

# Single-cell RNA sequencing reveals chemokine self-feeding of myeloma cells promotes extramedullary metastasis

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**In this study, we aimed to determine the mechanisms underlying the initial extramedullary translocation of myeloma cells from bone marrow into peripheral blood. We found that clonal circulating plasma cells (cPCs) are more frequently detected by flow cytometry in extramedullary plasmacytoma (EMP) patients and worsen their prognosis. It is technically much easier to collect single cPCs using FACS than it is to perform EMP biopsy. Therefore, combining EMP imaging with cPC detection may be a promising strategy for prognostic stratification. Here, using single-cell transcriptome analysis, we found that the chemokine CXCL12, a key molecule involved in CXCR4-dependent cell retention in the bone marrow, is abnormally upregulated in cPCs and might initially enable cPCs to evade bone marrow retention and translocate into the bloodstream.**

**Keywords:** chemokine self-feeding; circulating plasma cells; extramedullary plasmacytoma; multiple myeloma

Multiple myeloma (MM) is a malignant proliferation of monoclonal plasma cells that produce monoclonal immunoglobulins and is defined by the presence of 10%

of clonal plasma cells (PCs) in the bone marrow or a biopsy-proven extramedullary plasmacytoma (EMP). MM remains an incurable disease, representing the

## Abbreviations

BMMCs, bone marrow myeloma cells; CD, cluster differentiation; cPCs, circulating plasma cells; DEG, differentially expressed genes; EMP, extramedullary plasmacytoma; MM, multiple myeloma; OS, overall survival; PBMCs, peripheral blood mononuclear cells; PCA, principal component analysis.

second most common hematologic malignancy worldwide. Its incidence has been increasing in recent years [1,2]. MM often presents with hypercalcemia, renal function impairment, anemia, and osteolytic lesions [3,4]. Over the past decade, the median survival of myeloma patients has increased to 8 years. This remarkable improvement is mostly because of the use of high-dose therapy followed by autologous stem cell transplantation, in addition to the widespread incorporation of novel agents, including immunomodulatory drugs (thalidomide and lenalidomide) and proteasome inhibitors (bortezomib). MM is a heterogeneous entity; the overall survival (OS) time of MM ranges from several months to several years.

For most myeloma patients, the proliferation of PCs is restricted to the bone marrow. However, a subset of MM patients develops EMPs, defined by the presence of clonal PCs outside the bone marrow, which generally involve soft tissue, cortical bone, and centrum, and may be some of the most prominent clinical features of MM [5]. EMPs are present at the time of initial diagnosis in 9.6–19.6% of MM patients and develop during treatment in 6–20% patients [6,7]. MM patients with EMP invasion have a poor prognosis and are difficult to treat [5]. In such patients, late detection and delayed treatment can seriously diminish quality of life and can be life-threatening. Therefore, it is clinically important to optimize the detection of this entity.

To date, the mechanisms of extramedullary spread in MM are poorly understood. In one hypothetical model of EMP pathogenesis, metastatic myeloma cells initially exit the bone marrow, translocate into blood as clonal circulating plasma cells (cPCs), and finally settle in peripheral tissues and form an EMP [8–10]. However, the mechanistic evidence for this hypothesis is limited.

Here, we focused on the initial extramedullary translocation of myeloma cells from bone marrow into peripheral blood. The transcriptome can dynamically reflect intrinsic and environmental fluctuations [11,12]. We took transcriptome snapshots to provide insights into the translocation mechanism and applied single-cell RNA-seq to explore transcriptomic differences between myeloma cells in bone marrow (BMMCs) and peripheral cPCs in each EMP-positive patient. We found a consensus of upregulated auto-secretion of the chemokine CXCL12 in cPCs. This CXCL12 upregulation may contribute to the initial extramedullary translocation by evasion of CXCR4-dependent cell retention in bone marrow. Upregulation of another bone marrow retention chemokine, CXCL7, was previously published [13], further supporting the notion of

chemokine auto-secretion relating to extramedullary translocation in myeloma.

## Materials and methods

### Patients

The participants were 11 patients with primarily untreated MM (median age: 58 years, range: 49–76 years) and 10 patients with EMP (median age: 64.5 years, range: 44–75 years). All patients were diagnosed according to the 2009 International Myeloma Working Group (IMWG) consensus criteria [14]. The patients enrolled in this study were hospitalized during the period of October 2014 to June 2015 at the Peking University Third Hospital and Beijing Chao-Yang Hospital. The end point of the analyses was OS, which was defined as the time from diagnosis to death (regardless of the cause) or date of the last follow-up evaluation.

All patients received combination chemotherapy regimens, including BAD (bortezomib, doxorubicin, dexamethasone), VCD (bortezomib, cyclophosphamide, dexamethasone), TD (thalidomide, dexamethasone), RD (lenalidomide, dexamethasone), RCD (lenalidomide, cyclophosphamide, dexamethasone), and DCEP (dexamethasone, cyclophosphamide, etoposide, cisplatin). Some patients received additional radiation therapy, surgical therapy, and autologous hematopoietic stem cell transplantation. Efficacy criteria for MM were defined according to International Myeloma Working Group (IMWG) consensus criteria for response and minimal residual disease assessment in MM [15]. This research was approved by the ethics committee of Peking University Third Hospital (IRB00006761-2016120). All patients provided written informed consent in accordance with the Declaration of Helsinki.

### Sample collection and preparation

Typically, 4 mL bone marrow aspirate or peripheral blood was drawn from patients in a sterile manner. Peripheral blood mononuclear cells (PBMCs) and bone marrow cells were isolated by Ficoll-Paque Plus (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). A 2-min lysis of RBC (red blood cell) was performed if necessary. Samples were passed through 30- $\mu$ m nylon mesh to remove cell clumps. Filters were wetted with buffer before use. Purification of CD138<sup>+</sup> plasma cells from the single-cell suspension was performed using human CD138 MACS MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Cell number was determined, and the cell suspension was centrifuged at 300 *g* for 10 min. The supernatant was aspirated, and the cell pellet was resuspended in 80  $\mu$ L of buffer per  $2 \times 10^7$  total cells. Then, 20  $\mu$ L of CD138 MicroBeads per  $2 \times 10^7$  total cells was added, and mixed well, and cells were incubated for

15 min in the a refrigerator (2–8 °C). Cells were washed by adding 1–2 mL of buffer per  $2 \times 10^7$  total cells and centrifuging at 300 *g* for 10 min. The supernatant was then aspirated, and up to  $10^8$  cells were resuspended in 500  $\mu$ L of buffer. The column was placed in the magnetic field of a MACS Separator, and the MS column was prepared by rinsing with 500  $\mu$ L of buffer. Cell suspension was applied onto the column, and unlabeled cells that passed through were collected. The column was washed three times with 500  $\mu$ L of buffer, and total eluent was collected; this eluent is the unlabeled cell fraction. The column was removed from the separator and placed on a collection tube. Onto the column, 1 mL of buffer was pipetted and the magnetically labeled CD138<sup>+</sup> cells were immediately flushed out by firmly pushing the plunger into the column. The single-cell suspension was then stained with antibodies and analyzed or sorted by flow cytometry.

### Flow cytometry

Antibodies [CD38 clone HB-7, CD138 clone DL-101, CD117 (c-kit) clone 104D2, CD56 (N-CAM) clone 5.1H11, CD45 clone 2D1, CD19 clone HIB19] and cell viability dyes (Zombie NIR and Zombie Aqua) were purchased from BioLegend (San Diego, CA, USA). Antibodies and dyes were titrated for optimal performance. Compensation matrices were generated with Ultra CompBeads (eBioscience/Thermo Fisher, Carlsbad, CA, USA) and confirmed with samples. Data were collected with Aria II SORP flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed with FLOWJO (FlowJo LLC, Franklin Lakes, NJ, USA). Single cells were index-sorted into 96-well plates with the same machine.

### Single-cell RNA sequencing and analysis

Single myeloma cells or cPCs were sorted into lysis buffer in 96-well plates with index sorting mode on FACS. The SmartSeq2 protocol was immediately performed to rescue mRNAs from cell lysate [16]. Libraries were constructed with a Tn5-based TruePrep DNA Library Prep Kit (Vazyme Biotech, Nanjing, China) and were sequenced on the HiSeq 2500 platform (Illumina, Inc., San Diego, CA, USA). Sequencing reads were aligned to the human reference genome GENCODE GRCh37 v25 transcriptome using SALMON v0.8.2 [17]. Cells with sufficient quality were selected (mitochondria reads < 20%, TPM > 1 genes over 1k). Thirty-two single BMMCs were prepared for every patient (Fig. 5); generally, > 50% cells passed QC.

Principal component analysis (PCA) was carried out using FactoMineR in R. Four hundred highly variable genes were selected from first four principal components (PCs). MM-related gene groups were chosen, and their loading value or score in PC1 and PC2 was plotted (Fig. 4C–E) and summarized (Fig. 4F,G). To explore the

homogeneity and heterogeneity of myeloma among patients, 126 BM cells were analyzed by SEURAT (version 2.3.4: New York Genome Center, New York, NY, USA, default parameters) for highly variable genes and unsupervised hierarchical clustering (Fig. 5A). Selected gene groups were plotted in patient-oriented ordering (Fig. 5B–D, Fig. S3). In addition to unsupervised PCA, differential expression analysis between cPCs and BMMCs in the same patient was performed with DESeq2 and plotted in GRAPHPAD PRISM (Fig. 6C–E). Statistics of genes related to BM ingress, BM adhesion, and BM egress are summarized in Fig. 6F. The same analysis was performed for Fig. S5.

### Statistical analysis

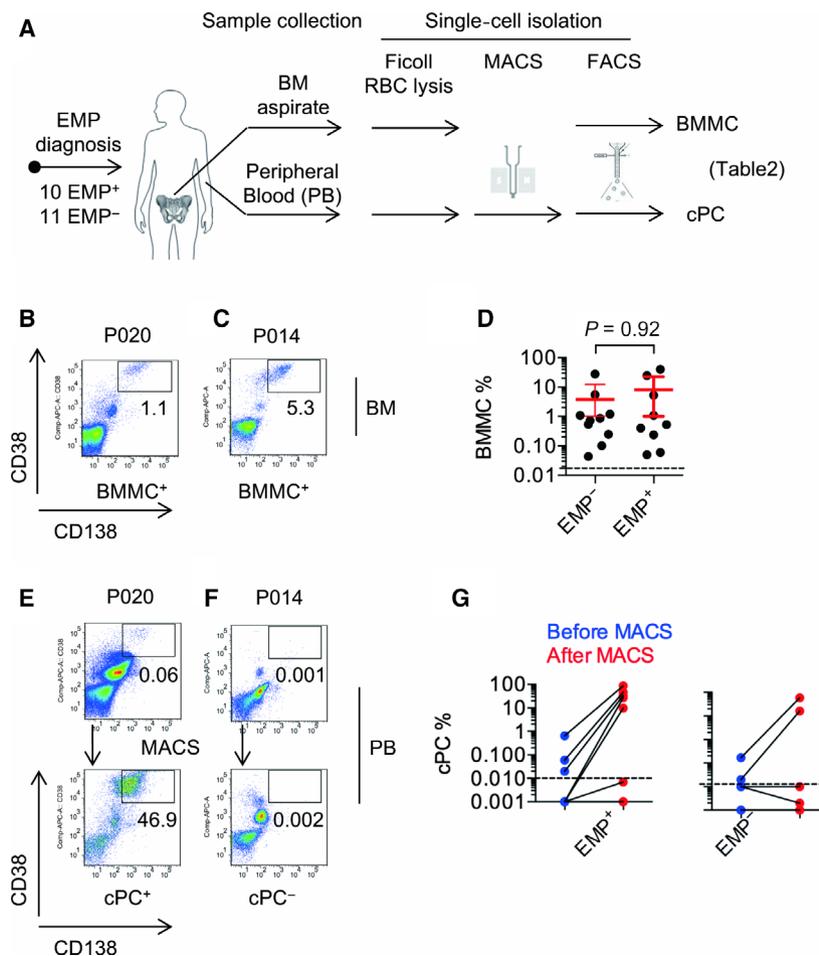
All calculations were performed using the Windows version of IBM SPSS software, version 18.0.1 (PASW Statistics for Windows; IBM, Armonk, NY, USA). OS and PFS were determined by the Kaplan–Meier analysis. Differences between survival curves were tested for statistical significance using 2-tailed log-rank tests. Numerical data were analyzed by GRAPHPAD PRISM (GraphPad Software, Inc., La Jolla, CA, USA). Adjusted *P*-values were analyzed in R (R Core Team, Vienna, Austria). Nonparametric Mann–Whitney *U*-test was used for two-group comparisons in Fig. 1D. *P* < 0.05 (log-rank test) was considered statistically significant.

## Results

### Circulating plasma cells worsen prognosis for EMP-positive patients

Twenty-one MM patients were enrolled. In Table 1, clinical characteristic analysis is provided for the 10 EMP-positive and 11 EMP-negative patients. Compared with EMP<sup>−</sup> patients, more EMP<sup>+</sup> patients were female (80% versus 27.3%). There was an even distribution of immunoglobulin subtype, similar ISS stages, and higher ESR-LDH-ALB detection. EMP was located primarily in soft tissue, centrum, and the chest wall.

To explore the hypothesis that EMP occurrence might have been associated with BMMCs or cPCs, 19 bone marrow aspirate samples and 16 peripheral blood samples were successfully collected. Both BMMCs and cPCs expressed high levels of malignant activation marker CD38 and plasma cell marker CD138, as expected (Fig. S1). Consistent with MM diagnosis, BMMCs were positive in all 19 bone marrow aspirates, but there was no difference between EMP-positive and EMP-negative aspirate samples. cPCs were identified after MACS enrichment. Five out of 9 (55.5%) EMP-positive patients had detectable cPCs in



**Fig. 1.** Circulating plasma cells were detected more frequently in EMP<sup>+</sup> patients. (A) Scheme of sample collection and process. (B, C) BMMC detection in BM aspirates from EMP<sup>+</sup> P020 (B) and P014 (C). (D) Statistical analysis of BMMC percentage in aspirates. Dashed line represents detection limit. Mean  $\pm$  SD. (E, F) cPC enrichment and detection in peripheral blood (PB) from EMP<sup>+</sup> P020 (E) and P014 (F). P020 is cPC-positive. P014 is cPC-negative. (G) Statistical analysis of cPC percentage in peripheral blood. Dashed line represents detection limit.

blood, whereas 2 out of 7 (28.6%) EMP-negative patients had detectable cPCs in blood (Fig. 1, Table 2). These results indicate that cPCs may be correlated with EMP progression from MM.

Circulating plasma cells have predictive power for survival of newly diagnosed MM [18]. However, it is still unknown whether cPCs contribute to EMP prognosis [19]. To address this issue, we first summarized OS of 21 patients. EMP-positive patients had significantly shorter survival than did EMP-negative patients (median survival: 12 months versus 74 months,  $P = 0.0002$ ). The same survival pattern was observed from retrospective data of 180 MM patients in our hospital during the period from 2008 to 2016 (median survival: 21 months versus 74 months,  $P = 0.0001$ ), suggesting minimal biased sampling of these 21 patients. In contrast, simply checking the presence of cPCs failed to predict survival in a significant way ( $P = 0.20$ ), whereas combining EMP and cPC information showed significant improvement in prognosis ( $P = 0.017$ ) (Fig. 2). EMP-negative patients showed maximal survival, regardless

of cPCs presence. EMP-positive patients without cPCs in blood showed similar survival to 180 retrospective data with median survival of 21 months. However, EMP-positive patients with cPCs had extremely shortened median survival of 8 months, and all passed away within 2 years. Therefore, cPCs may provide a stratification insight for EMP as well as MM patients. Together, these results indicate that cPCs were more frequently detected in EMP-positive patients, and worsened their prognosis.

### Circulating plasma cells showed a cluster differentiation marker phenotype similar to that of BM myeloma cells

Compared with normal plasma cells, myeloma cells dynamically regulate expression of surface cluster differentiation (CD) markers, such as down-regulation of B-cell markers CD45 and CD19, up-regulation of adhesion molecules CD56 (N-CAM) and CD81, and variable regulation of tumorigenic marker CD117 (c-kit) [20]. As expected, BMMCs were

**Table 1.** Clinical characteristics of 21 patients with MM. ESR, erythrocyte sedimentation rate;  $\beta$ 2-MG,  $\beta$ 2 microglobulin; LDH, lactate dehydrogenase; CRP, C-reactive protein; ALB, serum albumin

Characteristics	MM	EMP(-)	EMP(+)
Number	21	11	10
Age	62 (44–76)	58 (49–76)	64.5 (44–75)
Gender			
Male	47.6% (10/21)	72.7% (8/11)	20.0% (2/10)
Female	52.4% (11/21)	27.3% (3/11)	80.0% (8/10)
M component at diagnosis			
Ig G	61.9% (13/21)	54.5% (6/11)	70.0% (7/10)
Ig A	28.6% (6/21)	45.5% (5/11)	10.0% (1/10)
Ig D	4.8% (1/21)	0	10.0% (1/10)
Light chain	4.8% (1/21)	0	10.0% (1/10)
Stage of ISS			
I	23.8% (5/21)	18.2% (2/11)	30.0% (3/10)
II	33.3% (7/21)	27.3% (3/11)	40.0% (4/10)
III	42.9% (9/21)	54.5% (6/11)	30.0% (3/10)
Anemia	71.4% (15/21)	81.8% (9/11)	60.0% (6/10)
ESR > 20 mm·h <sup>-1</sup>	78.3% (16/21)	63.6% (7/11)	90.0% (9/10)
Hypercalcemia > 2.75 mmol·L <sup>-1</sup>	9.5% (2/21)	18.2% (2/11)	0
Creatinine > 177 $\mu$ mol·L <sup>-1</sup>	14.3% (3/21)	18.2% (2/11)	10.0% (1/10)
$\beta$ 2-MG > 1.8 mg·L <sup>-1</sup>	91.3% (20/21)	90.9% (10/11)	100% (10/10)
LDH > 245 U·L <sup>-1</sup>	19.0% (4/21)	9.1% (1/11)	30.0% (3/10)
CRP > 5 mg·L <sup>-1</sup>	4.8% (1/21)	9.1% (1/11)	0
ALB < 40 g·L <sup>-1</sup>	78.3% (16/21)	63.6% (7/11)	90.0% (9/10)

**Table 2.** Sample collection and cell detection. BM, bone marrow; PB, peripheral blood; +, collected or detected; N.C., not collected; -, not detected

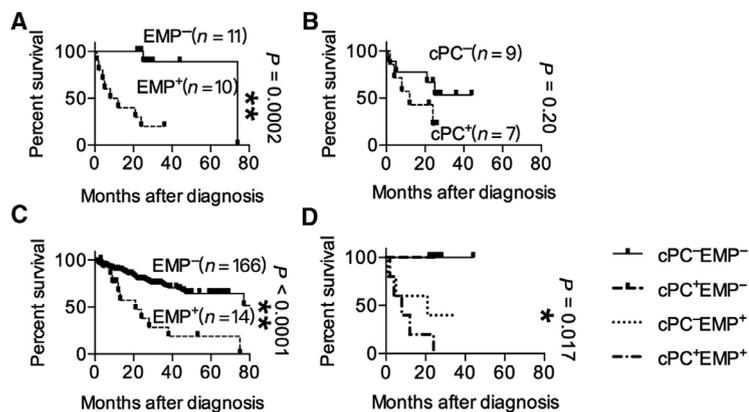
	Patient	BM collected	BMMC detected	PB collected	cPCs detected
EMP <sup>+</sup> (n = 10)	P002	+	+	+	+
	P014	+	+	+	-
	P015	+	+	+	-
	P016	+	+	+	-
	P017	+	+	+	+
	P020	+	+	+	+
	P024	+	+	+	-
	P023	+	+	N.C.	-
	P008	N.C.	-	+	+
	P018	N.C.	-	+	+
EMP <sup>-</sup> (n = 11)	P001	+	+	+	-
	P006	+	+	+	+
	P010	+	+	+	-
	P012	+	+	+	-
	P013	+	+	+	+
	P011	+	+	+	-
	P019	+	+	+	-
	P004	+	+	N.C.	-
	P007	+	+	N.C.	-
	P009	+	+	N.C.	-
	P021	+	+	N.C.	-
Total 21	(BMMC detected/BM collected) %		(cPCs detected/PB collected) %		
	100% (19/19)		EMP <sup>+</sup> 55.5% (5/9) EMP <sup>-</sup> 28.6% (2/7)		

CD45<sup>-</sup>/dimCD19<sup>-</sup>/dimCD117<sup>-</sup>CD56<sup>+</sup>CD81<sup>+</sup> phenotype (Fig. 3).

To explore the possibility that CD markers may contribute to cPCs' exit from bone marrow, we compared cPCs with BMMCs in the same patient (P017 and P020 are EMP-positive; P013 is EMP-negative) (Table 2, Fig. 3). In general, CD markers were similarly expressed in both BMMCs and cPCs, and CD56 (N-CAM) was slightly downregulated in cPCs in P017 (BMMC 93800 to cPC 34100 in MFI, 2.8-fold) and P020 (BMMC 9093 to cPC 6524 in MFI, 1.4-fold), confirming existing reports of lower CD56 expression in cPCs [20]. The finding that cPCs are similar to BMMCs in terms of surface CD markers does not help in understanding the extramedullary translocation. We then applied single-cell RNA-seq (scRNA-seq) to these cells, making effort to discover the major difference between cPCs and BMMCs through whole-transcriptome snapshots, overcoming limited marker space of immunophenotyping.

### Single-cell RNA-seq identifies myeloma cells from normal cells

Each cell contains only a few pgRNAs, which are easy to degrade and sensitive to contaminations [21]. We used the SmartSeq2 protocol to capture and amplify single-cell transcriptomes for sequencing. SmartSeq2



**Fig. 2.** Circulating plasma cells shortened EMP<sup>+</sup> patients' survival. (A) Survival by EMP in 21 patients. (B) Survival by EMP in 180 patients during 2008–2016. (C) Survival by cPCs. (D) Survival by cPCs and EMP. Data are shown in Kaplan–Meier curves. Log-rank (Mantel–Cox) test was used to calculate differences.

has been shown to have high sensitivity, high reproducibility, and full-length profiling ability [16,22]. These features were confirmed by using MM cell lines H929 and U266 and observing high gene recovery, highly reproducible gene expression results, and high coverage and evenness across the gene body (Fig. S2).

Twenty-one out of 24 sequenced single CD38<sup>+</sup>CD138<sup>+</sup> BMMCs, with 5 out of 6 sequenced CD38<sup>-</sup>CD138<sup>-</sup> nonmyeloma control cells, were plotted. In PCA, PC1 (principal component 1) represented 22.6% of the total variance and provided separation between the two types of cells (Fig. 4A). The hierarchical clustering of the highly variable genes among cells showed that the two types of cells have distinct gene expression patterns and tend to cluster separately, as expected (Fig. 4B, Table S1).

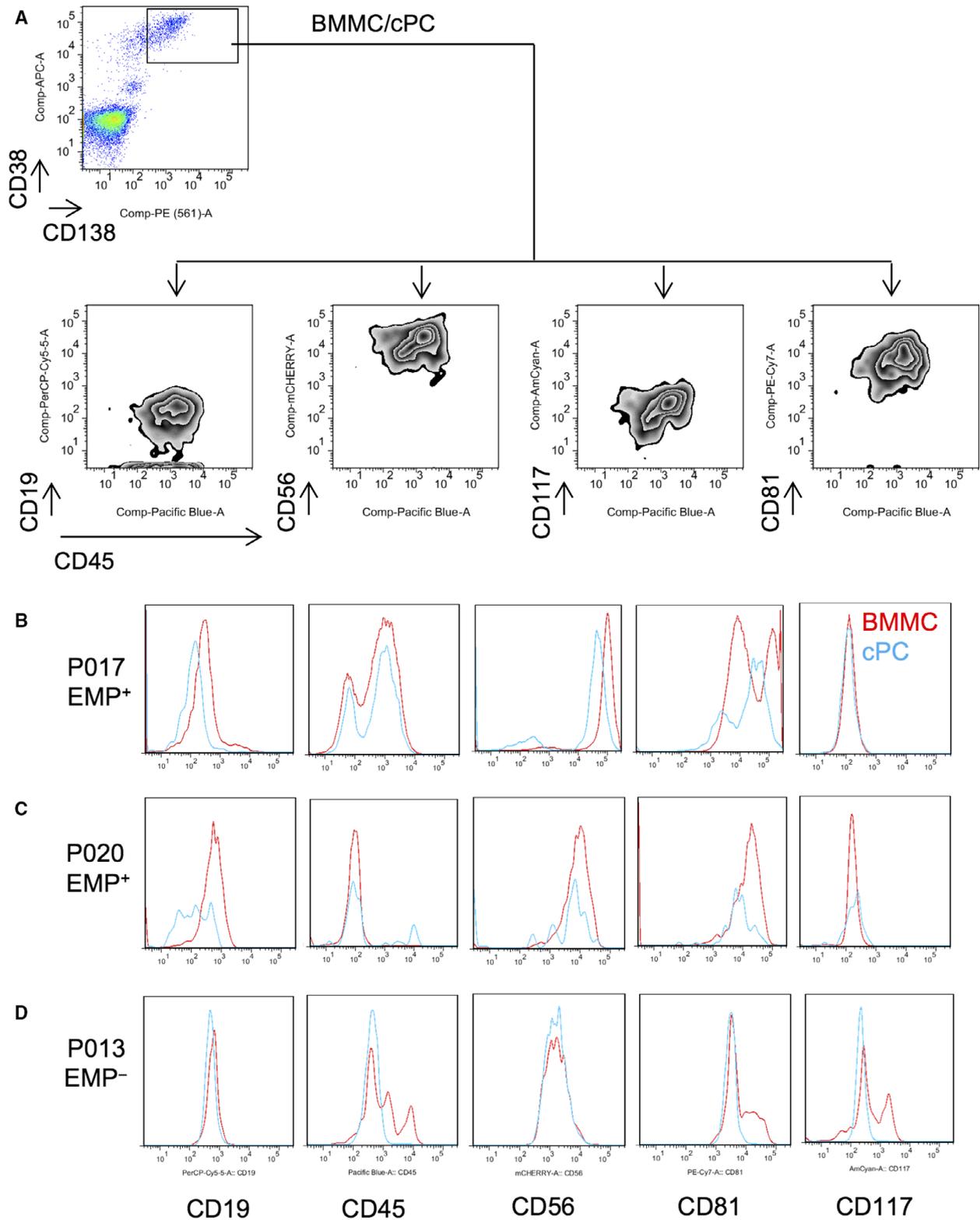
We further validated several crucial gene groups for myeloma, including myeloma markers, essential signaling pathway proteins/transcription factors/growth factors, and tumorigenic genes. As expected, all genes strongly correlated with PC1 (loading value > 0.5) (Fig. 4C–E). Unsurprisingly, FACS markers, such as CD38, CD138, CD56, CD19, and CD117, also contributed to define BMMCs in the transcriptome, and CD45 negatively correlated with PC1, confirming CD45 downregulation in myeloma cells [18]. Interleukin-6 (IL-6) signaling molecules, including IL-6 signal transducer (IL6st), receptor (IL6R), and transcriptional factor STAT3, necessary for myeloma survival and progression [23–25], were upregulated in BMMCs and contribute to PC1. Transcriptional factors specific for myeloma cells, such as IRF4, Blimp-1 (PRDM1), and XBP1, are also transcribed in BMMCs [26–29]. Upregulation of nourishing growth factors for myeloma, such as HGF, IGF1, and VEGF [30–33], was also confirmed. Furthermore, tumorigenic genes correlated with PC1 [34–39] (Fig. 4F,G). Together, these results validate our scRNA-seq data for further explorations.

### Patient-oriented clustering of single BMMCs

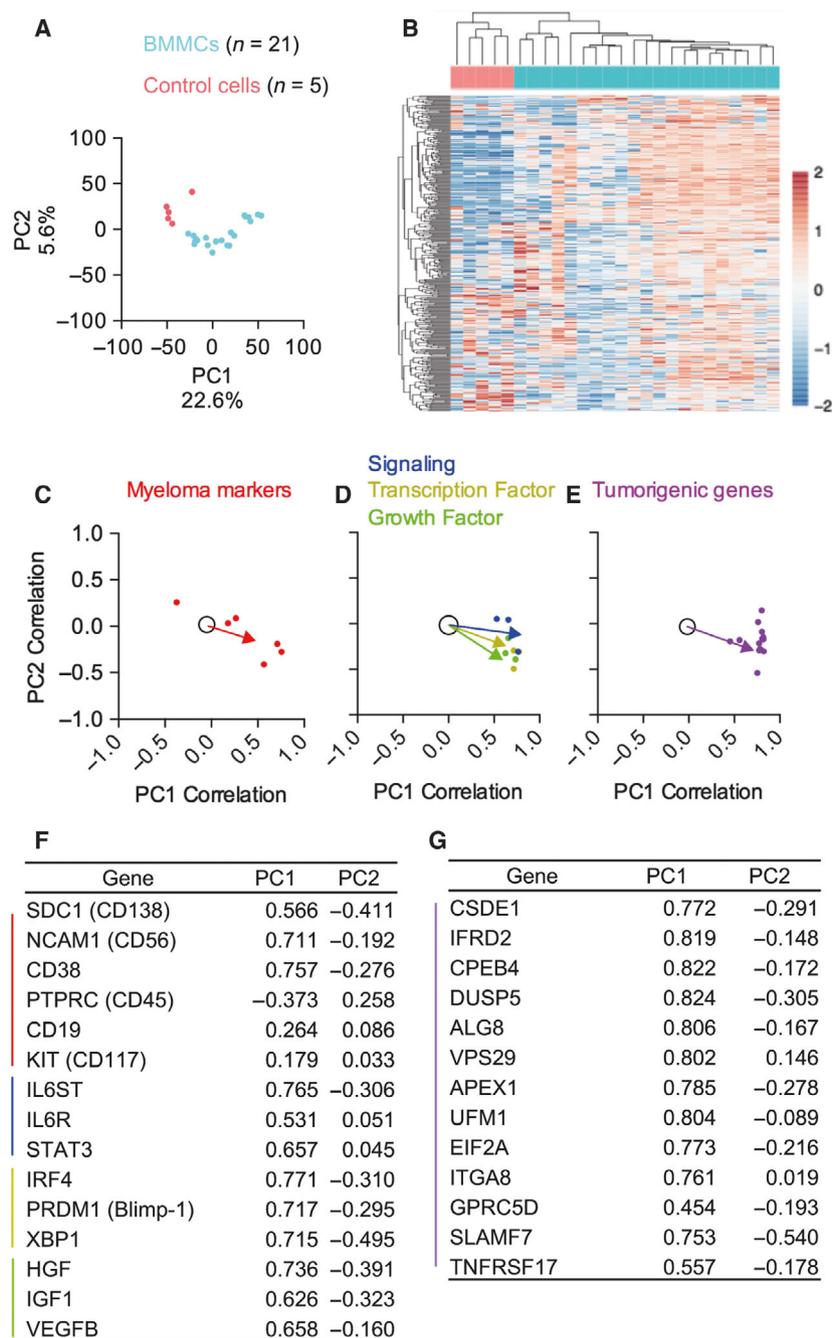
Next, we explored whether EMP patients have common traits in the single-cell transcriptome, which may in turn benefit EMP diagnosis or intervention. Single-cell analysis simultaneously monitors disease heterogeneity and homogeneity in multidimensional comparisons, such as between disease situations (EMP-positive or EMP-negative), between patients, and between single cells within the same patient. Therefore, to achieve a global view of myeloma, single-cell RNA-seq was performed on 126 single BMMCs from six patients (four EMP-positive, two EMP-negative), and hierarchical clustering of their highly variable genes was analyzed (Fig. S3, Table S2). The heat map depicts the structure of subgroups with both cells and their marker genes. In general, most of the cells clustered according to the patients. Each patient had a unique set of marker genes. Thus, no obvious similarity was observed among EMP-positive patients (P014, P017, P020, and P023) or between EMP-negative patients (P019 and P021). BMMCs clustered together within individual patients.

To further examine this patient-oriented clustering, single BMMCs were grouped and ordered by patient, and the expression levels of gene groups crucial for myeloma were checked. First, myeloma markers were confirmed. CD138 (SDC1), CD38, Blimp-1 (PRDM1), IRF4, XBP1, and IL-6R signaling genes (IL6R, IL6ST, and STAT3) were transcribed in BMMCs (Fig. 5A). Lack of CD117 (c-Kit, KIT) transcription confirmed our FACS observation that CD117 was rarely expressed on myeloma cells, regardless of EMP status. Expression of CD45 (PTPRC), CD19, and CD56 (NCAM1) was more dynamic, as expected, and patient-oriented.

We then analyzed the expression of immunoglobulin (Ig) genes, the hallmarks of clonal plasma cells. Constant regions of both Ig heavy chain (IGHG1 and IGHG2) and light chain (IGKC and IGLC1) were



**Fig. 3.** Circulating plasma cells were similar to BM myeloma cells on the surface. (A) Multifunctional immunophenotyping of BMMCs and cPCs. CD38<sup>+</sup>CD138<sup>+</sup> single malignant plasma cells were stained with CD45, CD19, CD56, CD117, and CD81 simultaneously. (B) EMP<sup>+</sup> patient P017's phenotype. (C) EMP<sup>+</sup> patient P020's phenotype. (D) EMP<sup>-</sup> patient P013's phenotype. Data are shown in overlay. Negative populations were below 1000 MFI.

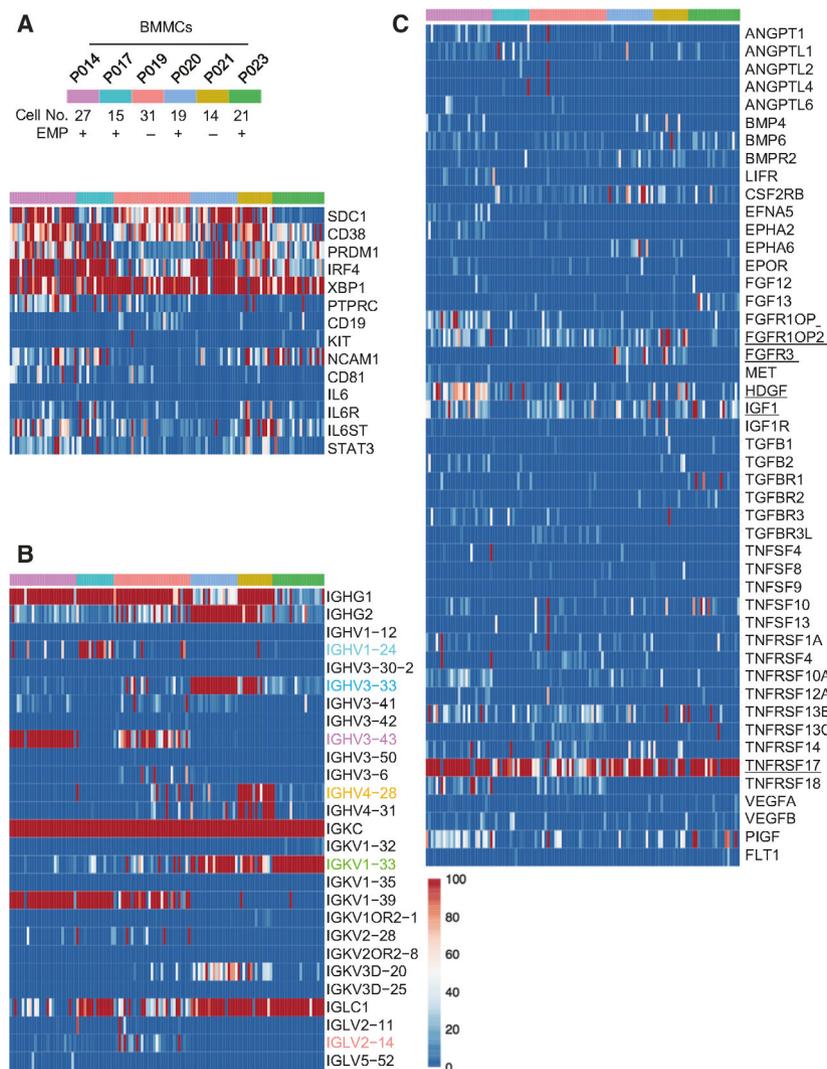


**Fig. 4.** scRNA-seq depicted BMMCs. (A) Cell distribution in PCA. PC1 explained 22.6% of all differences between cells, and PC2 explained 5.6%. (B) Unsupervised clustering of 22 single CD38<sup>+</sup>CD138<sup>+</sup> BMMCs and six CD38<sup>-</sup>CD138<sup>-</sup> control cells with PCA. A total of 400 DEG of first 4 principal dimensions were plotted (100 DEGs for each principal dimension). (C–G) Myeloma-associated genes pointed in PC1 direction. Correlations between each gene and PC1/PC2 were calculated (F, G) and are plotted (C–E).

transcribed robustly (Fig. 5B). Variable regions of heavy chain (IGHV) and light chain (IGKV and IGLV) showed clonal expansion for individual patients. We observed that the variable regions were transcribed in a patient-specific way. For example, in patient P014, BMMCs transcribed heavy chain IGHV3-43 and light chain IGKV1-39. In P017, IGHV1-24 and IGKV1-39 were transcribed. In P019, clone-type was more heterogeneous as expression of IGHV3-43, IGKV1-39, and IGLV2-14 could be seen. In P020, major clone-

were IGHV3-33, IGKV1-33, and 3D-20. In P021, IGHV4-28, IGKV1-33, and 3D-20 were transcribed. In P023, most BMMCs transcribed only IGKV1-33. Thus, individual patients showed unique Ig origins, and single BMMCs were clonal in the same patients.

Growth factors were reported to feed myeloma progression through surface receptors [40]. Universal transcription of FGFRs (fibroblast growth factor receptor 3), HDGF (hepatoma-derived growth factor), IGF1 (insulin-like growth factors 1), and TNFRSF17 (B-cell



**Fig. 5.** Patient-oriented clustering of BMMCs. (A–C) Expression plot of myeloma gene sets. Cells were ordered for each patient. Scale bar shows expression level. (A) Myeloma markers. (B) Immunoglobulins (Ig). Representative V region IDs are labeled as patient color. (C) Growth factors and receptors. Universal expressed genes are underlined.

maturation antigen, BCMA) emphasized their values in myeloma diagnosis and intervention. Among these genes, we found no obvious differentiations between EMP-positive and EMP-negative patients (Fig. 5C).

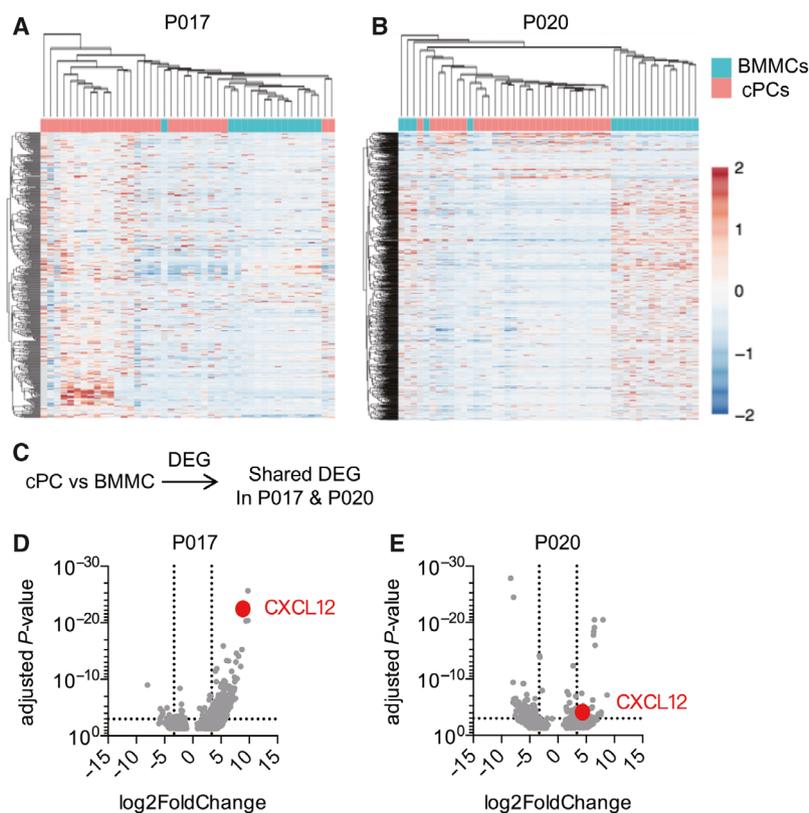
Oncogenes and tumor suppressors are also relevant to myeloma progression, and we also observed patient-oriented clustering of single BMMCs (Fig. S4). We further compared our data with previously published gene expression profiles GEP92 [41] and GEP70 [42] using microarrays and confirmed that the gene expression of single BMMCs is highly patient-dependent (Fig. S5).

#### Abnormal chemokine auto-secretion may contribute to cPC extramedullary translocation from the bone marrow

As stated above, extramedullary translocation is the initial step of cPC egress from the bone marrow. To

explore possible factors related to this translocation, we compared BMMCs and cPCs within the same patient (P017 and P020, both EMP-positive). In general, cPCs and BMMCs tended to cluster within the same cell type (Fig. 6A,B). Next, differentially expressed genes (DEG) were identified by DEG2 analysis, comparing cPCs to BMMCs. Then, shared DEGs of both EMP-positive patients were analyzed (Fig. 6C, Table S3). Interestingly, among shared genes, CXCL12 was identified, indicating its significance (Fig. 6D,E).

Chemokine CXCL12 and its sole receptor CXCR4 play pivotal roles in bone marrow cell retention [43,44]. CXCL12, functionally designated as stromal-derived factor-1 (SDF-1), is typically expressed in bone marrow stromal cells or vascular endothelial cells to attract CXCR4<sup>+</sup> cells in bone marrow and brain [45–47], but is not expressed in immune cells, such as normal plasma cells. Therefore, this novel finding of abnormal



**Fig. 6.** CXCL12 was upregulated in cPCs compared with BMMCs. (A) Unsupervised clustering of cPCs ( $n = 29$ ) and BMMCs ( $n = 15$ ) of EMP<sup>+</sup> patient P017. (B) Unsupervised clustering of cPCs ( $n = 29$ ) and BMMCs ( $n = 19$ ) of EMP<sup>+</sup> patient P020. Differential expressed genes in cPCs versus BMMCs were compared in DESeq2. (C) DEG were identified with cPCs versus BMMCs. Shared DEGs were identified in both P017 and P020. (D, E) Volcano plot of P017 (D) and P020 (E), cPCs versus BMMCs. Horizontal dashed line indicates adjusted  $P$ -value at 0.01. Vertical dashed lines indicate fold change equals to plus/minus 5. (F) Chemokine secretion pathway and key molecules. (G) Secretion pathway in cPCs versus BMMCs. log2FC, log<sub>2</sub> fold change. Adj- $P$ , adjusted  $P$ -value.

**F**

Nucleus  
Golgi  
Secretive vesicle  
Cell membrane

VAMPs  
Rabs  
Rac1/CDC42  
SNAP-23  
Syntaxins

**G**

Genes	P017		P020		
	log <sub>2</sub> FC	Adj- $P$	log <sub>2</sub> FC	Adj- $P$	
VAMPs	VAMP7	2.323	0.029	0.952	
	VAMP8	3.150	0.007	0.990	
	VAMP5	0.927	0.922	3.682	0.068
Rabs	RAB11B	3.442	0.001	5.953	10 <sup>-6</sup>
	RAB27A	2.874	0.055	2.921	0.141
RAC1	-1.135	0.436	-1.497	0.468	
CDC42	0.937	0.678	0.199	0.872	
SNAP23	0.771	0.655	0.960	0.656	
Syntaxins	SNX2	4.121	0.000	-2.331	0.183
	SNX11	3.922	0.032	1.635	0.772
	SNX20	1.648	0.572	6.203	0.009

CXCL12 transcription in cPCs may be utilized to circumvent CXCR4-dependent bone marrow retention.

Chemokine secretion utilizes a specific pathway, shown in Fig. 6F. With cPCs versus BMMCs, we found a global upregulation of secretion-related genes, including significantly over-expressed Rab11b in both P017 and P020. Furthermore, VAMP7, VAMP8, and Syntaxin-2/11 were upregulated in P017 cPCs, and SNX20 was upregulated in P020 cPCs (Fig. 6G). This upregulation of chemokine-secretive machinery in

cPCs compared with BMMCs indicates robust CXCL12 auto-secretion.

As abnormal upregulation of chemokine CXCL12 expression in cPCs was found in both patients, this finding suggests a self-feed loop generated by cPCs, consequently releasing themselves from the CXCR4-dependent bone marrow retention and translocating freely into the circulation.

To further support this chemokine auto-secretion and self-feed notion, we incorporated analysis of a published

dataset. Pioneer genetic interrogation of cPCs at single-cell resolution has been reported [13]. Although no information about EMP diagnosis was provided, we can still compare the transcriptomes between cPCs and BMBCs across patients for common genes, as in Fig. 6. Both patients showed the distinction between cPCs and BMBCs by unsupervised clustering. Common genes in cPCs versus BMBCs were analyzed with the same significance criteria ( $FC > 10$  and  $P < 10^{-5}$ ). We found only one gene, CXCL7 (pro-platelet basic protein, PPBP), which was another bone marrow retention-related chemokine that is normally expressed by endothelial cells and platelets, but not by immune cells, including normal plasma cells (Fig. S6).

Although CXCL7's direct link to myeloma is unknown, it has been reported to be expressed in cancer cells [48]. Furthermore, its chemokine receptors CXCR1 and CXCR2 are related to bone marrow retention and extramedullary translocation of immune cells, such as neutrophils. Similar to CXCL12, the chemokine secretive pathway was upregulated for CXCL7 (Fig. S6). Again, auto-secretion of CXCL7 generated a possible self-feed loop that provided additional freedom for cPC extramedullary translocation.

## Discussion

To explore the initiation of EMP pathogenesis through extramedullary translocation, we monitored the correlation of cPCs with EMP-positive patients in terms of detection frequency and survival prognosis. By comparing the single-cell transcriptome between cPCs and BMBCs, CXCL12 emerged in EMP-positive patients and was strongly correlated with cell retention in bone marrow. Previously published data identified that CXCL7, another bone marrow attracting chemokine, was auto-secreted in cPCs. Although the transient extramedullary translocation event was not captured in this study, since the very first alteration of myeloma cells into circulating metastatic cells occurs long before EMP diagnosis, the same mechanism may exist.

Establishment of a causal relationship between chemokine auto-secretion and EMP incidence is still challenging. A possible validation method is to block target chemokines, such as CXCL12 or CXCL7, in peripheral blood (e.g., with monoclonal antibody), and then test cPC presence and EMP incidence. The development of animal models for EMP would also benefit from validation of this mechanism.

As rare as 1/10 000 to 1/1000 in PBMCs, cPC detection is an invaluable method for MM diagnosis, follow-up, and prognosis [49–54]. Compared with bone marrow biopsy, cPC detection is cost-efficient and feasible

for multitesting, which is crucial for following treatment response. Compared with imaging-based EMP diagnosis, the single-cell resolution of cPC detection increases diagnostic sensitivity and may contribute to identification of early metastasis. In addition, it is technically much easier to collect single cPCs using FACS than it is to perform an EMP biopsy. We have shown that cPCs are more frequently detected in EMP-positive patients, confirming their clinical significance. Furthermore, by combining imaging EMP diagnosis and cPC detection, we found a possible strategy for prognostic stratification. Larger scale studies on this leading correlation between cPCs and EMP diagnosis are yet to be done.

Understanding of cancer biology has recently been brought into single-cell resolution. scRNA-seq has become a powerful tool with which to dissect the cellular state at the finest scale. This approach retains the benefits of conventional bulk RNA-seq in providing a global yet detailed view of the transcriptome and surpasses bulk RNA-seq by generating much more informative data reflecting intercellular heterogeneity, which is highly biomedically relevant.

Interventions targeting chemokines instead of receptors should be explored. In MM, antagonists and antibodies targeting CXCR4 are under clinical trials, with the rationale that after chemotherapy and irradiation, hematopoietic stem cells are retained in bone marrow by the CXCL12/CXCR4 axis, delaying the exit of mature cells, such as neutrophils and monocytes, to the periphery, and disrupting this axis will benefit patients with faster blood recovery. However, here we provided evidence that chemokines, not chemokine receptors, are related to EMP metastasis, emphasizing the long-neglected significance of chemokine upstream regulators, such as hypoxia, as targets.

## Conclusions

In summary, an abnormal chemokine auto-secretion was found to be upregulated specifically in cPCs. Both chemokines (CXCL12 and CXCL7) are strongly related to bone marrow retention and extramedullary translocation. Therefore, we deduce that a chemotactic self-feed mechanism may contribute to initial extramedullary translocation of cPCs from bone marrow, and the eventual formation of soft-tissue EMP. Our findings increase understanding of metastatic MM and identify a new chemokine target for intervention.

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### Author contributions

SG, JW, YH, and HJ designed the study and prepared the manuscript. JW, JZ, YZ, and HJ contributed to patient enrollment and follow-up. SG, XL, and WW carried out single-cell RNA-seq analysis. XZ and FW carried out scRNA-seq data analysis. YP, JW, YH, and HJ critically reviewed the manuscript.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Gating strategy of flow cytometry.

**Fig. S2.** Protocol validation.

**Fig. S3.** Patient-oriented clustering of BMMCs.

**Fig. S4.** Oncogenes, tumor suppressor genes supported patient-oriented clustering of BMMCs.

**Fig. S5.** Gene expression profile (GEP) genes supported patient-oriented clustering of BMMCs.

**Fig. S6.** CXCL7 were upregulated in cPCs compared with BMMCs in published data.

**Table S1.** PCA result for Fig. 4B.

**Table S2.** Seurat result for Fig. S3.

**Table S3.** DEG for Fig. 6C.