Catalano A, Iland H, et al. Response of myeloma to the proteasome inhibitor bortezomib is correlated with the unfolded protein response regulator XBP-1. *Haematologica* **2012**; 1: 64
28. Schewe DM, Aguirre-Ghiso JA. Inhibition of eIF2α Dephosphorylation Maximizes Bortezomib Efficiency and Eliminates Quiescent Multiple Myeloma Cells Surviving Proteasome Inhibitor Therapy. *Cancer Research* **2009**; 4: 1545-1552
29. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* **2001**; 7: 881-891
30. Wilson C, Maxwell PJ, Longley DB, Wilson RH, Johnston PG, Waugh DJ. Constitutive and treatment-induced CXCL8-signalling selectively modulates the efficacy of anti-metabolite therapeutics in metastatic prostate cancer. *PLoS One* **2012**; 5: e36545
31. Sharma B, Nawandar DM, Nannuru KC, Varney ML, Singh RK. Targeting CXCR2 Enhances Chemotherapeutic Response, Inhibits Mammary Tumor Growth, Angiogenesis, and Lung Metastasis. *Molecular Cancer Therapeutics* **2013**; 5: 799
32. Zeng Z, Xi Shi Y, Samudio IJ, Wang R-Y, Ling X, Frolova O, et al. Targeting the leukemia microenvironment by CXCR4 inhibition overcomes resistance to kinase inhibitors and chemotherapy in AML. *Blood* **2009**; 24: 6215-6224
33. Singh S, Srivastava SK, Bhardwaj A, Owen LB, Singh AP. CXCL12–CXCR4 signalling axis confers gemcitabine resistance to pancreatic cancer cells: a novel target for therapy. *British Journal of Cancer* **2010**; 11: 1671-1679

34. Li J, Jiang K, Qiu X, Li M, Hao Q, Wei L, et al. Overexpression of CXCR4 is significantly associated with cisplatin-based chemotherapy resistance and can be a prognostic factor in epithelial ovarian cancer. *BMB reports* **2014**; 1: 33-38

35. Johnson EL, Singh R, Johnson-Holiday CM, Grizzle WE, Partridge EE, Lillard JW, et al. CCR9 interactions support ovarian cancer cell survival and resistance to cisplatin-induced apoptosis in a PI3K-dependent and FAK-independent fashion. *Journal of Ovarian Research* **2010**; 1: 15

36. Kato T, Fujita Y, Nakane K, Mizutani K, Terazawa R, Ehara H, et al. CCR1/CCL5 interaction promotes invasion of taxane-resistant PC3 prostate cancer cells by increasing secretion of MMPs 2/9 and by activating ERK and Rac signaling. *Cytokine* **2013**; 1: 251-257

37. Qiang Y-W, Ye S, Chen Y, Buros AF, Edmonson R, van Rhee F, et al. MAF protein mediates innate resistance to proteasome inhibition therapy in multiple myeloma. *Blood* **2016**; 25: 2919

38. Nair B, van Rhee F, Shaughnessy JD, Jr., Anaissie E, Szymonifka J, Hoering A, et al. Superior results of Total Therapy 3 (2003-33) in gene expression profiling-defined low-risk multiple myeloma confirmed in subsequent trial 2006-66 with VRD maintenance. *Blood* **2010**; 21: 4168-4173

39. Hurt EM, Wiestner A, Rosenwald A, Shaffer AL, Campo E, Grogan T, et al. Overexpression of c-maf is a frequent oncogenic event in multiple myeloma that promotes proliferation and pathological interactions with bone marrow stroma. *Cancer Cell* **2004**; 2: 191-199

40. Moller C, Stromberg T, Juremalm M, Nilsson K, Nilsson G. Expression and function of chemokine receptors in human multiple myeloma. *Leukemia* **2003**; 1: 203-210

41. Uneda S, Hata H, Matsuno F, Harada N, Mitsuya Y, Kawano F, et al. Macrophage inflammatory protein-1 alpha is produced by human multiple myeloma (MM) cells and its expression correlates with bone lesions in patients with MM. *British Journal of Haematology* **2003**; 1: 53-55

42. Vallet S, Raje N, Ishitsuka K, Hideshima T, Podar K, Chhetri S, et al. MLN3897, a novel CCR1 inhibitor, impairs osteoclastogenesis and inhibits the interaction of multiple myeloma cells and osteoclasts. *Blood* **2007**; 10: 3744-3752

43. Moreaux J, Hose D, Kassambara A, Reme T, Moine P, Requirand G, et al. Osteoclast-gene expression profiling reveals osteoclast-derived CCR2 chemokines promoting myeloma cell migration. *Blood* **2011**; 4: 1280-1290
44. Roussou M, Tasidou A, Dimopoulos MA, Kastritis E, Migkou M, Christoulas D, et al. Increased expression of macrophage inflammatory protein-1α on trephine biopsies correlates with extensive bone disease, increased angiogenesis and advanced stage in newly diagnosed patients with multiple myeloma. *Leukemia* **2009**; 11: 2177-2181
45. Seifert R, Wenzel-Seifert K. Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors. *Naunyn-Schmiedeberg's Archives of Pharmacology* **2002**; 5: 381-416
46. Gilliland CT, Salanga CL, Kawamura T, Trejo J, Handel TM. The Chemokine Receptor CCR1 Is Constitutively Active, Which Leads to G Protein-independent, β-Arrestin-mediated Internalization. *The Journal of Biological Chemistry* **2013**; 45: 32194-32210
47. Noborio-Hatano K, Kikuchi J, Takatoku M, Shimizu R, Wada T, Ueda M, et al. Bortezomib overcomes cell-adhesion-mediated drug resistance through downregulation of VLA-4 expression in multiple myeloma. *Oncogene* **2009**; 2: 231-242
48. Bjorklund CC, Baladandayuthapani V, Lin HY, Jones RJ, Kuiatse I, Wang H, et al. Evidence of a role for CD44 and cell adhesion in mediating resistance to lenalidomide in multiple myeloma: therapeutic implications. *Leukemia* **2014**; 2: 373-383
49. Damiano JS, Cress AE, Hazlehurst LA, Shtil AA, Dalton WS. Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. *Blood* **1999**; 5: 1658-1667
50. Blaustein M, Pérez-Munizaga D, Sánchez MA, Urrutia C, Grande A, Risso G, et al. Modulation of the Akt pathway reveals a novel link with PERK/eIF2α, which is relevant during hypoxia. *PloS one* **2013**; 7: e69668-e69668
51. Sanchez-Alvarez M, del Pozo MA, Bakal C. AKT-mTOR signaling modulates the dynamics of IRE1 RNAse activity by regulating ER-mitochondria contacts. *Scientific Reports* **2017**; 1: 16497
52. Mimura N, Hideshima T, Shimomura T, Suzuki R, Ohguchi H, Rizq O, et al. Selective and Potent Akt Inhibition Triggers Anti-Myeloma Activities and

Enhances Fatal Endoplasmic Reticulum Stress Induced by Proteasome Inhibition. *Cancer Research* **2014**; 16: 4458

53. Que W, Chen J, Chuang MA, Jiang D. Knockdown of c-Met enhances sensitivity to bortezomib in human multiple myeloma U266 cells via inhibiting Akt/mTOR activity. *APMIS* **2012**; 3: 195-203

54. Hideshima T, Catley L, Yasui H, Ishitsuka K, Raje N, Mitsiades C, et al. Perifosine, an oral bioactive novel alkylphospholipid, inhibits Akt and induces in vitro and in vivo cytotoxicity in human multiple myeloma cells. *Blood* **2006**; 10: 4053-4062

Supplementary Table 3.1. Real-time qPCR human and mouse primer sequences

	Forward	Reverse
ACTB	5′ - GATCATTGCTCCTCCTGAGC - 3′	5′ - GTCATAGTCCGCCTAGAAGCAT - 3′
ActB	5′ - TTGCTGACAGGATGCAGAAG - 3′	5′ - AAGGGTGTAAACGCAGTTC - 3′
ERN1	5′ - CGGGAGAACATCACTGTCCC - 3′	5′ - CCCGGTAGTGGTGCTTCTTA - 3′
Ern1	5′ - CCCTGATAGGTTGAATCCTGGCTATGTG - 3′	5′ - AATCTATGCGCTAATCTGCTGGCCTCTG - 3′
XBP1	5′ - TTGTCACCCCTCCAGAACATC - 3′	5′ - TCCAGAATGCCCA ACAGGAT - 3′
Xbp1	5′ - TGGCCGGGTCTGCTGAGTCCG - 3′	5′ - GTCCATGGGAAGATGTTCTGG - 3′
XBP1s	5′ - TGCTGAGTCCGCAGCAGGTG - 3′	5′ - GCTGGCAGGCTCTGGGGAAG - 3′
Xbp1s	5′ - CTGAGTCCGAATCAGGTGCAG - 3′	5′ - GTCCATGGGAAGATGTTCTGG - 3′
DNAJC3	5′ - GGCTCGGTATTCCCCTTCCT - 3′	5′ - AGTAGCCCTCCGATAATAAGCAA - 3′
Dnajc3	5′ - AAGGGAAGCTTGACGAAGCA - 3′	5′ - TAGCAGCAGTGTAATCGCA - 3′
CHOP	5′ - AGAACCAGGAAACGGAAACAGA - 3′	5′ - TCTCCTTCATGCGCTGCTTT - 3′
Chop	5′ - CCACCACACCTGAAAGCAGAA - 3′	5′ - AGGTGAAAGGCAGGGACTCA - 3′
HSPA5	5′ - TGTTCAACCAATTATCAGCAAACTC - 3′	5′ - TTCTGCTGTATCCTCTTCACCAGT - 3′
Hspa5	5′ - TTCAGCCAATTATCAGCAAACTCT - 3′	5′ - TTTTCTGATGTATCCTCTTCACCAGT - 3′
ATF4	5′ - TCTCATTCAGGCTTCTCACGGCAT - 3′	5′ - AAGCTCATTTCGGTCATGTTGCGG - 3′
Atf4	5′ - CCTAGGTCTCTTAGATGACTATCTGGAGG - 3′	5′ - CCAGGTCATCCATTCGAAACAGAGCATCG - 3′
CCL3	5′- CTGGTTTCAGACTTCAGAAGGAC - 3′	5′ - GTAGTCAGCTATGAAATTCTGTGG - 3′

Chapter 4:

Chemokine receptor profile in multiple myeloma plasma cells: expression of CCR10 is associated with poor prognosis in newly diagnosed patients

Statement of Authorship

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Contribution to the Paper	Primary author of manuscript Designed and performed experiments Data analysis and interpretation		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Chemokine receptor profile in multiple myeloma plasma cells: expression of CCR10 is associated with poor prognosis in newly diagnosed myeloma patients

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Running title:

CCR10 is associated with poor prognosis in multiple myeloma

4.1. Abstract

The chemokine receptors are a family of nineteen G-protein coupled receptors that modulate cell migration and trafficking via their specificity for chemoattractant cytokines, known as chemokines. Previous studies have shown functional roles for some chemokine receptors in the pathogenesis of the haematological malignancy multiple myeloma (MM), but a comprehensive assessment of the association between elevated MM plasma cell (PC) chemokine receptor expression and patient prognosis has not been conducted. We have previously identified that the C-C chemokine receptor 1 (CCR1) is associated with poorer prognosis in newly diagnosed MM patients. Here, we examined the expression of the full repertoire of chemokine receptors expressed in CD138-selected BM MM PCs from newly diagnosed MM patients and investigated the association between increased expression and overall survival. Initially, we interrogated a publicly available microarray dataset consisting of BM PC gene expression analysis from 142 newly diagnosed MM patients (E-TABM-1138) and found that *CCR2*, *CCR5* and *CXCR4* were expressed in the majority of patients, *CCR1*, *CCR10*, *CXCR1*, *CXCR3* and *CX3CR1* were expressed in a subset of patients, while *CCR3*, *CCR4*, *CCR6*, *CCR7*, *CCR8*, *CCR9*, *CXCR2*, *CXCR5*, *CXCR6*, *CXCR7* and *XCR1* had low or undetectable expression in most patients. Assessment of the association between elevated receptor expression with overall survival found that apart from *CCR1*, only *CCR10* expression was associated with poorer prognosis. Approximately 50% of patients expressed *CCR10,* and patients with above median *CCR10* expression had significantly decreased overall survival when compared with patients without elevated levels (p=0.034, hazard ratio: 2.3 [95% CI: 1.1-4.8]). We further assessed CCR10 protein expression using flow cytometry on $CD38^{++}/CD138^{+/-}/CD45^{lo}/CD19^{-}$ BM MM PCs from 27 newly diagnosed MM patients and found that CCR10 was highly expressed in 26.9% (8/27) of MM patients. Furthermore, there was a trend towards elevated expression of CCR10 protein being associated with decreased overall survival, although this did not reach statistical significance (p=0.051, hazard ratio: 4.4 [95% CI: 0.99-19]). CCR10 expression was also not significantly different between tumour cells from patients with the precursor condition monoclonal gammopathy of undetermined significance (MGUS) and those with MM as assessed by flow cytometry (n=6 MGUS and 27 MM patients) and microarray analysis (n=5 MGUS and 155 MM, E-MTAB-363; n=11 MGUS and 133 MM, E-GEOD-16122). In addition, CCR10 expression, as assessed by flow cytometry and microarray analysis (E-TABM-1138), did not correlate with high risk clinical

prognostic factors or cytogenetic subgroups. Collectively, our studies suggest that CCR10 may be a novel independent prognostic marker in MM. Future studies are warranted to confirm the potential utility of CCR10 as a novel prognostic marker in patients and to investigate the functional role of CCR10 in MM pathogenesis.

4.2. Introduction

Chemokines are small, chemoattractant cytokines that bind to specific G-protein-coupled 7-span transmembrane receptors and play a critical role in the selective recruitment of leukocytes to target tissues.[1] Chemokines can be classified into four groups (XC, CC, CXC and CX3C) based on the number and arrangement of conserved cysteine (C) residues near the N-terminus. Their respective receptors are classified based on their affinity for the classes of ligand: one known XC chemokine receptor (XCR1), ten CC receptors (CCR1-10), seven CXC receptors (CXCR1-7), and one CX3C receptor (CX3CR1). Chemokine ligands have been shown to have redundancy in their affinity for the various receptors, while one chemokine receptor may be able to transduce the signals of multiple ligands (e.g. the CC chemokine receptor 10 (CCR10) has two ligands, CC chemokine ligands CCL27 and CCL28), ligands may be able to bind to multiple receptors (e.g. CCL3 is a ligand for both CCR1 and CCR5). To achieve specificity and direct leukocyte recruitment, chemokine expression is regulated in a temporal and spatial manner, and receptor expression is often tissue- and/or cell-type specific.[2]

Chemokines and their receptors have been shown to be involved in the migration and growth of tumour cells in multiple haematological malignancies and solid tumours.[3-4] Multiple myeloma plasma cells (MM PCs) express a variety of chemokine receptors and secrete several chemokines,[5] highlighting the need to assess the role of these receptors in MM pathogenesis and identify which receptors would be useful therapeutic targets to slow disease progression or novel prognostic markers. BM MM PCs from patients have been demonstrated to express CCR1, CCR2, CCR5, CXCR3 and CXCR4, while MM cell lines have been shown to express CCR1, CCR2, CCR6, CCR10, CXCR3, CXCR4 and CXCR6.[5-11] Previous studies have demonstrated key roles for chemokine receptors expressed on MM PCs in tumour cell migration and proliferation. The interaction of CXCR4 with its ligand CXCL12 (also known as stromal cell-derived factor 1 (SDF-1)) is well-characterised to be crucial in the homing of MM PCs from the circulation to the BM[12,13] and their subsequent retention within the BM.[14,15] BM stromal cells (BMSCs) abundantly express CXCL12,[16] creating a concentration gradient that directs entry of MM PCs into the BM[12,13] and mediates strong MM PC-BMSC adhesion.[14,15] In Chapter 2, we demonstrated that CCR1 is a driver of MM PC dissemination *in vivo.* In multiple studies, CCL3, the ligand for CCR1 and CCR5, has

been shown to be a potent inducer of MM PC migration *in vitro*.[5,9] Similarly, the ligands for CCR2 (CCL2, also known as monocyte chemotactic protein 1 (MCP-1), and MCP-2 and -3),[7,17] CXCR1 (CXCL8, also known as interleukin 8 (IL-8)),[18] CXCR3 (CXCL9, also known as monocyte/macrophage-activating IFN-γ-inducible protein (Mig), [19]; CXCL10, also known as IFN-γ-inducible 10 kDa protein (IP10),[5,19]; and CXCL11, also known as interferon-inducible T-cell alpha chemoattractant (I-TAC),[19]) and CXCR6 (CXCL6)[6] have also been shown to induce the chemotaxis of MM cell lines and patient-derived MM PCs *in vitro*. In addition, there is some evidence that chemokines can increase MM PC proliferation *in vitro*. For example, CXCL8 has also been shown to induce MM cell line and patient-derived MM PC proliferation *in vitro,*[18] while CXCL12 induces a modest increase in proliferation in MM cell lines and patient MM PCs.[18,20] While we demonstrated in the preceding chapters that CCL3 does not affect MM PC proliferation in two MM cell lines *in vitro*, CCL3 has also been suggested to induce proliferation of some MM cell lines *in vitro*.[21] Additionally, CXCL12/CXCR4 has been shown to drive resistance to chemotherapy, through cell-adhesion mediated resistance.[22,23] Furthermore, we found that CCR1 expression decreases the sensitivity of MM cell lines to bortezomib therapy (Chapter 3). While these studies suggest that some chemokines and their receptors may play a functional role in MM, the role of the other chemokine receptors in MM pathogenesis remains to be elucidated. Furthermore, a systematic analysis of the expression of all chemokine receptors in MM patients and their association with patient survival has not been conducted. We have previously shown using *in silico* analysis that elevated BM MM PC *CCR1* expression is associated with a poorer prognosis in newly diagnosed MM patients.[9] In contrast, newly diagnosed patients with detectable BM MM PC CCR1 expression by flow cytometry were shown to have a better prognosis than CCR1-negative patients.[24] Additionally, previous studies suggest that elevated MM PC expression of CXCR4,[24,25] or CCR2[24], as assessed by flow cytometry, was associated with better prognosis in newly diagnosed MM patients. Here, we systematically analysed the expression of chemokine receptors in newly diagnosed MM patients using microarray analyses and identified CCR10 as a novel independent prognostic marker. We further assessed CCR10 protein expression in MGUS and MM patients using multicolour flow cytometry. Lastly, we assessed whether CCR10 expression is associated with high-risk clinical features or cytogenetic subgroups.

4.3. Materials and Methods

4.3.1. Publicly available microarray data

Microarray analysis of publicly available data was conducted as previously described.[26] For analysis of *CCR1, CCR2*, *CCR3, CCR4*, *CCR5*, *CCR6, CCR7, CCR8, CCR9, CCR10*, *CX3CR1, CXCR1*, *CXCR2, CXCR3*, *CXCR4*, *CXCR5*, *CXCR6*, *CXCR7* and *XCR1* expression in CD138-selected BM plasma cells from newly diagnosed MM patients and analysis of the association of elevated receptor expression with overall survival the dataset E-TABM-1138 (n=142)[27] was used. For this dataset, expression of $\log_2(250)$ was used as the cut-off for high or low expression. For analysis of *CCR10* expression in CD138-selected BM PCs from newly diagnosed MGUS or MM patients or normal controls, two independent microarray datasets were used: E-GEOD-16122 (normal, n=5; MGUS, n=11; MM, n=133)[28] and E-MTAB-363 (normal, n=5; MGUS, n=5; MM, n=155).[29] E-MTAB-363 and E-TABM-1138 were conducted on Affymetrix GeneChip Human Genome U133 plus 2.0 arrays; E-GEOD-16122 was conducted on U133A arrays. Raw microarray data (CEL files) were downloaded from ArrayExpress (EMBL-EBI) or Gene Expression Omnibus (GEO; NCBI) and were normalised by RMA using the bioconductor package (affy)[30] in R (version 3.03) and $\log_2$ transformed, as previously described.[9]

4.3.2. Publicly available RNA sequencing data

RNA-sequencing data was obtained from the Multiple Myeloma Research Foundation (MMRF) CoMMpass (MMRF-COMMPASS) dataset, accessed via the NIH NCI GDC Data Portal (9 August 2019). Gene expression data (expressed as fragments per kilobase per million mapped reads [FPKM]) for CD138-selected BM PC was included from 762 MM patients at initial diagnosis who had not received previous therapy [median age: 63 years (range 27–88); male:female ratio 1.69:1].

4.3.3. Patient samples

Ethical approval for this study was obtained from the University of Freiburg Medical Centre Ethics Review Committee and all patients provided written, informed consent, in accordance with the Declaration of Helsinki. Posterior superior iliac spine BM aspirates (n=27) were collected from symptomatic MM patients at initial diagnosis that had not received previous therapy [median age: 68 years (range 49–84); male:female ratio 1.15:1]. All MM patients fulfilled the diagnostic criteria for active MM.[31] A further 6

patients, defined as MGUS[31] were also examined. The patients presented at the University of Freiburg Medical Centre (Freiburg, Germany) between 15 June 2007 and 30 April 2019. BM was collected in K-EDTA tubes and BM mononuclear cells (BMMNC) were isolated by density gradient centrifugation (FICOLL) and were cryopreserved prior to use as described previously.[32] Overall survival was calculated from the date of BM collection until death, by any cause. Patients who were lost to follow up were censored at the date of last contact. For MM patients with follow up available (n=25), median follow up was 23 months (range: 4 – 133). Information regarding patient outcomes was last updated in January 2020.

4.3.4. Flow cytometry

Cell surface CCR10 expression was assessed and viable $CD38^{++}/CD138^{+/-}/CD45^{lo}/CD19^{-}$ malignant PCs were sorted from BM aspirates of MGUS (n=6) and MM (n=27) patients by multicolour flow cytometry (FACSARIA III; BD Biosciences) as previously described.[9] Briefly, $3x10^5$ mononuclear cells per test were stained with an anti-CCR10-PE (REA326; Miltenyi Biotech, Bergisch Gladbach, Germany) antibody, or no PE-conjugated primary antibody [fluorescence minus one (FMO) control], in combination with CD38-PE-Cy7 (HIT2; BioLegend, San Diego, CA), CD138-AlexaFluor-647 (B-A38; BioRad, Hercules, CA) CD45-FITC (J.33; Beckman Coulter, Brea, CA), and CD19-Brilliant Violet 421 (HIB19; BioLegend) antibodies. Cells were stained with the viability dye hydroxystilbamidine (FluoroGold; Invitrogen, Life Technologies, Carlsbad, CA) immediately before analysis. Viable, single cells were gated on the basis of FSC and SSC characteristics and FluoroGold negativity and MM PCs were identified as CD38-bright and CD138-positive or CD138-negative cells. As CD138 is frequently downregulated on frozen specimens, CD138 positivity was not absolutely required for defining the MM PC population. Contaminating cells were subsequently excluded by gating on the CD45-low and CD19-negative population. A minimum of 100 cells was required to fulfil these criteria to be identified as a PC population. A minimum of $5x10^4$ total nucleated cells were analysed per test. CCR10 expression was quantitated as the change in the median fluorescence intensity (ΔMFI), defined as the difference in MFI between the CCR10-stained sample and the FMO control. High CCR10 expression, delineated based on two distinct populations of MM PCs, was defined as ΔMFI above 750.

4.3.5. Real-time quantitative PCR (qPCR)

Total RNA was isolated from a minimum of 5 x 10^4 viable $CD38^{++}/CD138^{+/-}/CD45^{lo}/CD19^{-}$ malignant PCs sorted from BM aspirates of MGUS (n=3) and MM (n=12) by multicolour flow cytometry (FACSARIA III; BD Biosciences). Isolated RNA was DNAse treated using the RNeasy Micro Kit as per manufacturer's instructions (Qiagen, Hilden, Germany), and cDNA was synthesised using Sensiscript RT Kit as per manufacturer's instructions (Qiagen). qPCR was performed using a CFX Connect Real-Time PCR machine (BioRad, Hercules, CA) using the primers for *ACTB* (Fwd 5'-GATCATTGCTCCTCCTGAGC -3; Rev 5'- GTCATAGTCCGCCTAGAAGCAT -3') and *CCR10* (Fwd 5'- CCTGCTGCTGGATACTGCC -3'; Rev 5'-TCACCAGCAGTGCGACAT -3'). Changes in gene expression were calculated relative to *ACTB* using the $2^{-\Delta Ct}$ method.[33]

4.3.6. Statistical Analyses

Unless otherwise described, statistical analysis was performed using GraphPad Prism (version 8.1; GraphPad Software). Overall and progression-free survival was assessed using Kaplan–Meier curves; comparisons between groups were made using the logrank (Mantel–Cox) test and the Mantel–Haenszel hazard ratio. For comparison between patient groups, groups were compared using a Kruskal-Wallis or Mann-Whitney test. For all other experiments, groups were compared using a Mann-Whitney test. Univariate and multivariate analyses were conducted using SPSS version 26. Initially, univariate Cox regressions were conducted to identify factors that were significantly associated with survival ($p < 0.1$). These factors were then included in multivariate Cox proportional hazards models to determine if these factors affected the association between CCR10 expression and overall survival using the dataset E-TABM-1138. Differences were considered to be statistically significant when $p < 0.05$.

4.4. Results

4.4.1. Chemokine receptor expression in MM PCs isolated from newly diagnosed MM patients

Using *in silico* analysis of the publicly available microarray dataset E-TABM-1138 consisting of 142 newly diagnosed MM patients enrolled in the Total Therapy 3 (TT3) trial,[27] we systematically assessed the expression of chemokine receptors on BM CD138$^+$ PCs isolated from newly diagnosed MM patients (Figure 4.1). We found that *CCR2*, *CCR5* and *CXCR4* were expressed (defined as > $\log_2(250)$ expression) in the majority of MM patients (*CCR2*: n=138/142 patients, 97.2%; *CCR5*: n=138/142, 97.2%; *CXCR4*: n=141/142, 99.3%). Furthermore, *CCR1* (n=67/142, 47.0%), *CCR10* (n=70/142, 49.3%), *CXCR1* (n=49/142, 34.5%), *CXCR3* (n=41/142, 28.9%) and *CX3CR1* (n=18/142, 12.7%) were expressed in a moderate number of patients. In contrast, *CCR3* (n=0/142, 0%), *CCR4* (n=4/142, 2.8%), *CCR6* (0/142, 0%), *CCR7* (n=4/142, 2.8%), *CCR8* (0/142, 0%), *CCR9* (0/142, 0%), *CXCR2* (0/142, 0%), *CXCR5* (6/142, 4.2%) *CXCR6* (0/142, 0%), *CXCR7* (0/142, 0%), and *XCR1* (0/142, 0%) were expressed in a minority of patients or were not detectable in any patients analysed. Similar results were observed in an independent cohort of MM patients (n = 762) analysed by RNA-sequencing (CoMMpass dataset; Supplementary Figure 4.1).

4.4.2. CCR10 expression in BM MM PCs is associated with poorer prognosis in newly diagnosed MM patients

We have shown that 64.3% (18/28) (Chapter 3) to 81% (13/16)[9] of newly diagnosed MM patients express detectable CCR1 by flow cytometry on isolated BM MM PCs. Furthermore, using this dataset, we have shown that elevated *CCR1* expression in CD138$^+$ BM PCs is associated with poorer prognosis in newly diagnosed MM patients.[9] To determine whether expression of other chemokine receptor(s) have prognostic value in MM, we investigated whether BM PC expression of chemokine receptors was a significant predictor of overall survival in newly diagnosed patients. For highly expressed receptors *CCR2*, *CCR5* and *CXCR4*, which were expressed in the majority (>97%) of patients, patients were grouped into high- and low-expressing groups based on median expression for that receptor. For all other receptors, which were expressed in less than 40% of patients, patients were classed into receptor-positive and -negative groups based on expression above the cut-off of $\log_2(250)$. These analyses showed that above median *CCR2* (p=0.14, HR=1.8 [95% CI: 0.84-3.7]; Figure 4.2A), *CCR5*

Figure 4.1. Expression of chemokine receptors in newly diagnosed MM patients. *In silico* analysis was performed on a publicly available microarray dataset (E-TABM-1138) analysing gene expression in $CD138^+$ PC isolated from newly diagnosed MM patients (n = 142 patients). Expression less than 7.966 ($log_2(250)$; in grey) was classed as not expressed. Graph depicts median with interquartile range, showing all data points.

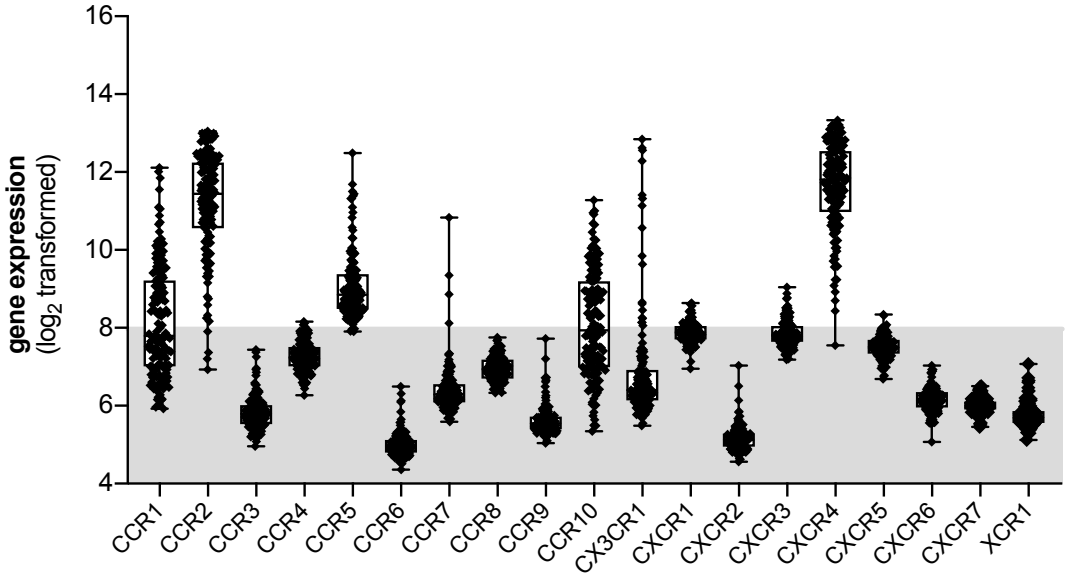
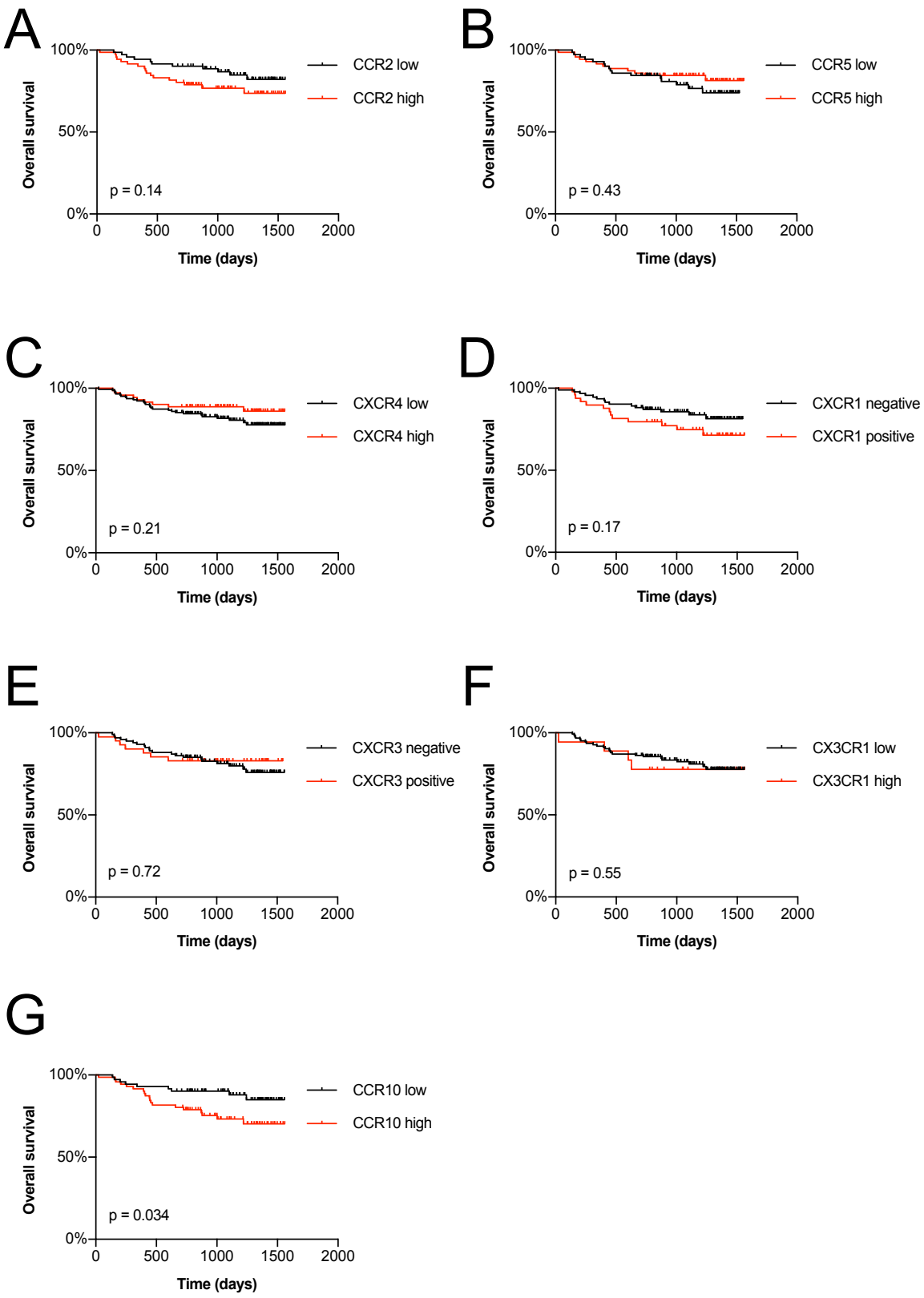

gene expression
(log2 transformed)
16
14
12
10
8
6
4
CCR1
CCR2
CCR3
CCR4
CCR5
CCR6
CCR7
CCR8
CCR9
CCR10
CX3CR1
CXCR1
CXCR2
CXCR3
CXCR4
CXCR5
CXCR6
CXCR7
XCR1

Figure 4.2. Association of chemokine receptors expressed on MM PCs with survival of newly diagnosed MM patients. Kaplan-Meier plots of overall survival are shown for newly diagnosed MM patients, stratified based on median $CD138^+$ PC expression of (**A**) *CCR2* (**B**) *CCR5* and (**C**) *CXCR4*, or based on positive- or negative-expression of (**D**) *CXCR1*, (**E**) *CXCR3* and (**F**) *CX3CR1* expression, or median expression of (**G**) *CCR10,* derived from microarray dataset E-TABM-1138 (n = 142 patients).


A
100%
50%
0%
Overall survival
p = 0.14
0
500
1000
1500
2000
Time (days)
CCR2 low
CCR2 high
B
p = 0.43
CCR5 low
CCR5 high
C
p = 0.21
CXCR4 low
CXCR4 high
D
p = 0.17
CXCR1 negative
CXCR1 positive
E
p = 0.72
CXCR3 negative
CXCR3 positive
F
p = 0.55
CX3CR1 low
CX3CR1 high
G
p = 0.034
CCR10 low
CCR10 high

(p=0.43, HR=0.74 [95% CI: 0.35-1.55]; Figure 4.2B) and *CXCR4* (p=0.21, HR=0.65 [95% CI: 0.33-1.3]; Figure 4.2C) expression were not significantly associated with overall survival. Additionally, expression of *CXCR1* (p=0.17, HR=1.7 [95% CI: 0.77-3.7; Figure 4.2D), *CXCR3* (p=0.72, HR=0.86 [95% CI: 0.38-2.0]; Figure 4.2E), *CX3CR1* (p=0.55, HR=1.2 [95% CI: 0.38-3.7]; Figure 4.2F) in MM patients was not associated with any effect on overall survival. Notably, however, above median *CCR10* expression was significantly associated with poor prognosis (p=0.034; HR=2.3 [95% CI: 1.1-4.8]; Figure 4.2G). The low number of positive patients precluded further investigation of the CCR4, CCR7 and CXCR5 chemokine receptors.

4.4.3. CCR10 is expressed in MGUS and MM patients and normal PCs

To further investigate *CCR10* expression in BM PCs of MGUS and MM patients as well as normal controls, we used *in silico* analysis of two independent publicly available microarray datasets E-MTAB-363 (n=5 MGUS, 155 MM patients and 5 normal controls) and GEOD16122 (n=11 MGUS,133 MM patients and 5 normal controls). *CCR10* was found to be consistently expressed across all groups of patients and normal controls (p=0.31, E-MTAB-363, Figure 4.3A; p=0.11, GEOD16122, Figure 4.3B). We confirmed this finding by sorting $CD38^{++}/CD138^{+/-}/CD45^{lo}/CD19^{-}$ malignant PCs (Figure 4.3C) from BM samples from 3 newly diagnosed MGUS and 12 newly diagnosed MM patients using flow cytometry. qPCR analysis showed that *CCR10* mRNA was detectable in all MGUS and 83.3% (10/12) of MM patients, with no difference in expression levels between patient groups (p=0.63, Figure 4.3D). To assess CCR10 protein expression, we utilised flow cytometry of $CD38^{++}/CD138^{+/-}/CD45^{lo}/CD19^{-}$ BM PCs (Figure 4.3C) in 27 newly diagnosed MM and 6 MGUS patients. MM patients were grouped into two distinct subgroups, with high CCR10 protein expression (ΔMFI > 750) being observed in 29.6% (8/27) of MM patients and 0% (0/6) of MGUS patients (Figure 4.4A). Notably, CCR10 protein expression was not significantly different between MGUS (ΔMFI median: 171.4, range: 29.5-489.8) and MM (ΔMFI median: 369.1, range: 76.80-2254) patients (p=0.0573, Figure 4.4B).

Our data suggests that elevated *CCR10* expression in BM MM PCs is associated with poor prognosis of newly diagnosed MM patients. In order to further analyse the association between CCR10 protein expression and poor survival, patients were delineated into those with high or low MM PC CCR10 expression, as determined by

Figure 4.3. *CCR10* is expressed by PCs from MGUS and MM patients and healthy donors. A. *In silico* analysis was performed on publicly available microarray dataset (E-MTAB-363) analysing gene expression of *CCR10* in $CD138^+$ PCs isolated from MGUS (n = 5) and MM (n = 155) patients and normal PCs from healthy donors (n = 5). Expression below 6.644 ($log_2(100)$; in grey) was classed as not expressed. **B**. *In silico* analysis was performed on publicly available dataset analysing gene expression of *CCR10* in $CD138^+$ PCs isolated from MGUS (n = 11) and MM (n = 133) patients and healthy controls (n = 5) (E-GEOD-16122). Expression below 6.644 ($log_2(100)$; in grey) was classed as not expressed. **C**. MM PCs were identified in the BM of newly diagnosed MGUS or MM patients as viable cells based on forward (FSC) and side (SSC) scatter characteristics and fluorogold-negativity. MM PCs were defined as $CD38^{++}CD138^{+/-}$ followed by exclusion of normal PCs that are CD45 and CD19 positive. A representative patient is shown. **D**. *CCR10* mRNA expression, normalised to *ACTB,* assessed by qPCR in $CD38^{++}/CD138^{+/-}/CD45^{lo}/CD19^-$ BM malignant PCs isolated from newly diagnosed MGUS (n=3) and MM (n=12) patients. Graphs depict median with interquartile range (**A**,**B**,**D**).

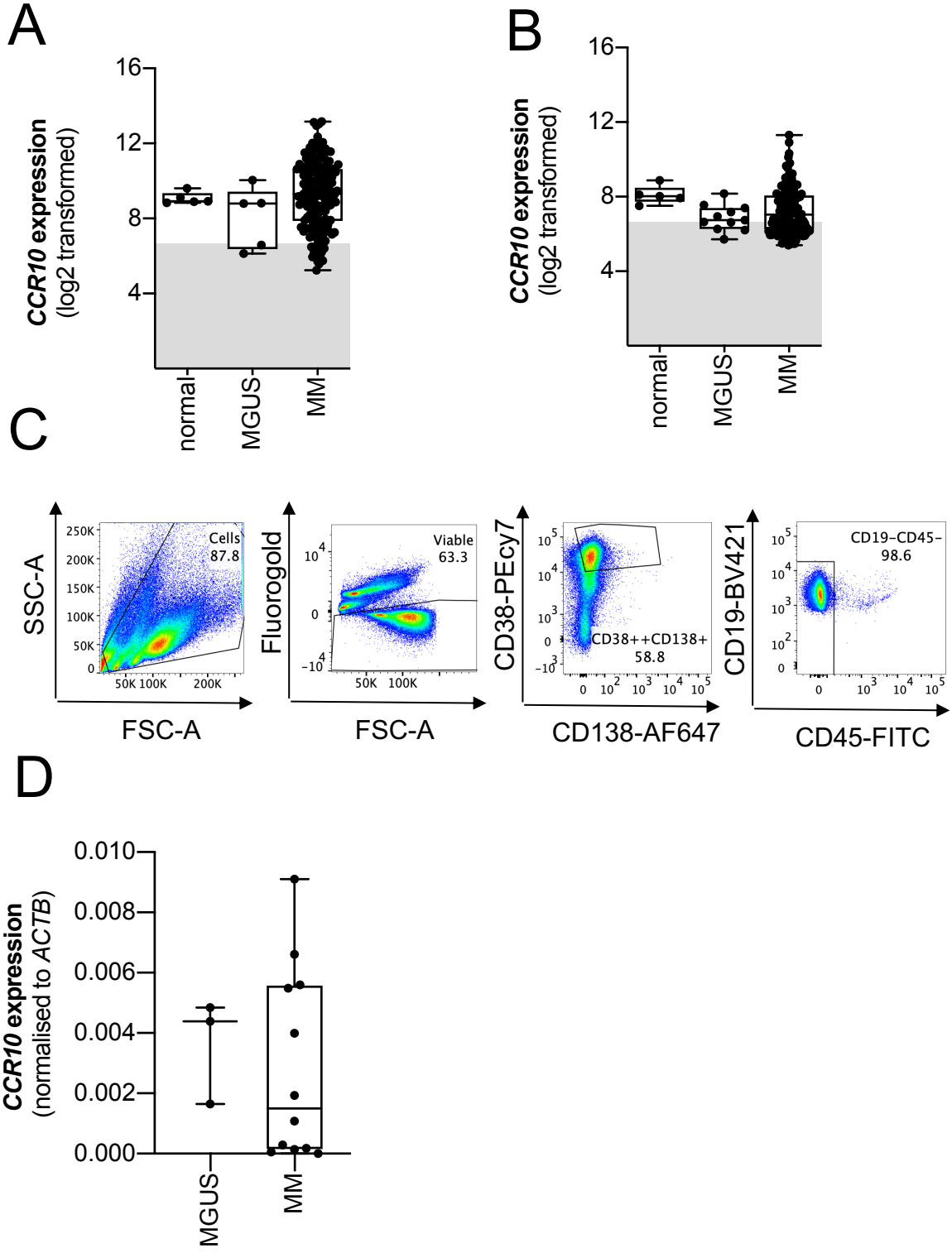

A
CCR10 expression
(log2 transformed)
16
12
8
4
normal
MGUS
MM
B
CCR10 expression
(log2 transformed)
16
12
8
4
normal
MGUS
MM
C
SSC-A
250K
200K
150K
100K
50K
0
50K 100K 200K
FSC-A
Cells
87.8
Fluorogold
Viable
63.3
FSC-A
CD38-PEcy7
CD38++CD138+
58.8
CD138-AF647
CD19-BV421
CD19-CD45-
98.6
CD45-FITC
D
CCR10 expression
(normalised to ACTB)
0.010
0.008
0.006
0.004
0.002
0.000
MGUS
MM

Figure 4.4. CCR10 protein expression is associated with decreased overall survival in newly diagnosed MM patients. A. CCR10 expression (unfilled histogram) and PE FMO control (filled histogram) analysed on $CD38^{++}/CD138^{+/-}/CD45^{lo}/CD19^{-}$ BM MM PCs is shown for 4 representative newly diagnosed MM patients. **B.** CCR10 protein expression (ΔMFI) on $CD38^{++}/CD138^{+/-}/CD45^{lo}/CD19^{-}$ BM malignant PCs is shown for newly diagnosed MGUS (n=6) and MM (n=27) patients analysed by flow cytometry. Expression below ΔMFI of 750 (based on two distinct MM PC populations; in grey) was classed as low CCR10 expression. Graph depicts median with interquartile range, showing all data points. **C**. Kaplan-Meier plot of overall survival is shown for 25 newly diagnosed MM patients stratified based on CCR10 protein expression analysed on $CD38^{++}/CD138^{+/-}/CD45^{lo}/CD19^{-}$ BM MM PCs by flow cytometry (**B**).

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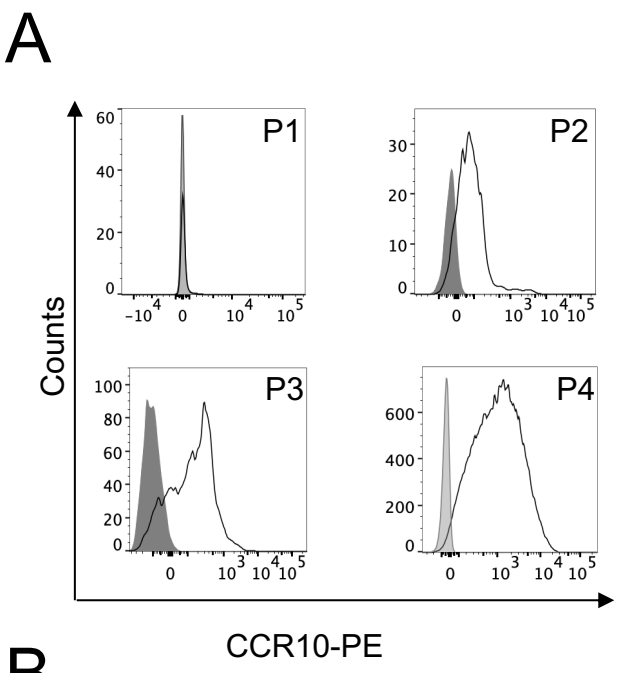
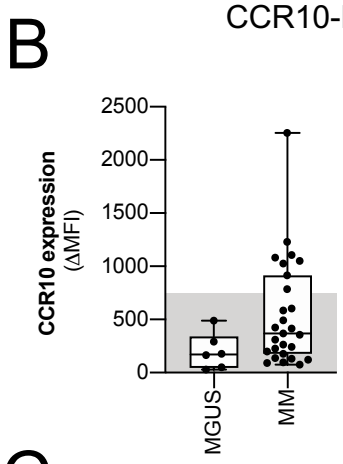
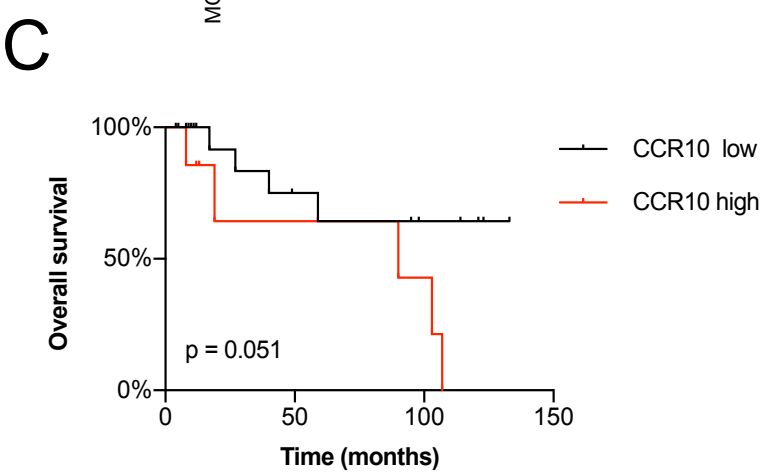

A
P1
P2
P3
P4
Counts
CCR10-PE


B
CCR10 expression
(ΔMFI)
MGUS
MM


C
Overall survival
p = 0.051
Time (months)
CCR10 low
CCR10 high

flow cytometry (using the cut-off ΔMFI 750), and patient overall survival was assessed. For the 25 patients with clinical follow up (median follow up: 23 months; range: 4–133), there was a trend towards high CCR10 expression and poorer overall survival, however this did not reach significance (p=0.051, HR=4.4 [95% CI: 0.99-19], median survival: 90 months vs not reached, Figure 4.4C).

4.4.4. CCR10 is an independent prognostic indicator in MM

We next assessed whether the poor prognosis associated with elevated CCR10 expression was associated with particular cytogenetic or clinical features in newly diagnosed MM patients. Recurring chromosomal abnormalities are a common feature in MM tumours, with t(4;14), t(14;16), t(14;20), del 17p and amp 1q being associated with poor prognosis, while t(11;14), t(6;14) and hyperdiploidy are associated with better prognosis.[35] Patients in the E-TABM-1138 gene expression dataset were subdivided based on the molecular subgroups described by the University of Arkansas for Medical Science (UAMS) molecular classification system: CD1 and CD2 (characterised by high *CCND1* and *CCND3* expression, corresponding to t(11;14) and t(6;14)), MF (characterised by high expression of *MAF* and *MAFB* proto-oncogenes, as seen in t(14;16) and t(14;20, respectively), MS (associated with high *FGFR3* and *MMSET* expression, associated with t(4;14)), HY (corresponding with the hyperdiploidy group), PR (associated with expression of numerous cell cycle and proliferation-related genes), and LB (Low bone disease; associated with lower number of magnetic resonance imaging (MRI)-defined focal lesions). While there were no statistically significant differences in the expression between UAMS subgroups in the E-TABM-1138 dataset, there were trends toward higher expression of *CCR10* in patients in the CD1, HY and PR subgroups (Figure 4.5). We found that *CCR10* was expressed (defined as > $\log_2(250)$ expression) in the majority of patients in the CD1, HY and PR subgroups (CD1: n=2/3 patients, 66.7%; HY: n=28/40, 70.0%; PR: n=10/15, 66.7%), a moderate proportion of patients in the CD2, MS and LB subgroups (CD2: n=4/11 patients, 36.4%; MS: n=6/13, 46.2%; LB: n=8/18, 44.4%), and in only one patient in the MF subgroup (n=1/10, 10%) (Figure 4.5).The relatively low incidence of poor prognostic cytogenetics precluded analysis of individual cytogenetic abnormalities in the CCR10 protein expression cohort (Table 4.1). However, we were able to search for any association between CCR10 expression and the presence of any poor cytogenetic prognostic markers. There was no significant association between CCR10 expression and overall

Figure 4.5. *CCR10* is expressed in most newly diagnosed MM cytogenetic subgroups. Expression of *CCR10* stratified based on UAMS subgroup: t(11;14) (CD1), t(6;14) (CD2), t(14;16) and t(14;20) (MF), t(14;4) (MS) translocations, hyperdiploidy (HY), low bone disease (LB) and proliferation (PR) groups. Derived from microarray dataset E-TABM-1138 (n = 142 patients). Graph depicts median with interquartile range.

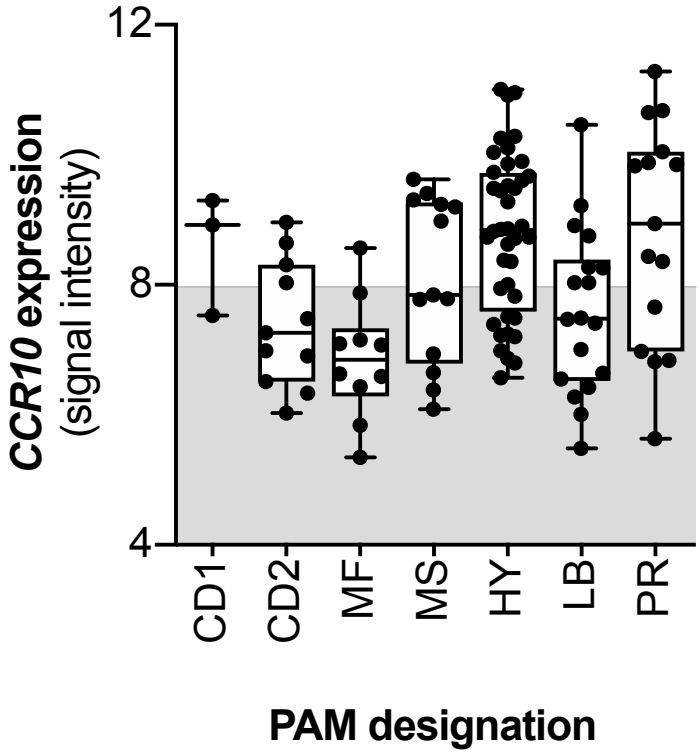

12
8
4
CCR10 expression
(signal intensity)
CD1
CD2
MF
MS
HY
LB
PR
PAM designation

Table 4.1. Baseline MM patient cytogenetic abnormalities grouped according to BM MM PC CCR10 protein expression

Cytogenetic abnormalities	Flow cohort (n=27)		
	CCR10 low (n=19)	CCR10 high (n=8)	p-value[#]
Hyperdiploidy			
Number	12/19	2/6	N/A
Percentage	63	33	
Deletion 13q			
Number	2/19	1/6	N/A
Percentage	10.5	16.7	
Amplification 1q			
Number	2/19	1/6	N/A
Percentage	10.5	16.7	
t(14;16)			
Number	0/19	1/6	N/A
Percentage	0	16.7	
t(11;14)			
Number	0/19	1/6	N/A
Percentage	0	16.7	
t(4;14)			
Number	2/19	0/6	N/A
Percentage	10.5	0	
Overall poor cytogenetics*			
Number	4/19	2/6	0.61
Percentage	21.1	33	

#χ^2 test or Fisher's exact test as appropriate

*based on the International Myeloma Working Group (IMWG) criteria (2016)

poor cytogenetics[35] (p=0.61, Table 4.1). To determine whether CCR10 is an independent prognostic indicator in MM, we assessed CCR10 expression, known clinical prognostic factors[34] and high-risk chromosomal abnormalities[35] for their association with overall patient survival (n=142 newly diagnosed patients, E-TABM-1138; Table 4.2). On univariable analysis, above median *CCR10*, elevated serum β2-microglobulin (> 5.5mg/L), anaemia (haemoglobin < 10g/dl) and high-risk gene expression signatures (MS, MF and PR subgroups) were associated with inferior survival ($p<0.05$, Table 4.2). These factors were then included in the multivariable analysis to determine if they affected the association between CCR10 expression and overall survival. Notably, in multivariate analysis, CCR10 retained its prognostic significance (p=0.014, Table 4.2). As CCR10 protein expression was not significantly associated with poorer prognosis, this precluded our ability to perform multivariable analysis on this cohort. However, we did observe that the incidence of none of the high-risk clinical features of patient age (>65 years), β2-microglobulin (>5.5mg/L), serum albumin (<35 g/L) or higher stage of the international staging system (ISS; based on β2-microglobulin and serum albumin) was significantly different in patients with high versus patients with low CCR10 protein expression (n=27 patients, $p>0.05$, Table 4.3).

Table 4.2. Univariable and multivariable analysis of overall survival associated with CCR10 and other factors

Factors	Univariable Analysis				Multivariable Analysis			
	p-value	HR	95% CI		p-value	HR	95% CI	
CCR10	0.04	2.30	1.04	5.09	0.014	2.81	1.24	6.37
Age > 65 years	0.62	0.77	0.32	1.97				
β-2-microglubulin > 5.5 mg/L	0.021	2.45	1.15	5.24	0.48	1.36	0.58	3.16
Serum albumin < 35 g/L	0.54	1.31	0.56	3.08				
Haemoglobin < 10 g/dl	0.001	3.52	1.67	7.45	0.006	3.34	1.41	7.90
High-risk cytogenetics* (MF, MS and PR subgroups#)	0.044	2.16	1.02	4.57	0.186	1.70	0.78	3.71

*based on the International Myeloma Working Group (IMWG) criteria (2016)
#UAMS molecular classification

Table 4.3. Baseline MM patient risk factors grouped according to BM MM PC CCR10 protein expression

Risk factors		Flow cohort (n=27)		
		CCR10 low (n=19)	CCR10 high (n=8)	p-value
Age >65 years				
Number		8/19	2/8	0.67
Percentage		42.1	25	
β-2-microglubulin >5.5 mg/L				
Number		5/18	2/8	>0.99
Percentage		27.8	25	
Serum albumin < 35 g/L				
Number		6/18	1/8	0.37
Percentage		33.3	12.5	
ISS Stage				
I	Number	6/17	5/8	
	Percentage	35.3	62.5	
II	Number	6/17	1/8	0.39
	Percentage	35.3	12.5	
III	Number	5/17	2/8	
	Percentage	29.4	25	
Paraprotein				
IgG	Number	14/19	3/8	0.10
	Percentage	73.7	37.5	
IgA	Number	3/19	1/8	>0.99
	Percentage	15.8	12.5	
Light chain	Number	2/19	4/8	0.04
	Percentage	10.5	50	

4.5. Discussion

Chemokine receptors expressed on MM PCs play functional roles in MM PC migration,[6,7,9,17,18] proliferation[18,20] and response to chemotherapy[22,23] (Chapter 3). Notably, CCR1, CCR2 and CXCR4 have previously been shown, by our group and others, to be prognostic markers in MM.[9,24,25] However, a systematic analysis of the association of chemokine receptor expression with survival in newly diagnosed MM patients has not been conducted. Here, we investigated the expression of all known chemokine receptors in BM PCs from newly diagnosed patients and determined whether their expression was associated with overall survival, in order to identify chemokine receptors that may play a previously unidentified functional role in MM pathogenesis. Of all chemokine receptors expressed in BM MM PCs, apart from *CCR1,* only *CCR10* expression was found to be significantly associated with poor prognosis in newly diagnosed MM patients.

Our analyses suggest that *CCR1* is expressed in almost half (47%) of newly diagnosed MM patients by microarray analysis. In accordance with the data presented here, studies from our group as well as others have shown that CCR1[5,9] is expressed in BM MM PCs from the majority of patients by flow cytometry. Furthermore, in Chapter 3 we showed that CCR1, as assessed by flow cytometry, is expressed in 64.3% (18/28) of newly diagnosed MM patients. In line with these studies showing a high proportion of CCR1 positivity, in Chapter 2 we demonstrated that CCR1 is crucial for the egress of MM PCs from the BM to the circulation during dissemination. Our analyses also suggested that *CXCR4* is expressed in a large proportion (99.3%) of newly diagnosed MM patients by microarray analysis. Studies from our group as well as others have shown that CXCR4[5,8,9] is expressed in BM MM PCs from the majority of patients by flow cytometry. In line with the consistent expression of CXCR4 observed here, MM PC expression of CXCR4 is well-established to be crucial for the homing to and subsequent retention within the BM, where BMSC produce high levels of the ligand CXCL12.[12-15] While CCR6 has been shown to be weakly expressed in some MM cell lines,[5] and its ligand CCL20 able to promote a chemotactic response in these MM cell lines,[5] previous studies assessing patient-derived BM MM PCs in small numbers of patients (4 to 5 patients per study)[5,8] were unable to detect CCR6 by flow cytometry. These findings are in accord with a lack of *CCR6* mRNA expression observed in the present study. Transcriptome profiling has previously revealed that elevated *CX3CR1* expression is

highly characteristic of patients with *MAF* or *MAFB* translocations (which make up approximately 6% of all MM patients),[36,37] in accordance with our finding that a relatively low number (12.7%) of MM patients express *CX3CR1*. While the functional role for CX3CR1 in MM is unclear, the human MM cell line RPMI-8226 has been shown to bind to CX3CL1 *in vitro* under shear flow, suggesting that it may play a role in adhesion to endothelial cells during MM PC homing or dissemination.[38] Our studies show that *CXCR3* is expressed in approximately 30% of patients, which contrasts two independent studies which showed that more that 90% of BM MM PCs from patients express CXCR3 expression as assessed by flow cytometry.[11,19] Supporting our findings, we also conducted analysis of RNA-sequencing data from the CoMMpass dataset and identified that *CXCR3* was expressed (FPKM > 1) in MM PCs from 32.9% (251/762) of patients (Supplementary Figure 4.1). These data suggesting that the high level of CXCR3 expression in previous flow cytometry studies may be due to antibody non-specificity as the same antibody clone was used in both studies. Previous studies have shown that the three CXCR3 ligands, CXCL9, CXCL10 and CXCL11, all induce the chemotaxis of MM PCs[5,19] and are able to increase the secretion of MMP-2 and MMP-9 by MM PCs *in vitro*,[19] suggesting that CXCR3 may be involved in MM PC migration within the BM. We also observed a high incidence (97.2% of MM patients) of *CCR5* expression. However, previous analysis of protein expression on BM MM PCs showed that only 25% (8/29) of patients express CCR5 protein,[10] which may be due to receptor internalisation or antibody non-specificity. Inhibition of CCR5 has been shown to decrease *in vivo* homing to the BM of $CCR5^{+}$ 5TMM MM cells[39] suggesting a role in dissemination of MM PCs. We also showed that the majority of MM patients (97.2%) express *CCR2* by microarray, in accordance with previous flow cytometry data which demonstrated that CCR2 is expressed in 82% (n=23/28) of MM patients.[7] Conditioned media from BM endothelial cells (BMECs), which contains CCL2 (ligand for CCR2),[17] is able to induce chemotaxis in MM cell lines and primary MM PCs *in vitro*,[7,17] suggesting that CCR2 may contribute to migration towards the vasculature. In support of this, migration of mouse 5T MM cells towards BMEC conditioned media can be blocked using an antibody against CCL2.[17] Notably, we demonstrated for the first time that *CXCR1* is expressed in approximately one third of MM patients. The ligand for CXCR1, namely CXCL8, has been shown to induce the chemotaxis and proliferation of MM cells *in vitro*.[18] While CXCR4, CCR2, CCR5, CXCR1, CX3CR1 and CXCR3 have been previously suggested to have functional roles in MM PCs, our analysis identified

no association between elevated BM MM PC expression of these receptors with survival of newly diagnosed MM patients.

Nakayama and colleagues have previously reported expression of *CCR10* mRNA in a panel of 4 human MM cell lines.[6] Here we report that *CCR10* is expressed in more than half of all newly diagnosed MM patients, as assessed by microarray and by qPCR. Our microarray analysis also suggested that *CCR10* is consistently expressed by normal PCs, as well as PCs from MGUS and MM patients. This finding concurs with previous microarray studies that showed that normal BM PCs have the highest expression of *CCR10* of any normal leukocyte subset analysed.[40] Additionally, BM $CD38^{++}$ PCs isolated from healthy donors have previously been shown to express an intermediate level of CCR10 by flow cytometry.[6] CCR10 has also been shown to be predominantly expressed on normal circulating IgA PCs found in the peripheral blood[41] or in the BM,[42] where they constitute approximately 40% of all BM PCs.[42] In this study, we assessed, for the first time, CCR10 protein expression in BM malignant PCs isolated from newly diagnosed MGUS and MM patients by flow cytometry and found that CCR10 was relatively lowly expressed in the majority of MGUS patients but was high in approximately one third of MM patients. While in normal PCs CCR10 is predominantly expressed by IgA PCs, we found no association between CCR10 expression and patients with IgA or IgG paraprotein in our cohort. Importantly, we also conducted multivariable analysis and found that elevated expression of CCR10, as assessed by microarray, retained its prognostic significance when known high-risk prognostic factors, including high-risk cytogenetics, anaemia or β2-microglobulin >5.5mg/L were taken into account, suggesting that CCR10 is an independent prognostic factor in MM. Furthermore, we showed that elevated BM MM PC *CCR10* expression is significantly associated with a poorer prognosis in newly diagnosed MM patients. Analysis of CCR10 protein expression on BM MM PCs showed similar results, with a trend towards high CCR10-expressing patients having a poorer outcome compared with low CCR10-expressing patients. Taken together, these data suggest that CCR10 may be a novel independent prognostic indicator in MM.

Previous studies suggest that CCR10 may play a role in the pathogenesis of solid tumours. For example, CCR10 has been shown to be expressed in 71% (63/89 patients) of human primary breast cancer tissue samples, and patients positive for CCR10 expression also presented with a higher tumour stage and increased incidence of local

invasion and lymph node metastasis.[43] Furthermore, one of the ligands for CCR10, namely CCL27, has been shown to promote migration and Matrigel invasion of a CCR10-positive breast cancer cell line *in vitro*.[43] Previous studies have shown that the ligands for CCR10, CCL27 and CCL28, also induce the chemotaxis of healthy donor BM PCs,[6] that CCL27 induces the chemotaxis of some, but not all, MM cell lines tested. In contrast, CCL28 was not found to induce the chemotaxis of MM cell lines *in vitro.*[44] CCL27 has been shown to be expressed by stromal cell lines and BM stromal cell cultures from MM patients, and CCL27 protein levels are increased in the BM of MM patients compared with healthy controls.[44] Taken together, these data suggest that CCL27 may play a role in migration of MM PCs towards stromal cell niches within the BM. Notably, elevated levels of CCL27 in BM plasma from MM patients, as assessed by ELISA, has been shown to be associated with shorter overall survival of MM patients, supporting our data on CCR10 expression.[44] As both ligands have been shown to have no effect on the proliferation of MM cell lines *in vitro*,[44] we hypothesise that CCR10 expression in MM patients promotes increased dissemination of MM PCs, and not increased tumour proliferation, leading to poorer prognosis.

While these data raise the possibility that BM CCL27 may play a role in MM pathogenesis, the most abundant sources of CCL27 and CCL28 are skin and mucosal tissues,[41,45-47] suggesting the potential role for CCR10 in driving dissemination to extramedullary sites. IgA positive PCs that express CCR10 selectively home to sites of high expression of the ligands; namely the oral and olfactory mucosa, salivary gland, mammary gland, small and large intestines, female reproductive tract, lungs and the trachea where epithelial cells are the primary source of CCL28,[41] and the skin where epidermal keratinocytes are the primary source for CCL27.[46] MM PCs are thought of as being strongly dependent on the BM microenvironment for their growth, however in 3-5% of MM patients at diagnosis dissemination of plasmacytomas to soft tissues occurs, known as extramedullary disease (EMD).[48] There is also a high incidence of EMD occurrence at relapse, with up to 20% of patients developing EMD lesions as detectable by PET scan,[49 50] while several studies report that two-thirds of MM patients have detectable EMD on autopsy.[51-53] The most common sites of extramedullary growth occur in skin/soft tissues (30%), spine (25%), liver (21%) and lymph nodes (21%), as assessed by PET scans.[50] Importantly, many of aforementioned tissues that CCR10-positive PCs home to including the lungs,[50] breast,[50] stomach,[54] large and small

intestine[54] and oral cavity[50] have been reported in retrospective studies and case series as being recurrent sites for EMD. This suggests a potential mechanism whereby CCR10-expressing MM PC may disseminate to soft tissue sites via CCL27 and CCL28. In support of this, a case study showed that a MM patient who developed EMD in the skin at relapse had increased CCR10 expression in MM PCs isolated from the BM at relapse compared with diagnosis.[55] Furthermore, there was a higher level of *CCR10* mRNA expressed in peripheral blood tumour cells of patients with T-cell leukaemia/lymphoma that developed skin lesions (n=10) compared with patients without lesions (n=18), as assessed by qPCR.[56] In MM, the development of EMD is associated with highly aggressive and disseminated disease, reflected by increased numbers of circulating tumour cells and poorer prognosis compared with MM patients without EMD.[57] It can therefore be postulated that the prognostic disadvantage conferred by elevated CCR10 expression may, at least in part, be associated with development of EMD.[50] It is important to note though that as EMD only occurs in up to 20% of MM patients[49] and, of those, only up to 30% present with skin, soft tissue or mucosal EMD,[50,58] at either diagnosis or relapse, this is unlikely to be the only mechanism underlying the prognostic disadvantage of CCR10 expression in MM patients. Future studies are required to determine whether CCR10 plays a role in the development of EMD in MM.

Our studies have identified that elevated chemokine receptor CCR10 expression in BM MM PCs is associated with poor prognosis in newly diagnosed MM patients. This study suggests that CCR10 is a novel independent prognostic marker in MM, but future studies on other cohorts of patients are required to confirm these findings. While the role of elevated CCR10 in MM remains to be demonstrated, this study provides a basis for future studies to investigate the mechanisms that underpin the adverse prognostic significance of CCR10 expression in MM.

4.6. Acknowledgements

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4.7. References

1. Rot A, von Andrian UH. Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. *Annu Rev Immunol* **2004**; 891-928
2. Vallet S, Anderson KC. CCR1 as a target for multiple myeloma. *Expert Opinion on Therapeutic Targets* **2011**; 9: 1037-1047
3. Balkwill F. Cancer and the chemokine network. *Nature Reviews Cancer* **2004**; 540
4. Müller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, et al. Involvement of chemokine receptors in breast cancer metastasis. *Nature* **2001**; 50
5. Moller C, Stromberg T, Juremalm M, Nilsson K, Nilsson G. Expression and function of chemokine receptors in human multiple myeloma. *Leukemia* **2003**; 1: 203-210
6. Nakayama T, Hieshima K, Izawa D, Tatsumi Y, Kanamaru A, Yoshie O. Cutting edge: profile of chemokine receptor expression on human plasma cells accounts for their efficient recruitment to target tissues. *J Immunol* **2003**; 3: 1136-1140
7. Vande Broek I, Asosingh K, Vanderkerken K, Straetmans N, Van Camp B, Van Riet I. Chemokine receptor CCR2 is expressed by human multiple myeloma cells and mediates migration to bone marrow stromal cell-produced monocyte chemotactic proteins MCP-1, -2 and -3. *Br J Cancer* **2003**; 6: 855-862
8. Durig J, Schmucker U, Duhrsen U. Differential expression of chemokine receptors in B cell malignancies. *Leukemia* **2001**; 5: 752-756
9. Vandyke K, Zeissig MN, Hewett DR, Martin SK, Mrozik KM, Cheong CM, et al. HIF-2a Promotes Dissemination of Plasma Cells in Multiple Myeloma by Regulating CXCL12/CXCR4 and CCR1. *Cancer Research* **2017**; 20: 5452-5463
10. Trentin L, Miorin M, Facco M, Baesso I, Carraro S, Cabrelle A, et al. Multiple myeloma plasma cells show different chemokine receptor profiles at sites of disease activity. *British Journal of Haematology* **2007**; 5: 594-602
11. Giuliani N, Bonomini S, Romagnani P, Lazzaretti M, Morandi F, Colla S, et al. CXCR3 and its binding chemokines in myeloma cells: expression of isoforms and potential relationships with myeloma cell proliferation and survival. *Haematologica* **2006**; 11: 1489

12. Alsayed Y, Ngo H, Runnels J, Leleu X, Singha UK, Pitsillides CM, et al. Mechanisms of regulation of CXCR4/SDF-1 (CXCL12)-dependent migration and homing in multiple myeloma. *Blood* **2007**; 7: 2708-2717
13. Roccaro AM, Mishima Y, Sacco A, Moschetta M, Tai YT, Shi J, et al. CXCR4 Regulates Extra-Medullary Myeloma through Epithelial-Mesenchymal-Transition-like Transcriptional Activation. *Cell Rep* **2015**; 4: 622-635
14. Sanz-Rodriguez F, Hidalgo A, Teixido J. Chemokine stromal cell-derived factor-1alpha modulates VLA-4 integrin-mediated multiple myeloma cell adhesion to CS-1/fibronectin and VCAM-1. *Blood* **2001**; 2: 346-351
15. Parmo-Cabañas M, Bartolomé RA, Wright N, Hidalgo A, Drager AM, Teixidó J. Integrin α4β1 involvement in stromal cell-derived factor-1α-promoted myeloma cell transendothelial migration and adhesion: role of cAMP and the actin cytoskeleton in adhesion. *Experimental Cell Research* **2004**; 2: 571-580
16. Kortesidis A, Zannettino A, Isenmann S, Shi S, Lapidot T, Gronthos S. Stromal-derived factor-1 promotes the growth, survival, and development of human bone marrow stromal stem cells. *Blood* **2005**; 10: 3793-3801
17. Vanderkerken K, Vande Broek I, Eizirik DL, Van Valckenborgh E, Asosingh K, Van Riet I, et al. Monocyte chemoattractant protein-1 (MCP-1), secreted by bone marrow endothelial cells, induces chemoattraction of 5T multiple myeloma cells. *Clinical & Experimental Metastasis* **2002**; 1: 87-90
18. Pellegrino A, Ria R, Di Pietro G, Cirulli T, Surico G, Pennisi A, et al. Bone marrow endothelial cells in multiple myeloma secrete CXC-chemokines that mediate interactions with plasma cells. *Br J Haematol* **2005**; 2: 248-256
19. Pellegrino A, Antonaci F, Russo F, Merchionne F, Ribatti D, Vacca A, et al. CXCR3-binding chemokines in multiple myeloma. *Cancer Letters* **2004**; 2: 221-227
20. Hideshima T, Chauhan D, Hayashi T, Podar K, Akiyama M, Gupta D, et al. The biological sequelae of stromal cell-derived factor-1alpha in multiple myeloma. *Molecular cancer therapeutics* **2002**; 7: 539
21. Lentzsch S, Gries M, Janz M, Bargou R, Dorken B, Mapara MY. Macrophage inflammatory protein 1-alpha (MIP-1 alpha) triggers migration and signaling cascades mediating survival and proliferation in multiple myeloma (MM) cells. *Blood* **2003**; 9: 3568-3573

22. Ghobrial I, Perez R, Baz R, Richardson PG, Anderson K, Sabbatini P, et al. Phase Ib Study of the Novel Anti-CXCR4 Antibody Ulocuplumab (BMS-936564) in Combination with Lenalidomide Plus Low-Dose Dexamethasone, or with Bortezomib plus Dexamethasone in Subjects with Relapsed or Refractory Multiple Myeloma. *Blood* **2014**; 21:
23. Azab AK, Runnels JM, Pitsillides C, Moreau A-S, Azab F, Leleu X, et al. CXCR4 inhibitor AMD3100 disrupts the interaction of multiple myeloma cells with the bone marrow microenvironment and enhances their sensitivity to therapy. *Blood* **2009**; 18: 4341-4351
24. Van de Broek I, Leleu X, Schots R, Facon T, Vanderkerken K, Van Camp B, et al. Clinical significance of chemokine receptor (CCR1, CCR2 and CXCR4) expression in human myeloma cells: the association with disease activity and survival. *Haematologica* **2006**; 2: 200-206
25. Bao L, Lai Y, Liu Y, Qin Y, Zhao X, Lu X, et al. CXCR4 is a good survival prognostic indicator in multiple myeloma patients. *Leukemia Research* **2013**; 9: 1083-1088
26. Cheong CM, Chow AW, Fitter S, Hewett DR, Martin SK, Williams SA, et al. Tetraspanin 7 (TSPAN7) expression is upregulated in multiple myeloma patients and inhibits myeloma tumour development in vivo. *Experimental Cell Research* **2015**; 1: 24-38
27. Shaughnessy JD, Jr., Qu P, Usmani S, Heuck CJ, Zhang Q, Zhou Y, et al. Pharmacogenomics of bortezomib test-dosing identifies hyperexpression of proteasome genes, especially PSMD4, as novel high-risk feature in myeloma treated with Total Therapy 3. *Blood* **2011**; 13: 3512-3524
28. Agnelli L, Mosca L, Fabris S, Lionetti M, Andronache A, Kwee I, et al. A SNP microarray and FISH-based procedure to detect allelic imbalances in multiple myeloma: an integrated genomics approach reveals a wide gene dosage effect. *Genes, Chromosomes and Cancer* **2009**; 7: 603-614
29. Reme T, Hose D, Theillet C, Klein B. Modeling risk stratification in human cancer. *Bioinformatics* **2013**; 9: 1149-1157
30. Gautier L, Cope L, Bolstad BM, Irizarry RA. affy--analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics* **2004**; 3: 307-315

31. Rajkumar SV. Updated Diagnostic Criteria and Staging System for Multiple Myeloma. *American Society of Clinical Oncology Educational Book* **2016**; 36: e418-e423
32. Zannettino ACW, Farrugia AN, Kortesidis A, Manavis J, To LB, Martin SK, et al. Elevated serum levels of stromal-derived factor-1α are associated with increased osteoclast activity and osteolytic bone disease in multiple myeloma patients. *Cancer Research* **2005**; 5: 1700-1709
33. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods* **2001**; 4: 402-408
34. Iriuchishima H, Saitoh T, Handa H, Isoda A, Matsumoto M, Sawamura M, et al. A new staging system to predict prognosis of patients with multiple myeloma in an era of novel therapeutic agents. *European Journal of Haematology* **2015**; 2: 145-151
35. Sonneveld P, Avet-Loiseau H, Lonial S, Usmani S, Siegel D, Anderson KC, et al. Treatment of multiple myeloma with high-risk cytogenetics: a consensus of the International Myeloma Working Group. *Blood* **2016**; 24: 2955-2962
36. Hurt EM, Wiestner A, Rosenwald A, Shaffer AL, Campo E, Grogan T, et al. Overexpression of c-maf is a frequent oncogenic event in multiple myeloma that promotes proliferation and pathological interactions with bone marrow stroma. *Cancer Cell* **2004**; 2: 191-199
37. Zhan F, Huang Y, Colla S, Stewart JP, Hanamura I, Gupta S, et al. The molecular classification of multiple myeloma. *Blood* **2006**; 6: 2020-2028
38. Cederblad L, Rosengren B, Ryberg E, Hermansson N-O. AZD8797 is an allosteric non-competitive modulator of the human CX3CR1 receptor. *Biochemical Journal* **2016**; 5: 641
39. Menu E, De Leenheer E, De Raeve H, Coulton L, Imanishi T, Miyashita K, et al. Role of CCR1 and CCR5 in homing and growth of multiple myeloma and in the development of osteolytic lesions: a study in the 5TMM model. *Clin Exp Metastasis* **2006**; 5-6: 291-300
40. Kawano Y, Kakoki M, Inada Y, Nishimura N, Okuno Y, Hata H, et al. Altered Expression of CCR10 Indicates Malignant Transformation of Bone Marrow Plasma Cells. *Blood* **2017**; Supplement 1: 1782-1782

41. Kunkel EJ, Kim CH, Lazarus NH, Vierra MA, Soler D, Bowman EP, et al. CCR10 expression is a common feature of circulating and mucosal epithelial tissue IgA Ab-secreting cells. *The Journal of clinical investigation* **2003**; 7: 1001-1010

42. Mei HE, Yoshida T, Sime W, Hiepe F, Thiele K, Manz RA, et al. Blood-borne human plasma cells in steady state are derived from mucosal immune responses. *Blood* **2009**; 11: 2461-2469

43. Lin HY, Sun SM, Lu XF, Chen PY, Chen CF, Liang WQ, et al. CCR10 activation stimulates the invasion and migration of breast cancer cells through the ERK1/2/MMP-7 signaling pathway. *Int Immunopharmacol* **2017**; 124-130

44. Thangavadivel S, Zelle-Rieser C, Olivier A, Postert B, Untergasser G, Kern J, et al. CCR10/CCL27 crosstalk contributes to failure of proteasome-inhibitors in multiple myeloma. *Oncotarget* **2016**; 48: 78605-78618

45. Eksteen B, Miles A, Curbishley SM, Tselepis C, Grant AJ, Walker LSK, et al. Epithelial Inflammation Is Associated with CCL28 Production and the Recruitment of Regulatory T Cells Expressing CCR10. *J Immunol* **2006**; 1: 593-603

46. Homey B, Alenius H, Muller A, Soto H, Bowman EP, Yuan W, et al. CCL27-CCR10 interactions regulate T cell-mediated skin inflammation. *Nat Med* **2002**; 2: 157-165

47. Hieshima K, Kawasaki Y, Hanamoto H, Nakayama T, Nagakubo D, Kanamaru A, et al. CC Chemokine Ligands 25 and 28 Play Essential Roles in Intestinal Extravasation of IgA Antibody-Secreting Cells. *The Journal of Immunology* **2004**; 6: 3668-3675

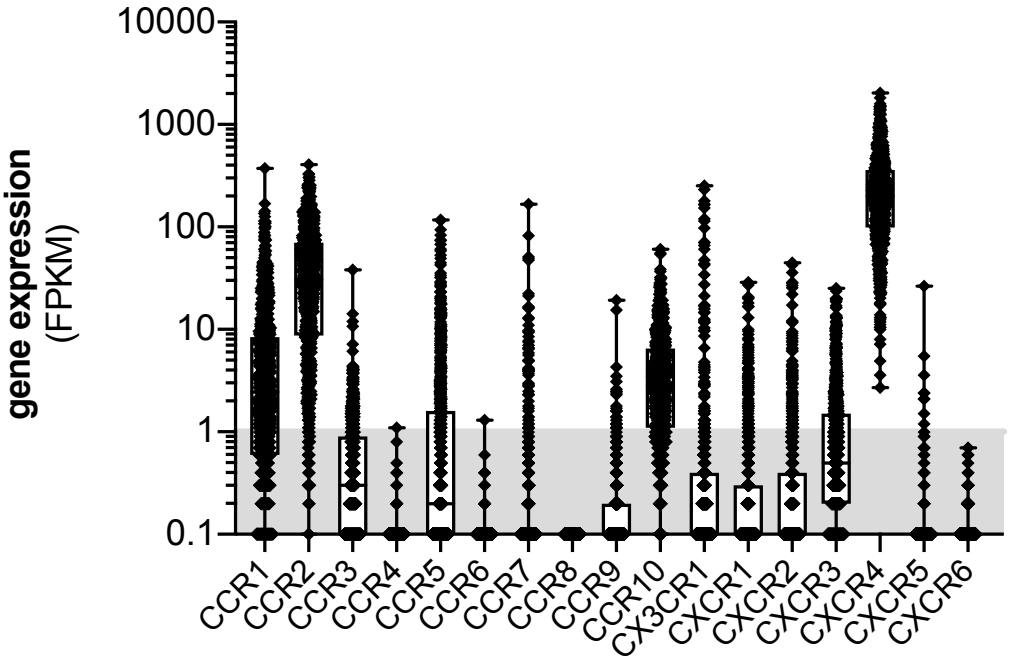
48. Jagosky MH, Usmani SZ. Extramedullary Disease in Multiple Myeloma. *Current Hematologic Malignancy Reports* **2020**;

49. Bladé J, Fernández de Larrea C, Rosiñol L, Cibeira MT, Jiménez R, Powles R. Soft-Tissue Plasmacytomas in Multiple Myeloma: Incidence, Mechanisms of Extramedullary Spread, and Treatment Approach. *Journal of Clinical Oncology* **2011**; 28: 3805-3812

50. Usmani SZ, Heuck C, Mitchell A, Szymonifka J, Nair B, Hoering A, et al. Extramedullary disease portends poor prognosis in multiple myeloma and is overrepresented in high risk disease even in era of novel agents. *Haematologica* **2012**; haematol.2012.065698

51. Pasmantier MW, Azar HA. Extraskeletal spread in multiple plasma cell myeloma: A review of 57 autopsied cases. *Cancer* **1969**; 1: 167-174
52. Oshima K, Kanda Y, Nannya Y, Kaneko M, Hamaki T, Suguro M, et al. Clinical and pathologic findings in 52 consecutively autopsied cases with multiple myeloma. *American Journal of Hematology* **2001**; 1: 1-5
53. Kapadia SB. Multiple myeloma: a clinicopathologic study of 62 consecutively autopsied cases. *Medicine* **1980**; 5: 380-392
54. Talamo G, Cavallo F, Zangari M, Barlogie B, Lee CK, Pineda-Roman M, et al. Clinical and biological features of multiple myeloma involving the gastrointestinal system. *Haematologica* **2006**; 7: 964
55. Marchica V, Accardi F, Storti P, Mancini C, Martella E, Dalla Palma B, et al. Cutaneous localization in multiple myeloma in the context of bortezomib-based treatment: how do myeloma cells escape from the bone marrow to the skin? *International Journal of Hematology* **2017**; 1: 104-108
56. Harasawa H, Yamada Y, Hieshima K, Jin Z, Nakayama T, Yoshie O, et al. Survey of chemokine receptor expression reveals frequent co-expression of skin-homing CCR4 and CCR10 in adult T-cell leukemia/lymphoma. *Leukemia & Lymphoma* **2006**; 10: 2163-2173
57. Sher T, Miller KC, Deeb G, Lee K, Chanan-Khan A. Plasma cell leukaemia and other aggressive plasma cell malignancies. *British Journal of Haematology* **2010**; 4: 418-427
58. Gagelmann N, Eikema D-J, Iacobelli S, Koster L, Nahi H, Stoppa A-M, et al. Impact of extramedullary disease in patients with newly diagnosed multiple myeloma undergoing autologous stem cell transplantation: a study from the Chronic Malignancies Working Party of the EBMT. *Haematologica* **2018**; 5: 890

Supplementary Figure 4.1. Expression of chemokine receptors in newly diagnosed MM patients. Gene expression data (FPKM) for CD138-selected BM MM PC isolated from newly diagnosed patients (CoMMpass dataset, n=762 patients). Expression less than 1 (in grey) was classed as not expressed. Graph depicts median with interquartile range, showing all data points.


gene expression
(FPKM)
10000
1000
100
10
1
0.1
CCR1
CCR2
CCR3
CCR4
CCR5
CCR6
CCR7
CCR8
CCR9
CCR10
CX3CR1
CXCR1
CXCR2
CXCR3
CXCR4
CXCR5
CXCR6

Chapter 5:

Discussion

5.1. Clinical implications of targeting CCR1 to prevent tumour dissemination in MM

Multiple myeloma (MM) is characterised by the growth of malignant plasma cells (PCs) within the bone marrow (BM).[1] MM is preceded by precursor diseases of monoclonal gammopathy of undetermined significance (MGUS) and smouldering MM (SMM), which are characterised by elevated clonal PCs in the BM, but no evidence of MM-related pathologies such as hypercalcemia, anaemia, osteolytic lesions and renal insufficiency.[1] One of the key characteristic features of MM is the presence of multiple MRI-detectable tumours throughout the skeleton,[1,2] suggesting that MM disease progression is dependent on the ability of MM PCs to leave a peripheral lymphoid organ, enter the peripheral circulation and disseminate to distal BM sites. In support of this, increased numbers of circulating tumour cells, present in the peripheral blood (PB) of MGUS and SMM patients, is associated with faster progression to symptomatic MM.[3-5] With this in mind, it could be hypothesised that targeting dissemination provides an opportunity to prevent or slow the progression of disease. In support of this, increased numbers of MM PCs in the PB of newly diagnosed MM patients is associated with poorer overall survival.[6-10] In Chapter 3, CCR1 expression in BM malignant PCs was shown to be upregulated in MM patients compared with MGUS patients, as assessed by microarray analysis and flow cytometry. As dissemination is one of the key drivers of disease development, this suggests that increased CCR1 expression may underlie the increased dissemination seen in MM patients compared with MGUS patients (as reflected by elevated circulating malignant PC).[11] In support of this, a recent RNA sequencing study of matched PB and BM MM PCs from 3 MM patients, revealed that transcriptional programs related to hypoxia are upregulated in PB MM PCs compared with their BM counterparts.[12] We have previously shown that CCR1 is the most highly upregulated gene when MM cell lines are subjected to hypoxia.[13] This may suggest a model whereby malignant PC growth within the BM increases BM hypoxia, leading to increased CCR1 expression and resulting in malignant PC dissemination and progression of disease. This raises the possibility that by inhibiting CCR1 during the asymptomatic precursor stages, it may lead to a delay in the transition to active MM.

To the best of our knowledge, the studies in Chapter 2 are the first to show that an inhibitor of CCR1 can slow spontaneous MM PC dissemination *in vivo.* To do this, we established mouse models of MM, in which treatment with the small molecule CCR1

inhibitor CCX9588, commencing at 3 days post-tumour cell injection, could reduce the development of secondary tumours *in vivo*. However, despite reducing MM PC dissemination, mice treated with CCX9588 still developed large secondary tumours over the course of treatment. This may be due to early dissemination events which occurred prior to initiation of therapy. Alternatively, this may reflect the fact that the treatment regimen used was unable to completely block MM PC dissemination. While the dose selected was based on pharmacokinetic data suggesting that trough PB levels of CCX9588 were sufficient to block CCR1, we did not achieve this dose for some animals, suggesting that a higher dose may be required. Furthermore, the concentration achieved in the BM may be lower than that achieved in the PB. In addition, the dose used was well-tolerated by the animals and could be increased in a subsequent study to determine if a complete blockade of dissemination could be achieved with a higher dose of CCX9588. Another option would be the development and testing of a CCR1 inhibitor with higher potency than CCX9588. In terms of the process of dissemination, subsequent homing and establishment of MM PCs is required for the colonisation and growth of new secondary tumours. This suggests that combination therapy, using an anti-dissemination therapy like CCR1-targeting to prevent circulating MM PC, in conjunction with an anti-homing agent to prevent the establishment of secondary tumours, may be more effective at slowing disease progression. Previous studies have demonsrated that anti-homing agents are effective at slowing homing and thereby slowing disease progression in *in vivo* MM models. Inhibition, either therapeutically or using stable knockdown, of molecules involved in adhesion of MM PCs to endothelial cells such as P-selectin glycoprotein-1,[14] CD44,[15] CD166[16] and N-cadherin[17,18] slowed *in vivo* homing of MM PCs and decreased tumour burden. The studies described in this thesis highlight the feasibility of targeting CCR1 to inhibit mobilisation of MM PCs to the BM. Further studies are warranted to determine whether an optimised CCR1 inhibitor dosing, the use of a more potent inhibitor, or combination with anti-homing strategies can slow MM disease progression.

While anti-dissemination therapy alone may not be sufficient to slow disease progression, it may extend the remission phase post-therapy. Following each round of therapy relapse occurs progressively sooner, highlighting the importance of extending the remission phase following front-line therapy to achieve better progression-free survival rates and extend patient overall survival.[19,20] In support of this, there is

evidence to suggest that dissemination of therapy-resistant clones may promote the development of overt relapse. Multiple studies have shown that time to progression is dramatically shorter in patients with detectable circulating PCs at baseline.[21,22] For example, a lack of detectable cells in the circulation following treatment is associated with a significantly longer time to progression (581 days), compared with patients whose circulating MM PC numbers increased following therapy (51 days).[21] This is further supported by studies suggesting that the expansion of MM PC is important for disease relapse following therapy,[23] with the appearance of focal tumour growth at new sites following disease relapse, as detected by MRI, being observed in ~30% of patients.[23] Lastly, MM tumours are found in disseminated sites other than the BM, termed EMD, in approximately 3-5% of MM patients at diagnosis, but this increases up to 20% at relapse, suggesting that during the development of relapse, MM PCs are disseminating and leading to the development of more aggressive forms of disease.[24] These studies suggest that the recolonisation of the BM at new sites by a dominant, therapy-resistant clone is associated with a rapid disease relapse. Therefore, anti-dissemination agents may potentially be an effective maintenance therapy to extend the remission phase and delay relapse. Future studies should address if the use of a CCR1 inhibitor as a maintenance therapy after front-line treatment can extend progression-free survival in *in vivo* models. Furthermore, studies could be conducted to determine if this therapeutic strategy could be harnessed for the treatment of $CCR1^{+}$ MM patients to determine if this leads to an increase in progression-free and overall survival. Patients who present with highly aggressive and disseminated disease (as assessed by increased circulating tumour cells, EMD and/or indications of plasma cell leukemia (PCL)),[25-28] may stand to benefit the most from anti-dissemination therapy as a maintenance therapy.

The findings of this thesis could also have implications for the treatment of other cancers. In addition to being frequently expressed in MM tumours, CCR1 is commonly expressed in T- and B-cell lymphomas (follicular lymphoma, diffuse large B-cell lymphoma, anaplastic large cell lymphoma peripheral T-cell lymphoma),[29] acute myeloid leukaemia,[29] and in tumour biopsies from breast[30] and ovarian cancer[31] patients. Additionally, previous studies have shown that expression of CCR1 promotes invasion of non-small cell lung cancer,[32] hepatocellular carcinoma,[33] breast cancer,[30]

ovarian cancer[34] and prostate cancer[35] cell lines *in vitro*. Thus, it is possible that CCR1 inhibitors may be therapeutically useful in preventing metastasis of solid tumours.

5.2. Clinical implications of targeting CCR1 to increase drug sensitivity

The use of the proteasome inhibitor, bortezomib, as a front-line or maintenance therapy, has led to significant improvements in MM patient survival.[36] Currently, bortezomib is used as an upfront therapy in approximately 50% of MM patients in the USA and 75% of patients in Europe, the Middle East, and Africa.[37-41] Notably, bortezomib is a mainstay of treatment for MM in Australia, where is used as induction therapy in approximately 85% of MM patients.[42] While responses to bortezomib-based regimens are effective in the majority of cases, approximately 20% of patients present with intrinsic therapeutic resistance and do not respond to front-line bortezomib-based therapy.[28] Importantly, these patients have a median overall survival of only 2 years,[28] compared with 6-10 years for other patients.[36,43,44] Of those patients who do respond, emergence of secondary bortezomib resistance is common, with approximately 40-50% of bortezomib-treated patients not responding to retreatment at relapse.[45,46] As bortezomib is used in second-line regimens in approximately 25% of cases in the USA,[37] this highlights the importance of achieving a high depth of response to induction therapy for long-term patient survival prospects.[19,20] In Chapter 3, CRISPR-Cas9 deletion of CCR1 in MM cells significantly increased their response to initial bortezomib therapy, suggesting that CCR1 expression increases intrinsic resistance to bortezomib. Furthermore, we also found that patients with either elevated BM MM PC CCR1 expression at diagnosis, or with induction of CCR1 expression at relapse, have a poorer prognosis compared with patients with low MM PC CCR1 expression throughout treatment. It can therefore be postulated that inclusion of a CCR1 inhibitor to front-line bortezomib-based therapy might sensitise the tumour cells and achieve a greater depth of response in $CCR1^+$ patients. As CCR1 is expressed in more than 70% of MM patients[13] (Chapter 3), inclusion of a CCR1 inhibitor, if effective, could be beneficial for the majority of MM patients.

CXCR4 expressed on MM PCs is critical for homing to and subsequent retention within the BM. CXCL12 is abundantly produced by BM stromal cells (BMSCs),[47] forming a concentration gradient that directs PC entry into the BM, but also increases expression of integrin α4β1 on MM PCs to mediate adhesion to BMSCs and retention in the

niche.[48] In a recent clinical trial, the CXCR4 inhibitor, plerixafor, was used in combination with dexamethasone and bortezomib in relapsed/refractory MM patients with the aim to decrease adhesion of MM PCs to BMSCs, mobilising MM PCs from the protective niche and therefore increase the response to therapy.[49] Plerixafor was shown to cause at least a partial response to subsequent chemotherapy in 31% (18/58) of patients.[49] While this suggested a potential increase in response rates, it is important to note that treatment with plerixafor also mobilised tumour cells into the blood, which may therefore increase the chance of subsequent MM PC dissemination. In this thesis, we showed that CCR1 inhibition can slow MM PC dissemination and that loss of CCR1 expression increases sensitivity to bortezomib therapy. Therefore, treatment with a CCR1 inhibitor may prove more effective than plerixafor, as the tumour cells will be sensitised to therapy with no added risk of spread to distal sites. This suggests another potential therapeutic strategy, whereby CCR1 inhibitors could be used in combination with front-line therapy to enhance sensitivity and retained as maintenance therapy to limit spread of any treatment-resistant tumours. Future studies are warranted to investigate whether this strategy would increase depth of response to therapy and subsequently extend progression-free survival.

CCR1 expression has also been shown to be elevated in a chemotherapy-resistant prostate cancer cell line,[35] suggesting that CCR1 may play a role in the sensitivity of other cancer cells to therapeutics. Therefore, CCR1 inhibitors may also increase the efficacy of anti-tumour drugs in other cancers and warrants further investigation.

5.3. Use of CCR1 inhibitors in the clinic

CCR1 plays a role in the activation and trafficking of immune cells, including neutrophils, monocytes, and lymphocytes.[50] On the basis of these roles, CCR1 inhibitors have entered clinical trials for the treatment of a range of inflammatory diseases. For example, the CCR1 inhibitors BX471 and AZD-4818 have been assessed in phase II trials for multiple sclerosis and chronic obstructive pulmonary disease, respectively; however, these studies demonstrated a lack of clinical benefit in these conditions.[51] In contrast, several studies have demonstrated some efficacy for CCR1 inhibition in the treatment of rheumatoid arthritis (RA). The small molecule inhibitor CP-481,715 entered Phase II clinical trials for the treatment of RA and was found to significantly reduce macrophage and other $CCR1^+$ cell infiltration into the synovial membrane of the

knee joint.[52] Importantly, there was a clinical improvement in a third of treated patients, but this did not reach statistical significance in this small study. Similarly, the small molecule inhibitor CCX354, an analogue of the inhibitor CCX9588 used in the studies described in this thesis, entered Phase II trials for the treatment of RA and showed biological and clinical efficacy.[53] In contrast, MLN3897 also entered a Phase II proof-of-concept study for the treatment of RA but was no longer pursued as it did not show discernible activity, possibly due to insufficient CCR1 blockade.[54] Importantly, CCR1 targeting has been shown to be generally well tolerated in all of the above-mentioned clinical trials.[52-55]

In the treatment of MM, a number of CCR1 inhibitors have shown promise in pre-clinical mouse models with regard to the prevention of the development of severe osteolytic lesions.[56,57] Osteolytic lesions are a major debilitating symptom of MM,[58,59] associated with bone pain and pathological fractures, and prevention of the formation of overt lesions would be extremely beneficial for patients. CCR1 is expressed on osteoclasts, with studies showing that CCL3 produced by MM PCs activates CCR1 on pre-osteoclasts to promote their differentiation into mature osteoclasts and thereby increasing tumour associated bone destruction.[60,61] Notably, inhibition of CCR1 has previously been demonstrated to inhibit tumour associated bone loss in mouse models of MM. In a MM mouse model, the CCR1 inhibitor MLN3897 was shown to decrease osteoclast formation and activity.[62] CCR1 inhibition using the small molecule inhibitor CCX721 resulted in a reduction in the number of osteoclasts per bone surface compared with controls in an MM mouse model, a reduction that was comparable to using the bisphosphonate zoledronic acid.[56] Furthermore, this study showed that CCR1 inhibition reduced tumour burden in mice compared with controls. Similarly, a study by Menu and colleagues showed that treatment of mice with the CCR1 inhibitor BX471 reduced the number of osteolytic lesions and tumour burden compared with controls.[57] Notably, these *in vivo* studies utilised the MM cell lines 5TMM and 5TGM1 that express very little or no CCR1 on their cell surface but produce CCL3.[56,57] Therefore, the effects on tumour burden are suggested to be secondary to inhibition of osteoclasts, which are a known source of MM PC growth factors such as IL-6.[63] Taken together with previous studies, the findings presented in this thesis suggest that targeting of CCR1 may be beneficial to target multiple aspects of MM pathogenesis: reducing dissemination and

tumour burden, reducing the severity of osteolytic lesions, and potentially increasing the efficacy of bortezomib.

5.4. CCR10 as a novel prognostic factor in MM

MM PCs have been shown to express a variety of chemokine receptors and express a range of chemokine ligands,[64] but the role of many of these receptors as therapeutic targets or biomarkers in MM remains unclear. Our previous study showed that elevated BM MM PC *CCR1* expression was associated with poorer prognosis of newly diagnosed MM patients.[13] In this thesis, several molecular mechanisms that may be involved in the prognostic disadvantage of CCR1 in MM were identified. To further investigate the role of chemokine receptors in MM and identify other receptors which may play a role in MM pathogenesis, a systematic analysis of the association between MM PC chemokine receptor expression prior to therapy and survival in MM patients was conducted (Chapter 4). These analyses identified that elevated *CCR10* expression is associated with poorer prognosis of newly diagnosed MM patients. Furthermore, eCCR10 expression, as assessed by microarray, was not associated with any known prognostic markers. These studies suggest that CCR10 is a novel independent prognostic marker in MM, although this requires validation in an independent patient cohort.

While MM PCs are thought of as being strongly dependent on the BM microenvironment for their growth, the growth of plasmacytomas in soft tissues, known as EMD, occurs in about 3-5% of MM patients at diagnosis,[24] with the most common sites of growth occurring in skin/soft tissues (23-30%), kidney (27%), spine (14-25%), lymph nodes (17-21%) and liver (8-21%), as assessed by PET scans.[65,66] Furthermore, in up to 20% of patients, EMD develops at relapse,[24] with sites of common growth being liver (34%), spine (23%) and skin/soft tissues (14%), as assessed by PET scans.[65] Importantly, patients with EMD often present with highly aggressive disease and have a poorer prognosis compared with MM patients without EMD.[27] Despite the need for targeted therapeutics to prevent the development of EMD, what signals expressed within soft tissues enables MM PC dissemination to these sites remains unclear. The ligands for CCR10, CCL27 and CCL28, are highly expressed in and dictate the infiltration of $CCR10^+$ inflammatory cells to the skin and mucosal surfaces, respectively.[67] Furthermore, CCL27 has been shown to induce the chemotaxis of MM

cell lines *in vitro*.[68,69] These studies suggest that extramedullary spread of CCR10$^+$ MM PCs to the skin and mucosal surfaces may be mediated in part by CCR10 and its ligands, identifying one potential mechanism underpinning the prognostic disadvantage of elevated CCR10 expression in MM patients. While further investigation of this mechanism is needed, inhibition of CCR10 may represent a novel targeted therapy for the prevention skin and mucosal surface EMD development in MM. Furthermore, while PET-detectable skin and mucosal infiltration is generally a rare event in MM patients, being observed in less than 2% of MM patients,[65] the actual frequency of dissemination to these sites may be more frequent than is commonly identified in studies that rely on imaging or clinical diagnosis to identify EMD. For example, while lung EMD is reported in less than 1% of MM patients clinically, it has been observed in 3%-15% of all MM patients on autopsy,[70-73] suggesting that the true contribution of soft tissue infiltration of MM PCs may be higher than realised. Future studies should investigate the association of CCR10 expression and incidence of EMD in patients and determine if CCR10 drives MM PC dissemination and EMD development *in vivo*.

5.5. Concluding Remarks

While the majority of MM patients currently have a median overall survival of 6-10 years,[36,43,44] approximately 20% of MM patients have a median overall survival of only 2 years.[28] These patients are classified as high-risk and are characterised by poor cytogenetic features, elevated numbers of circulating tumour cells and poorer responses to front-line therapy leading to shorter time to relapse.[28] This suggests that both intrinsic therapeutic resistance and increased circulating tumour cells contribute to poorer prognosis in MM. We have previously shown that elevated CCR1 expression is associated with increased numbers of circulating tumour cells and poorer prognosis in newly diagnosed MM patients.[13] This thesis definitively demonstrated that CCR1 is crucial for the egress of MM PCs from the BM to the circulation in MM mouse models. Furthermore, we are the first, to our knowledge, to show that targeting of dissemination *in vivo* is feasible, with results indicating that therapeutic inhibition of CCR1 slowed the dissemination of MM PCs. While these studies suggest that anti-dissemination therapy alone may not be effective at slowing disease progression, CCR1 inhibitors may be beneficial as a maintenance therapy to prevent the dissemination of therapy-resistant clones in CCR1$^+$ patients. We further demonstrated that CCR1 expression decreased the sensitivity of MM PCs to bortezomib therapy, suggesting that CCR1 inhibitors may

synergise with bortezomib to increase anti-tumour effects. Taken together, our findings on the role of CCR1 in dissemination and therapeutic resistance coupled with previous studies showing that CCR1 inhibitors can reduce the development of osteolytic lesions in mouse models of MM[56,57,62] suggest that inhibition of CCR1 may be an effective strategy to target multiple aspects of MM pathogenesis.

This thesis further investigated the association of chemokine receptors expressed in patient-derived MM PCs with patient survival to identify receptors that play a role in MM pathogenesis and/or potential biomarkers. Using patient specimens and interrogation of large patient datasets, this thesis showed that elevated CCR10 expression in BM MM PCs is associated with poorer prognosis of newly diagnosed MM patients. CCR10 expression did not correlate with known prognostic markers, suggesting that CCR10 may represent a novel independent prognostic marker in MM, although this requires confirmation in an independent cohort of patients. This thesis research has provided a basis for future studies to determine the mechanisms underlying the prognostic disadvantage of elevated CCR10 in MM pathogenesis.

5.6. References

1. Rajkumar SV. Updated Diagnostic Criteria and Staging System for Multiple Myeloma. *American Society of Clinical Oncology Educational Book* **2016**; 36: e418-e423
2. Ghobrial IM. Myeloma as a model for the process of metastasis: implications for therapy. *Blood* **2012**; 1: 20-30
3. Bianchi G, Kyle RA, Larson DR, Witzig TE, Kumar S, Dispenzieri A, et al. High levels of peripheral blood circulating plasma cells as a specific risk factor for progression of smoldering multiple myeloma. *Leukemia* **2013**; 3: 680-685
4. Gonsalves WI, Rajkumar SV, Dispenzieri A, Dingli D, Timm MM, Morice WG, et al. Quantification of circulating clonal plasma cells via multiparametric flow cytometry identifies patients with smoldering multiple myeloma at high risk of progression. *Leukemia* **2017**; 1: 130-135
5. Kumar S, Rajkumar SV, Kyle RA, Lacy MQ, Dispenzieri A, Fonseca R, et al. Prognostic value of circulating plasma cells in monoclonal gammopathy of undetermined significance. *J Clin Oncol* **2005**; 24: 5668-5674
6. Dingli D, Nowakowski GS, Dispenzieri A, Lacy MQ, Hayman SR, Rajkumar SV, et al. Flow cytometric detection of circulating myeloma cells before transplantation in patients with multiple myeloma: a simple risk stratification system. *Blood* **2006**; 8: 3384-3388
7. Chakraborty R, Muchtar E, Kumar SK, Jevremovic D, Buadi FK, Dingli D, et al. Risk stratification in myeloma by detection of circulating plasma cells prior to autologous stem cell transplantation in the novel agent era. *Blood Cancer Journal* **2016**; 12: e512
8. Gonsalves WI, Rajkumar SV, Gupta V, Morice WG, Timm MM, Singh PP, et al. Quantification of clonal circulating plasma cells in newly diagnosed multiple myeloma: implications for redefining high-risk myeloma. *Leukemia* **2014**; 10: 2060-2065
9. Nowakowski GS, Witzig TE, Dingli D, Tracz MJ, Gertz MA, Lacy MQ, et al. Circulating plasma cells detected by flow cytometry as a predictor of survival in 302 patients with newly diagnosed multiple myeloma. *Blood* **2005**; 7: 2276-2279

10. Witzig TE, Gertz MA, Lust JA, Kyle RA, O'Fallon WM, Greipp PR. Peripheral blood monoclonal plasma cells as a predictor of survival in patients with multiple myeloma. *Blood* **1996**; 5: 1780-1787
11. Sanoja-Flores L, Flores-Montero J, Garcés JJ, Paiva B, Puig N, García-Mateo A, et al. Next generation flow for minimally-invasive blood characterization of MGUS and multiple myeloma at diagnosis based on circulating tumor plasma cells (CTPC). *Blood cancer journal* **2018**; 12: 117-117
12. Garces JJ, Simicek M, Vicari M, Brozova L, Burgos L, Bezdekova R, et al. Transcriptional profiling of circulating tumor cells in multiple myeloma: a new model to understand disease dissemination. *Leukemia* **2020**; 2: 589-603
13. Vandyke K, Zeissig MN, Hewett DR, Martin SK, Mrozik KM, Cheong CM, et al. HIF-2a Promotes Dissemination of Plasma Cells in Multiple Myeloma by Regulating CXCL12/CXCR4 and CCR1. *Cancer Research* **2017**; 20: 5452-5463
14. Azab AK, Quang P, Azab F, Pitsillides C, Thompson B, Chonghaile T, et al. P-selectin glycoprotein ligand regulates the interaction of multiple myeloma cells with the bone marrow microenvironment. *Blood* **2012**; 6: 1468-1478
15. Asosingh K, Günthert U, De Raeve H, Van Riet I, Van Camp B, Vanderkerken K. A unique pathway in the homing of murine multiple myeloma cells: CD44v10 mediates binding to bone marrow endothelium. *Cancer research* **2001**; 7: 2862
16. Xu L, Machal C, Wu H, Poteat B, Crean CD, Cardoso AA, et al. Role Of CD166 In Multiple Myeloma Cell Homing To The Bone Marrow Microenvironment and Disease Progression. *Blood* **2013**; 21: 3102-3102
17. Mrozik KM, Cheong CM, Hewett D, Chow AW, Blaschuk OW, Zannettino AC, et al. Therapeutic targeting of N-cadherin is an effective treatment for multiple myeloma. *Br J Haematol* **2015**; 3: 387-399
18. Groen RWJ, de Rooij MFM, Kocemba KA, Reijmers RM, de Haan-Kramer A, Overdijk MB, et al. N-cadherin-mediated interaction with multiple myeloma cells inhibits osteoblast differentiation. *Haematologica* **2011**; 11: 1653
19. Martinez-Lopez J, Wong SWK, Shah N, Bahri N, Zhou K, Martin TG, et al. Minimal residual disease clinical monitoring and depth of response in multiple myeloma. *Journal of Clinical Oncology* **2019**; 15_suppl: 8026-8026
20. Lahuerta J-J, Paiva B, Vidriales M-B, Cordón L, Cedena M-T, Puig N, et al. Depth of Response in Multiple Myeloma: A Pooled Analysis of Three

PETHEMA/GEM Clinical Trials. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **2017**; 25: 2900-2910

21. Peceliunas V, Janiulioniene A, Matuzeviciene R, Zvirblis T, Griskevicius L. Circulating plasma cells predict the outcome of relapsed or refractory multiple myeloma. *Leukemia & Lymphoma* **2012**; 4: 641-647
22. Chakraborty R, Muchtar E, Kumar SK, Jevremovic D, Buadi FK, Dingli D, et al. Serial measurements of circulating plasma cells before and after induction therapy have an independent prognostic impact in patients with multiple myeloma undergoing upfront autologous transplantation. *Haematologica* **2017**; 8: 1439-1445
23. Keats JJ, Chesi M, Egan JB, Garbitt VM, Palmer SE, Braggio E, et al. Clonal competition with alternating dominance in multiple myeloma. *Blood* **2012**; blood-2012-2001-405985
24. Jagosky MH, Usmani SZ. Extramedullary Disease in Multiple Myeloma. *Current Hematologic Malignancy Reports* **2020**;
25. Ganzel C, Rouvio O, Avivi I, Magen H, Jarchowsky O, Herzog K, et al. Primary plasma cell leukemia in the era of novel agents for myeloma - a multicenter retrospective analysis of outcome. *Leuk Res* **2018**; 9-14
26. Qu X, Chen L, Qiu H, Lu H, Wu H, Qiu H, et al. Extramedullary Manifestation in Multiple Myeloma Bears High Incidence of Poor Cytogenetic Aberration and Novel Agents Resistance. *BioMed Research International* **2015**;
27. Sher T, Miller KC, Deeb G, Lee K, Chanan-Khan A. Plasma cell leukaemia and other aggressive plasma cell malignancies. *British Journal of Haematology* **2010**; 4: 418-427
28. Palumbo A, Avet-Loiseau H, Oliva S, Lokhorst HM, Goldschmidt H, Rosinol L, et al. Revised International Staging System for Multiple Myeloma: A Report From International Myeloma Working Group. *J Clin Oncol* **2015**; 26: 2863-2869
29. Anderson MW, Zhao S, Ai WZ, Tibshirani R, Levy R, Lossos IS, et al. C-C chemokine receptor 1 expression in human hematolymphoid neoplasia. *Am J Clin Pathol* **2010**; 3: 473-483
30. Shin SY, Lee DH, Lee J, Choi C, Kim J-Y, Nam J-S, et al. C-C motif chemokine receptor 1 (CCR1) is a target of the EGF-AKT-mTOR-STAT3 signaling axis in breast cancer cells. *Oncotarget* **2017**; 55: 94591-94605

31. Scotton C, Milliken D, Wilson J, Raju S, Balkwill F. Analysis of CC chemokine and chemokine receptor expression in solid ovarian tumours. *Br J Cancer* **2001**; 6: 891-897
32. Wang CL, Sun BS, Tang Y, Zhuang HQ, Cao WZ. CCR1 knockdown suppresses human non-small cell lung cancer cell invasion. *J Cancer Res Clin Oncol* **2009**; 5: 695-701
33. Li Y, Wu J, Zhang P. CCL15/CCR1 axis is involved in hepatocellular carcinoma cells migration and invasion. *Tumour Biol* **2016**; 4: 4501-4507
34. Long H, Xie R, Xiang T, Zhao Z, Lin S, Liang Z, et al. Autocrine CCL5 signaling promotes invasion and migration of CD133+ ovarian cancer stem-like cells via NF-kappaB-mediated MMP-9 upregulation. *Stem Cells* **2012**; 10: 2309-2319
35. Kato T, Fujita Y, Nakane K, Mizutani K, Terazawa R, Ehara H, et al. CCR1/CCL5 interaction promotes invasion of taxane-resistant PC3 prostate cancer cells by increasing secretion of MMPs 2/9 and by activating ERK and Rac signaling. *Cytokine* **2013**; 1: 251-257
36. Kumar SK, Dispenzieri A, Lacy MQ, Gertz MA, Buadi FK, Pandey S, et al. Continued improvement in survival in multiple myeloma: changes in early mortality and outcomes in older patients. *Leukemia* **2014**; 5: 1122-1128
37. Song X, Cong Z, Wilson K. Real-world treatment patterns, comorbidities, and disease-related complications in patients with multiple myeloma in the United States. *Current Medical Research and Opinion* **2016**; 1: 95-103
38. Raab MS, Cavo M, Delforge M, Driessen C, Fink L, Flinois A, et al. Multiple myeloma: practice patterns across Europe. *Br J Haematol* **2016**; 1: 66-76
39. Bergin K, McQuilten Z, Moore E, Wood E, Spencer A. Myeloma in the Real World: What Is Really Happening? *Clinical Lymphoma Myeloma and Leukemia* **2017**; 3: 133-144.e131
40. Mateos M-V, Ludwig H, Bazarbachi A, Beksac M, Bladé J, Boccadoro M, et al. Insights on Multiple Myeloma Treatment Strategies. *HemaSphere* **2018**; 1: e163-e163
41. Moreau P, San Miguel J, Sonneveld P, Mateos MV, Zamagni E, Avet-Loiseau H, et al. Multiple myeloma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* **2017**; suppl_4: iv52-iv61

42. Ho PJ, Moore EM, McQuilten ZK, Wellard C, Bergin K, Augustson B, et al. Renal Impairment at Diagnosis in Myeloma: Patient Characteristics, Treatment, and Impact on Outcomes. Results From the Australia and New Zealand Myeloma and Related Diseases Registry. *Clinical Lymphoma Myeloma and Leukemia* **2019**; 8: e415-e424
43. Kumar SK, Rajkumar SV, Dispenzieri A, Lacy MQ, Hayman SR, Buadi FK, et al. Improved survival in multiple myeloma and the impact of novel therapies. *Blood* **2008**; 5: 2516-2520
44. Wong R, Tay J. Economics of Multiple Myeloma. *Blood* **2018**; Supplement 1: 4773-4773
45. Petrucci MT, Giraldo P, Corradini P, Teixeira A, Dimopoulos MA, Blau IW, et al. A prospective, international phase 2 study of bortezomib retreatment in patients with relapsed multiple myeloma. *Br J Haematol* **2013**; 5: 649-659
46. Sood R, Carloss H, Kerr R, Lopez J, Lee M, Druck M, et al. Retreatment with bortezomib alone or in combination for patients with multiple myeloma following an initial response to bortezomib. *Am J Hematol* **2009**; 10: 657-660
47. Sanz-Rodriguez F, Hidalgo A, Teixido J. Chemokine stromal cell-derived factor-1alpha modulates VLA-4 integrin-mediated multiple myeloma cell adhesion to CS-1/fibronectin and VCAM-1. *Blood* **2001**; 2: 346-351
48. Sanz-Rodríguez F, Ruiz-Velasco N, Pascual-Salcedo D, Teixidó J. Characterization of VLA-4-dependent myeloma cell adhesion to fibronectin and VCAM-1. *British Journal of Haematology* **1999**; 4: 825-834
49. Ghobrial IM, Liu CJ, Zavidij O, Azab AK, Baz R, Laubach JP, et al. Phase I/II trial of the CXCR4 inhibitor plerixafor in combination with bortezomib as a chemosensitization strategy in relapsed/refractory multiple myeloma. *Am J Hematol* **2019**; 11: 1244-1253
50. Schaller MA, Kallal LE, Lukacs NW. A key role for CC chemokine receptor 1 in T-cell-mediated respiratory inflammation. *Am J Pathol* **2008**; 2: 386-394
51. Kerstjens HA, Bjermer L, Eriksson L, Dahlström K, Vestbo J. Tolerability and efficacy of inhaled AZD4818, a CCR1 antagonist, in moderate to severe COPD patients. *Respir Med* **2010**; 9: 1297-1303
52. Haringman JJ, Kraan MC, Smeets TJM, Zwinderman KH, Tak PP. Chemokine blockade and chronic inflammatory disease: proof of concept in patients with rheumatoid arthritis. *Ann Rheum Dis* **2003**; 715-721

53. Tak PP, Balanescu A, Tseluyko V, Bojin S, Drescher E, Dairaghi D, et al. Chemokine receptor CCR1 antagonist CCX354-C treatment for rheumatoid arthritis: CARAT-2, a randomised, placebo controlled clinical trial. *Ann Rheum Dis* **2013**; 3: 337-344
54. Vergunst CE, Gerlag DM, von Moltke L, Karol M, Wyant T, Chi X, et al. MLN3897 plus methotrexate in patients with rheumatoid arthritis: safety, efficacy, pharmacokinetics, and pharmacodynamics of an oral CCR1 antagonist in a phase IIa, double-blind, placebo-controlled, randomized, proof-of-concept study. *Arthritis Rheumatol* **2009**; 12: 3572-3581
55. Dairaghi DJ, Zhang P, Wang Y, Seitz LC, Johnson DA, Miao S, et al. Pharmacokinetic and pharmacodynamic evaluation of the novel CCR1 antagonist CCX354 in healthy human subjects: implications for selection of clinical dose. *Clin Pharmacol Ther* **2011**; 5: 726-734
56. Dairaghi DJ, Oyajobi BO, Gupta A, McCluskey B, Miao S, Powers JP, et al. CCR1 blockade reduces tumor burden and osteolysis in vivo in a mouse model of myeloma bone disease. *Blood* **2012**; 7: 1449-1457
57. Menu E, De Leenheer E, De Raeve H, Coulton L, Imanishi T, Miyashita K, et al. Role of CCR1 and CCR5 in homing and growth of multiple myeloma and in the development of osteolytic lesions: a study in the 5TMM model. *Clin Exp Metastasis* **2006**; 5-6: 291-300
58. Kyle RA. Multiple myeloma: review of 869 cases. *Mayo Clin Proc* **1975**; 1: 29-40
59. Lee OL, Horvath N, Lee C, Joshua D, Ho J, Szer J, et al. Bisphosphonate guidelines for treatment and prevention of myeloma bone disease. *Intern Med J* **2017**; 8: 938-951
60. Han JH, Choi SJ, Kurihara N, Koide M, Oba Y, Roodman GD. Macrophage inflammatory protein-1alpha is an osteoclastogenic factor in myeloma that is independent of receptor activator of nuclear factor kappaB ligand. *Blood* **2001**; 11: 3349-3353
61. Lentzsch S, Gries M, Janz M, Bargou R, Dorken B, Mapara MY. Macrophage inflammatory protein 1-alpha (MIP-1 alpha) triggers migration and signaling cascades mediating survival and proliferation in multiple myeloma (MM) cells. *Blood* **2003**; 9: 3568-3573

62. Vallet S, Raje N, Ishitsuka K, Hideshima T, Podar K, Chhetri S, et al. MLN3897, a novel CCR1 inhibitor, impairs osteoclastogenesis and inhibits the interaction of multiple myeloma cells and osteoclasts. *Blood* **2007**; 10: 3744-3752

63. Abe M, Hiura K, Wilde J, Shioyasono A, Moriyama K, Hashimoto T, et al. Osteoclasts enhance myeloma cell growth and survival via cell-cell contact: a vicious cycle between bone destruction and myeloma expansion. *Blood* **2004**; 8: 2484-2491

64. Moller C, Stromberg T, Juremalm M, Nilsson K, Nilsson G. Expression and function of chemokine receptors in human multiple myeloma. *Leukemia* **2003**; 1: 203-210

65. Usmani SZ, Heuck C, Mitchell A, Szymonifka J, Nair B, Hoering A, et al. Extramedullary disease portends poor prognosis in multiple myeloma and is overrepresented in high risk disease even in era of novel agents. *Haematologica* **2012**; haematol.2012.065698

66. Gagelmann N, Eikema D-J, Iacobelli S, Koster L, Nahi H, Stoppa A-M, et al. Impact of extramedullary disease in patients with newly diagnosed multiple myeloma undergoing autologous stem cell transplantation: a study from the Chronic Malignancies Working Party of the EBMT. *Haematologica* **2018**; 5: 890

67. Eksteen B, Miles A, Curbishley SM, Tselepis C, Grant AJ, Walker LSK, et al. Epithelial Inflammation Is Associated with CCL28 Production and the Recruitment of Regulatory T Cells Expressing CCR10. *J Immunol* **2006**; 1: 593-603

68. Thangavadivel S, Zelle-Rieser C, Olivier A, Postert B, Untergasser G, Kern J, et al. CCR10/CCL27 crosstalk contributes to failure of proteasome-inhibitors in multiple myeloma. *Oncotarget* **2016**; 48: 78605-78618

69. Nakayama T, Hieshima K, Izawa D, Tatsumi Y, Kanamaru A, Yoshie O. Cutting edge: profile of chemokine receptor expression on human plasma cells accounts for their efficient recruitment to target tissues. *J Immunol* **2003**; 3: 1136-1140

70. Pasmantier MW, Azar HA. Extraskeletal spread in multiple plasma cell myeloma: A review of 57 autopsied cases. *Cancer* **1969**; 1: 167-174

71. Oshima K, Kanda Y, Nannya Y, Kaneko M, Hamaki T, Suguro M, et al. Clinical and pathologic findings in 52 consecutively autopsied cases with multiple myeloma. *American Journal of Hematology* **2001**; 1: 1-5

72. Kapadia SB. Multiple myeloma: a clinicopathologic study of 62 consecutively autopsied cases. *Medicine* **1980**; 5: 380-392

73. Bross IDJ, Viadana E, Pickren JW. The metastatic spread of myeloma and leukemias in men. *Virchows Archiv A* **1975**; 2: 91-101