Osteoclast-gene expression profiling reveals osteoclast-derived CCR2 chemokines promoting myeloma cell migration

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Multiple myeloma is characterized by the clonal expansion of malignant plasma cells (multiple myeloma cells [MMCs]), in the bone marrow. Osteolytic bone lesions are detected in 80% of patients because of increased osteoclastic bone resorption and reduced osteoblastic bone formation. MMCs are found closely associated with sites of increased bone resorption. Osteoclasts strongly support MMC survival in vitro. To further elucidate the mechanisms involved in osteoclast/MMC interaction, we have identified 552 genes overexpressed in osteoclasts compared with other bone marrow cell subpopulations. Osteoclasts express specifically genes coding for 4 CCR2-targeting chemokines and genes coding for MMC growth factors. An anti-CCR2 monoclonal antibody blocked osteoclast chemotactic activity for MMC, and CCR2 chemokines are also MMC growth factors, promoting mitogen-activated protein kinase activation in MMC. An anti-insulin growth factor-1 receptor monoclonal antibody completely blocked the osteoclast-induced survival of MMC suppressing both osteoclast and MMC survival. Specific a proliferation-inducing ligand or IL-6 inhibitors partially blocked osteoclast-induced MMC survival. These data may explain why newly diagnosed patients whose MMC express high levels of CCR2 present numerous bone lesions. This study displays additional mechanisms involved in osteoclast/MMC interactions. This study suggests using CCR2 and/or insulin growth factor-1 targeting strategies to block this interaction and prevent drug resistance. (Blood. 2011;117(4):1280-1290)

Introduction

Multiple myeloma (MM) is a plasma cell neoplasm characterized by the accumulation of malignant plasma cells primarily in the bone marrow (BM). A majority of patients with MM develop osteolytic bone disease characterized by bone pain, pathologic fractures, and hypercalcemia resulting from the disruption of the coupling of osteoclastic bone resorption and osteoblastic bone formation.1 An increase in bone turnover rate was reported to precede progression from monoclonal gammopathy of undetermined significance to overt MM.1 In patients with intramedullary MM, MM cells (MMCs) develop in close interaction with the BM microenvironment, mainly BM stromal cells,2 endothelial cells,3 and osteoclasts.4,5 In particular, MMCS promote osteoclast formation directly4,6 or indirectly7,8 and osteoclasts support MMC survival, producing a proliferation-inducing ligand (APRIL) or interleukin-6 (IL-6) particularly.9,10 Several factors are involved in myeloma bone disease,11 mainly the receptor activator of nuclear transcription factor-B (RANKL),8 macrophage inflammatory protein-1-α (MIP-1α/CCL3),12-14 tumor necrosis factor-α,15 interleukin-1α (IL-1α),15 and IL-6.15 A shift in RANKL versus osteoprotegerin expression in patients with MM favors osteoclast generation and activation,16 and blocking RANKL decreases tumor burden and bone destruction in patients with MM.4 MIP-1α/CCL3 is produced by MMCs, stromal cells, monocytes, and osteoclasts.13,17 Both osteoclasts and MMCs express CCR1, a receptor for MIP-1α/CCL3 receptor, which promotes osteoclast formation and activation, and inhibition of MIP-1α/CCL3 decreases markedly both tumor burden and bone destruction in a murine model of MM.14,18

Given the importance of the interaction of MMCs and osteoclasts to promote both MMC growth and osteoclast formation, this study aims to further characterize the cell communication mechanisms between these 2 cell types. We show here that osteoclasts specifically express a set of chemokines targeting CCR2 receptors that are overexpressed by MMCs compared with normal plasma cells. At the same time, newly diagnosed MM patients with a high CCR2 gene expression on MMCs exhibit a higher number of bone lesions than patients with a low CCR2 expression on MMCs. The chemotactant activity of osteoclasts on MMCs is inhibited by an anti-CCR2 monoclonal antibody (mAb). These CCR2 chemokines are also myeloma growth factors (MGFs), promoting mitogen-activated protein kinase (MAPK) activation in MMCs. We also show that osteoclasts support MMCs by producing insulin growth factor-1 (IGF-1), APRIL, and IL-6. This study underscores the important role of osteoclasts in recruiting MMC and promoting their survival and emphasizes the interest of CCR2 targeting therapies.

Methods

XG-human myeloma cell lines (HMCLs) were obtained as described.19 SKMM, L363, OPM2, LP1, and RPMI8226 HMCLs were purchased from ATTC (LGC Promochem). MMCs were obtained in agreement with the...
French and German ethical laws. MMCs were purified from the BM of 206 patients with newly diagnosed MM (median age, 59 years) after written informed consent was given in accordance with the Declaration of Helsinki. The study has been approved by the ethics boards of Heidelberg University and Montpellier University Hospitals. These 206 patients were treated with high-dose therapy and autologous stem cell transplantation and were termed in the following Heidelberg-Montpellier series.\(^{20}\)

We also used Affymetrix data of a cohort of 345 purified MMCs from previously untreated patients from the Arkansas Cancer Research Center. The patients were treated with total therapy\(^ {21}\) and termed in the following Arkansas Cancer Research Center-TT2 series. These data are publicly available via the online Gene Expression Omnibus (Gene Expression Profile of Multiple Myeloma, accession number GSE2658; http://www.ncbi.nlm.nih.gov/geo; accessed June 1, 2006). Normal BM plasma cells (BMPCs) and whole BM cells were obtained from healthy donors after informed consent was given. Whole BM cells were collected after lysis of red blood cells with \(\text{NH}_4\text{Cl}\).

Myeloma, accession number GSE2658; http://www.ncbi.nlm.nih.gov/geo; Cancer Research Center-TT2 series. These data are publicly available via a Cell Titer Glo Luminescent Assay (Promega) with a Centro LB 960 luminometer (Berthold Technologies).

### Enzyme-linked immunosorbent assay

The concentrations of B-cell-activating factor of the tumor necrosis factor family (BAFF), APRIL, IL-10, IL-6, and IGF-1 were assayed with enzyme-linked immunosorbent assays purchased from Bender MedSystems for BAFF and APRIL,\(^ {22}\) from Diacline for IL-6 and IL-10 and from R&D Systems for IGF-1 in accordance with the manufacturer’s instructions.

### Study of apoptosis

IL-6–dependent HMCLs were starved of IL-6 for 3 hours and cultured in 24-well flat-bottomed microtiter plates at 10\(^5\) cells/well in RPMI 1640–10% FCS in the presence or not of osteoclasts (2.5 × 10\(^4\) cells/well), with or without B-E8 anti–IL-6 mAb (10 \(\mu\)g/mL), TACI-Fc (20 \(\mu\)g/mL), anti–IL-10 mAb (10 \(\mu\)g/mL), anti-CRR2 mAb (10 \(\mu\)g/mL), or IGF-1 receptor mAb (4 \(\mu\)g/mL; Calbiochem). Recombinant IL-6 (2 ng/mL) was added in one culture group as positive control. After 4 days of culture, cells were washed twice in phosphate-buffered saline, and apoptosis was assayed with FITC-conjugated annexin V labeling (Boehringer). Fluorescence was analyzed on a FACSscan flow cytometer (BD Biosciences).

### cRNA and microarray hybridization

RNA extraction was performed using the RNeasy kit (Qiagen), the SV-total RNA extraction kit (Promega), and Trizol (Invitrogen). Labeled cRNA was generated using the small sample-labeling protocol VII (Affymetrix), and hybridized to U133 2.0 plus arrays according to the manufacturer’s instructions. Fluorescence intensities were quantified and analyzed using GECOS Version 1.4 software (Affymetrix).

All microarray data presented in this paper have been deposited in the ArrayExpress public database, under accession numbers E-MEXP-2360 for BMPC\(^ {23}\) and E-TABM-937 for B cells, polyclonal plasmablasts, MMCs, and environment population samples.

### Real-time reverse-transcribed polymerase chain reaction

Total RNA was converted to cDNA using the Superscript II reverse transcriptase (Invitrogen). The assays-on-demand primers and probes for the \(\text{β}_2\)mRNA Universal Master Mix were used according to the manufacturer’s instructions (Applied Biosystems). The measurement of gene expression was performed using the ABI Prism 7000 Sequence Detection System and analyzed using the ABI Prism 7000 SDS Version 1.0 software. For each set of primers, serial dilutions of a standard cDNA were amplified to create a standard curve, and values of unknown samples were estimated relative to this standard curve to assess the polymerase chain reaction efficiency. Threshold cycle (\(\text{C}_t\)) values were obtained for glyceraldehyde-3-phosphate dehydrogenase and the respective genes of interest during log phase of the cycle. Gene of interest levels were normalized to glyceraldehyde-3-phosphate dehydrogenase for each sample (\(\text{ΔC}_t = \text{C}_t\) gene of interest – \(\text{C}_t\) glyceraldehyde-3-phosphate dehydrogenase) and compared with the values obtained for a known positive control using the following formula: 100 × 2\(^{\text{Δ(ΔC}_t)}\) where \(\text{ΔΔC}_t = \text{ΔC}_t\) unknown – \(\text{ΔC}_t\) positive control.
Western blot analysis

HMCLs were starved overnight in RPMI 1640–1% bovine serum albumin without IL-6 and then incubated with serum-free culture medium, recombiant IL-6 (30 ng/mL), recombinant CCL7, or CCL8 (2 μg/mL) for 10 and 30 minutes. Cells were then processed for Western blot analysis as detailed elsewhere.19 The primary antibodies (phospho-specific antibodies anti-ERK1/2, anti-STAT3, and anti-AKT; New England Biolabs) were diluted 1% bovine serum albumin Tris-buffered saline containing Tween 20 (1:1000 dilution). The primary antibodies were visualized with goat antirabbit (Sigma-Aldrich) or goat antigoat (Bio-Rad) peroxidase-conjugated antibodies using an enhanced chemiluminescence detection system. As a control for protein loading, we used anti-STAT3 (1:2000) (Transduction Laboratories), anti-ERK1/2 (1:2000) (Santa Cruz Biotechnology), and anti-AKT (New England Biolabs) antibodies.

Statistical analysis

Gene expression data were normalized with the MAS5 algorithm and analyzed with our bioinformatics platform (RAGE, http://rage.montp.inserm.fr; and Amazonia, http://amazonia.montp.inserm.fr), the Significance Analysis of Microarrays software (as previously described),19 and the Maxstat package used in R software (http://cran.r-project.org). Statistical comparisons were done with Mann-Whitney, χ², or unpaired or paired Student t tests.

Results

Identification of cell communication signals involved in MMC/osteoclast interactions

To identify osteoclast-associated factors that could promote MMC survival, we investigated genes overexpressed in osteoclasts compared with BM CD14 monocytes, BM CD15 polymorphonuclear cells, BM CD34 cells, BM stromal cells, or BM B cells using supervised Significance Analysis of Microarrays analysis (ratio ≥ 2, false discovery rate ≤ 1%, 1000 permutations). Genes overexpressed in osteoclasts compared with normal BM plasma cells or primary MMs were also determined. Crossing these gene lists yielded 552 genes expressed sequence tags significantly overexpressed in osteoclasts compared with the 7 BM cell populations (supplemental Table 1, available on the Blood Web site; see the Supplemental Materials link at the top of the online article). These genes were significantly (P < .05) enriched in genes encoding for 2 major pathways: “cellular function and maintenance” and “cell-to-cell signaling and interaction” as well as 10 other pathways (supplemental Figure 1).

Osteoclasts express genes coding for CCR2 chemokines specifically, and high CCR2 gene expression in myeloma cells is associated with increased bone lesions

Seven of the 552 osteoclast genes encoded for chemokines (Figure 1A; supplemental Table 1). Four of these chemokines (CCL2, CCL7, CCL8, and CCL13) target the CCR2 receptor expressed by myeloma cells.25,26 One gene encodes for CCL23 chemokine, which is a weak activator of CCR1,27 a chemokine receptor also expressed by myeloma cells.13,14,28 The other 2 chemokine genes encode for CXCL5 whose receptor CXCR2 is not expressed by MMC (http://amazonia.transcriptome.eu) and CCL10 whose receptor is unknown. Besides these osteoclast-specific genes, osteoclasts expressed additional chemokine genes also expressed by other components of the BM environment, in particular the MPI1-α/CCL3 gene expressed by monocytes, BM stromal cells, and MMs (supplemental Figure 2) as previously described.29 Given the very specific expression of genes coding for CCR2 chemokines by osteoclasts and that myeloma cells stimulate bone resorption,30 we questioned whether CCR2 gene expression of MMs from newly diagnosed patients with MM could be linked with increased bone lesions. The CCR2 gene expression on normal and malignant plasma cells is shown in Figure 1B. The reliability of the Affymetrix probe set (206978_at) to assay for CCR2 gene expression was validated characterizing protein expression by flow cytometry in HMCLs. A strong correlation between CCR2 gene and protein expression was found (r = .90, P < .001, supplemental Figure 3). Furthermore, CCR2 protein could not be detected in HMCLs with an absent Affymetrix call (supplemental Figure 3). CCR2 expression was not expressed in normal memory B cells in agreement with its reported suppression by Fx5 B-cell transcrip- tion factor.11 CCR2 was expressed by normal plasmablasts and BM plasma cells and was significantly increased in MMs compared with normal BMPCs (P ≤ .05, Figure 1B). We then investigated whether CCR2 expression in MMs could be linked with bone lesions. Using the Maxstat function of R package, a maximum difference in the proportion of patients with major bone structural damage or more than 3 osteolyses was obtained splitting patients into 2 groups with a CCR2 cutoff = 3735. Major bone structural damage or more than 3 osteolyses were observed in 55% of the patients with CCR2 signal in MMs more than 3735 versus 36% of the patients with CCR2 signal less than or equal to 3735 (P < .05, Tables 1, 2). This CCR2 signal cutoff is 2.7-fold the median value of CCR2 signal in normal plasma cells. Of note, because osteo- clasts specifically express the gene coding for CCL23 that activates CCR1 weakly, we also studied whether CCR1 expression in MMC could be linked with bone lesion extent. Contrarily to CCR2, no cutoff of CCR1 gene expression in MMs could identify a group of newly diagnosed patients with increased bone lesions. An explanation is that various cells in the BM environment could produce other CCR1-targeting chemokines. This is the case for MPI1-α/CCL3 whose gene is expressed by osteoclasts but also by monocytes, stromal cells, and MMs as mentioned in Supplemental Figure 2. These data suggest these chemokines could be important to recruit MMCs to the BM but not specifically close to osteoclasts.

Role of CCR2 chemokines in the osteoclast chemoattractant activity for myeloma cells

The chemoattractant activity of osteoclasts for MMs was evaluated using the XG-6, XG-19, and LP1 HMCLs. XG-19 and XG-6 myeloma cells express CCR2 gene and protein, unlike LP1 myeloma cells (Figure 2). Osteoclasts could efficiently attract XG-19 and XG-6 cells, unlike LP1 cells in a transwell assay. This chemoattractant activity was inhibited by a neutralizing mAb to CCR2 by 95% and 87% for the XG-19 and XG-6 cells, respectively (P < .05, Figure 2B). As osteoclasts express MPI1-α/CCL3 chemokine gene together with monocytes or BM stromal cells and specifically CCL23 gene, we also investigated a role of CCR1-targeting chemokines in the osteoclast chemoattractant activity. XG-19 and XG-6 cells expressed CCR1 and a mAb to CCR1 inhibited by 80% and 87% the osteoclast-induced migration of XG-19 and XG-6 cells, respectively (P < .05, Figure 2B). Addition of both anti-CCR1 and anti-CCR2 mAbs further inhibited the osteoclast-mediated migration of myeloma cells (Figure 2B). Similar data were obtained with primary myeloma cells of 5 patients (Figure 2C-D). Primary myeloma cells variably expressed CCR2 or CCR1 assayed by flow cytometry depending on patients’ sample. Osteoclasts could attract more than 4% primary myeloma...
Figure 1. Chemokine signature of osteoclasts. (A) Affymetrix CCL2, CCL7, CCL8, CCL13, CCL23, CXCL5, and CCL18 gene expression in BM CD34 cells (n = 5), BM stromal cells (n = 5), purified BM CD15 (n = 5), CD14 (n = 5), and CD3 cells (n = 5), osteoclasts (n = 7), normal memory B cells (n = 6), normal polyclonal plasmablasts (n = 7), normal BMPCs (n = 7), and purified myeloma cells from consecutive patients with MM (n = 206). (B) CCR2 expression in memory B cells, normal plasmablasts, and normal BM plasma cells from 7 healthy donors, 206 with MM, and 20 HMCL. The Affymetrix signal of the CCR2 probe set 206978_at is indicated on the y-axis. CCR2 expression in each sample is indicated by the height of the bar. Samples are ordered from the highest to lowest expression of CCR2 gene from left to right on the x-axis. The attached table shows the median value and range of Affymetrix signal and the percentage of samples with a present call.
cells for 3 of the 5 patients, the highest chemotactic effect being observed with primary myeloma cells with the highest CCR2 and CCR1 expression (Figure 2C-D). The osteoclast chemotactic activity was inhibited by a neutralizing anti-CCR2 mAb (P < .05, Figure 2D). It was also inhibited by a neutralizing anti-CCR1 mAb (P < .05, Figure 2D)

CCR2 chemokines activate MAP kinase pathway in myeloma cells and promote myeloma cell growth, and a MAP kinase inhibitor abrogates the chemotactic activity of osteoclasts to myeloma cells

The recombinant CCL7, CCL8, and CCL13 CCR2 chemokines significantly increased the growth of CCR2+ XG-19 cells and only CCL8 that of XG-6 cells (P < .05, Figure 3A). These chemokines did not stimulate the growth of CCR2- LP1 cells (Figure 3A). CCL7 and CCL8 chemokines, which had the highest effects on myeloma cell growth, were assayed for signal transduction. The 2 chemokines induced MAPK phosphorylation in XG-19 cells. They did not induce STAT3 or AKT phosphorylations, whereas IL-6 induced the phosphorylation of STAT3, MAPK, and AKT, in agreement with previous data. CCL7 or CCL8 did not induce MAPK phosphorylation in CCR1−CCR2− LP1 cells (Figure 3B). As the osteoclast chemotactic activity for MMC was inhibited by anti-CCR2 mAbs and as CCR2 chemokines activated MAPK pathway in MMC, we looked for the effect of a MAP kinase inhibitor on osteoclast chemotactic activity for MMC. The PD98059 MAP kinase inhibitor dramatically inhibited the migration of myeloma cells toward osteoclasts (Figure 3C).

Osteoclasts promote the survival of primary MMC

Osteoclasts overexpress genes encoding for previously reported MGFs, including IGF-1, IL-10, and APRIL (Figure 4A). IL-6 and BAFF genes are also highly expressed by osteoclasts but were not picked in the osteoclast gene list because they are also highly expressed by BM stromal cells (IL-6), monocytes, or polymorphonuclear cells (BAFF, Figure 4A). Osteoclasts produced IGF-1 or APRIL proteins. A total of 418 plus or minus 16 pg/mL of IGF-1 and 5 plus or minus 0.3 ng/mL of APRIL were detected in 3-day culture supernatants of osteoclasts (Figure 4B), concentrations that are active on myeloma cells. Osteoclasts also produced IL-6 (141 ± 13 pg/mL) and BAFF (1.6 ± 0.5 ng/mL) and produced a low amount of IL-10 (5.1 ± 1.1 pg/mL) compared with the known biologically active concentrations. A coinoculation of osteoclasts with XG-19 MMC did not significantly affect the production of IGF-1, IL-6, APRIL, BAFF, or IL-10 (Figure 4B).

To investigate the role of these various growth factors in the survival activity of MMC induced by osteoclasts, we used the XG-19 and XG-20 HMCLs that expressed IL-6R, IL-10R, IGF1R, TACI, and BCMA BAFF/APRIL receptors. On IL-6 deprivation, XG-19 and XG-20 cells rapidly apoptosed, and a coculture with osteoclasts protected them from apoptosis (P = .01). Fluorescence-activated cell sorter data from one representative experiment are shown in Figure 5A and the mean values plus or minus SD of 5 experiments in Table 3. The survival-promoting activity of osteoclasts was partially inhibited blocking IL-6 using the B-E8 anti-IL-6 mAb (P = .05 for XG-19 and P = .01 for XG-20) or BAFF/APRIL using TACI-Fc fusion protein (P = .01 for XG-19 and P = .02 for XG-20). Inhibiting IGF-1 using an anti-IGF-1R mAb completely blocked MMC survival supported by osteoclasts in serum-free culture medium (P = .003 for XG19 and P = .0005 for XG20; Figure 5A). The anti-IGF-1R mAb further increased the apoptosis rate observed without osteoclasts, probably by inhibiting the autocrine IGF-1/IGF-1R loop occurring in some HMCLs. A neutralizing anti-IL-10 mAb had no significant effect. The anti-CCR2 mAb did not either inhibit the MMC growth activity of osteoclasts, but this anti-CCR2 mAb partially blocked the growth activity of recombinant CCL7 or CCL8 (supplemental Figure 4), whereas it blocked efficiently their chemotactic activity (Figure 2B).

Discussion

The aim of this study was to identify some major cell communication signals involved in recruitment of MMC close to osteoclasts and subsequent support of MMC survival. We first identified 552 genes that are significantly up-regulated in osteoclasts compared with various cell components of the BM microenvironment and to MMC. Comparing our results
with previous gene array analyses of osteoclasts, 22 common genes coding for secreted or extracellular matrix proteins were identified in the Pederson et al study\(^3\) and in our current study, in particular CCL7, CCL8, and CXCL5 chemokine genes and RANK, IGF-1, and IL-10 genes (supplemental Table 2). This stringent method was initially chosen to limit the size of the osteoclast gene list, being aware that some genes that are highly expressed both in osteoclasts and in another cell population could be eliminated. Our data show that this method is of interest to find the signals that may attract MMC specifically to osteoclasts and promote MMC survival.

There are approximately 50 chemokines,\(^2\) some of them being already found to be produced in the BM milieu of patients with MM: mainly CXCL12/SDF-1 (targeting CXCR4 and produced by stromal cells, endothelial cells, and myeloma cells) and MIP1\(\alpha/\beta\) CCL3 (targeting CCR1 and CCR5 and produced by osteoclasts, BM stromal cells, osteoblasts, monocytes, and myeloma cells\(^2\)). These chemokines are important to recruit circulating MMCs into the BM as illustrated for CXCL12/SDF-1.\(^4\) They could be also critical to trigger the fate of a myeloma cell in the presence of gradients of multiple chemokines produced by various environment cells. The current data provide some answer regarding the chemokines targeting CCR1 or CCR2, both receptors being expressed by myeloma cells.\(^2\) These CCR1 or CCR2 chemokines are produced by osteoclasts, and we show that CCR1 and CCR2

\[\text{Figure 2. Gene and protein expression of CCR1 and CCR2 chemokine receptors.} (A) Data are Affymetrix signals for CCR1 (probe set 205098_at) and CCR2 (probe set 206978_at) in LP1, XG-19, and XG-6 HMCLs. Membrane expression of CCR1 or CCR2 was evaluated by flow cytometry using PE-conjugated anti-CCR1 or anti-CCR2 mAbs or isotype-related PE-conjugated control mAbs. The numbers in the panels are the RFI of the PE-conjugated anti-CCR1 or anti-CCR2 mAbs compared with the PE-conjugated control mAbs. (B) The chemoattractant activity of osteoclasts to myeloma cells was assayed using XG-19, XG-6, and LP1 HMCLs. Data are the fraction of MMC in the upper chamber of the transwell that could migrate to the lower chamber. Results are the mean values plus or minus SD of 3 experiments. *Significant difference of MMC migration in MMC/osteoclast cocultures compared with control using a paired Student t test (P < .05). **Significant difference of MMC migration in MMC/osteoclast cocultures with anti-CCR1 and/or anti-CCR2 mAb compared with MMC/osteoclast cocultures using a paired Student t test (P < .05). (C) The expression of CCR1 and CCR2 by CD138\(^+\) primary myeloma cells of patients was evaluated by flow cytometry using FITC-conjugated anti-CD138 mAb and PE-conjugated anti-CCR1 or anti-CCR2 mAbs labeling. Isotype-matched FITC-conjugated or PE-conjugated mAbs recognizing no human antigens were used as control mAb. The numbers in the panels are the RFI of the PE-conjugated anti-CCR1 or anti-CCR2 mAbs compared with the PE-conjugated control mAbs. (D) The chemoattractant activity of osteoclasts to myeloma cells was assayed using primary myeloma cells. Data are the fraction of primary MMC in the upper chamber of the transwell that could migrate to the lower chamber.\]
chemokines contribute to osteoclast chemoattractant activity for myeloma cells in vitro, in agreement with previous data for CCR1 chemokines. In addition, we show that the expression of CCR2 chemokines is very specific to osteoclasts, whereas it was previously shown that various cells, including osteoclasts, produce the MIP-1α/CCL3 CCR1 chemokine. This suggests that a myeloma cell that expresses CCR1 could be recruited close to the various cells producing MIP-1α/CCL3 (ie, osteoclasts, stromal...
cells, monocytes, or myeloma cells). But if the myeloma cell expresses CCR2, it could be attracted by osteoclasts specifically. Given that osteoclast can recruit and promote survival of CCR2^+ myeloma cells, and in turn, myeloma cells promote osteoclast recruitment and activation, it is hardly surprising that the bone lesion number in patients with newly diagnosed MM was associated with high CCR2 expression in myeloma cells, unlike CCR1 expression. This hypothesis should be further tested injecting CCR2^+ or CCR2^- myeloma cell lines in immunodeficient mice. If the specificity for human CCR2-targeting chemokine expression is kept by murine osteoclasts, one should expect that CCR2^+ myeloma cell lines could create more bone lesions than CCR2^- ones and that anti-CCR2 antibodies could prevent bone lesions and even tumor growth. Alternatively, this could be tested in the murine 5T2 MM model because these murine myeloma cells express CCR2.41

Chemokine receptors are G-protein-coupled receptors that can activate various signaling pathways depending on chemokine receptor and cell type: MAPK, PI-3/AKT, Src, JNK, and Rho.42,43 The CCR2 chemokines induced MAPK phosphorylation in myeloma cells and did not activate Akt or Stat3 pathways, in agreement with the inhibition of osteoclast chemoattractant activity.
for myeloma cells by the PD98059 MAPK inhibitor. High concentrations of recombinant CCR2 chemokines (≥125 ng/mL) can trigger the survival of CCR2⁺ MMC unlike CCR2⁻ MMC.

Osteoclasts also highly expressed genes encoding for some major MGFs and produced high levels of these MGFs: IGF-1, APRIL, and IL-6 and produced the corresponding proteins. A high expression of IL-10 gene was found, but no protein production. In agreement with growth factor production, the survival of MMC induced by osteoclast was partly inhibited by IL-6 and APRIL inhibitors, indicating their contribution to osteoclast-induced MMC survival. An interesting finding is the complete inhibition of osteoclast-induced survival of MMC by the anti-IGF-1R mAb. This may be for 2 reasons. First, IGF-1 is a critical factor for the generation and survival of osteoclasts⁴⁴ and the neutralizing anti-IGF-1R decreased osteoclast survival in our culture system (results not shown). Second, IGF-1 is a major MGF, both as a paracrine and autocrine growth factor. In particular, the MGF activities of IL-6, HGF, or EGF-family members are inhibited in part by IGF-1R inhibitors, being dependent of an autocrine IGF-1 loop produced by MMCs.³³
Bone lesions in MM are the result of an abnormal bone remodeling with increased osteoclast and decreased osteoblast activities. In healthy persons, bone remodeling occurs in closed bone remodeling compartments (BRCs) containing osteoblasts and osteoclasts. These BRCs are protected from BM cells by a canopy of flat cells with osteoblastic markers. Red blood cells are found in the BRCs, and radiolabeled ferritin is detected in BRCs 5 minutes after injection. This indicated that BRCs are linked to the vasculature through capillaries that are directly connected to the BRC canopy. This connection of closed BRCs with the vasculature should ensure the recruitment of osteoblasts or osteoclast progenitors while protecting BRC cells from other BM cells. In patients with MM, the canopy of BRCs is frequently disrupted in association with the extent of bone lesions. This canopy disruption makes a direct contact possible between osteoclasts and MMC.

The current data suggest that osteoclasts can directly recruit MMC by producing CCR2 chemokines, promote MMC survival, growth, and drug resistance by producing various growth factors. Vicious versa, MMC will further promote osteoclast progenitor recruitment and differentiation producing MIP1-α/CCL3, MIP-1β, and CXCL12 chemokines, IGF-1, and increasing RANKL production by stromal cells. Thus, osteoclasts and MMC can recruit each other and mutually promote their survival through various mechanisms. It will be interesting to investigate whether this strong cooperation between MMC and osteoclasts is a reflection of a cooperation occurring between osteoclasts and normal plasma cells. Indeed, CCR2 is a plasma cell marker, which is not expressed by B cells. The reason is that CCR2 gene expression is repressed by the Pax5 B-cell transcription factor. This negative control by Pax5 is removed when B cells differentiate into plasma cells because of the repression of Pax5 gene expression by the Blimp1 plasma cell transcription factor. After generation in the lymph node and exit into the circulation, plasma cells have to find a niche either in the BM or in mucosa. CXCR4 is one of the critical homing molecules for normal plasma cells to the BM by SDF-1 nicotinamide adenine dinucleotide phosphate (NADPH), and CXCR4 is one of the critical homing molecules for normal plasma cells to the BM by SDF-1 nicotinamide adenine dinucleotide phosphate (NADPH) receptor CXCR4. This connection of closed BRCs with the vasculature should ensure the recruitment of osteoblasts or osteoclast progenitors while protecting BRC cells from other BM cells. In patients with MM, the canopy of BRCs is frequently disrupted in association with the extent of bone lesions. This canopy disruption makes a direct contact possible between osteoclasts and MMC.

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