CC-Chemokine Ligand 20/Macrophage Inflammatory Protein-3α and CC-Chemokine Receptor 6 Are Overexpressed in Myeloma Microenvironment Related to Osteolytic Bone Lesions

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Abstract

The expression of the chemokine CC-chemokine ligand 20 (CCL20)/macrophage inflammatory protein (MIP)-3α and its receptor CC-chemokine receptor 6 (CCR6) by multiple myeloma (MM) and microenvironment cells and their potential relationship with osteoclast (OC) formation and osteolytic bone lesions in MM patients was investigated in this study. First, we found that MM cells rarely produce CCL20/MIP-3α but up-regulate its production by bone marrow (BM) osteoprogenitor cells and osteoblasts in coculture with the involvement of soluble factors as interleukin-1β and tumor necrosis factor α. MM cells also stimulate both CCL20/MIP-3α and CCR6 expression by OCs in coculture. Thereafter, we showed that CCL20/MIP-3α significantly increases both the number of multinucleated tartrate-resistant acid phosphatase–positive OCs and receptor activator of nuclear factor-κB–positive OC progenitor cells similar to CCL3/MIP-1α. Finally, we found that blocking anti-CCL20/MIP-3α and anti-CCR6 antibodies significantly inhibits MM-induced OC formation. *In vitro* data were further expanded *in vivo* analyzing a total number of 64 MM patients. Significantly higher CCL20/MIP-3α levels were detected in MM patients versus monoclonal gammopathy of uncertain significance (MGUS) subjects and in MM osteolytic patients versus nonosteolytic ones. Moreover, a significant increase of CCL20/MIP-3α–positive osteoblasts in osteolytic MM patients compared with nonosteolytic ones was observed. Interestingly, no significant difference in BM CCL20/MIP-3α expression and level was observed between MGUS and nonosteolytic MM patients. Our data indicate that CCL20/MIP-3α and its receptor CCR6 are up-regulated in the bone microenvironment by MM cells and contribute to OC formation and osteolytic bone lesions in MM patients. [Cancer Res 2008;68(16):6840–50]

Introduction

Multiple myeloma (MM) is a plasma cell malignancy characterized by the high capability to induce bone destruction (1).

Osteolytic bone lesions are the hallmark of MM due to the increase of osteoclast (OC) formation, recruitment, and activation that occur in close contact with MM cell infiltration (2, 3). The increase of OC formation and activity in MM is mainly induced by the imbalance of receptor activator of nuclear factor-κB ligand (RANKL) to OPG ratio in favor of the critical osteoclastogenic factor RANKL that is overexpressed in the bone microenvironment by the adherence of MM cells to the bone marrow (BM) stromal cells or osteoblasts (2, 3). Soluble factors may contribute to OC activation, including interleukin (IL)-6, tumor necrosis factor α (TNFα), IL-1β, and IL-3, through either a RANKL-dependent or RANKL-independent mechanism (2, 3). The chemokine macrophage inflammatory protein-1α (MIP-1α)/CCL3 has also been suggested as a potential OC-activating factor in MM (4–6).

Chemokines are small chemoattractant proteins involved in the regulation of leukocyte migration, adhesion, and proliferation (7–9). Chemokines are classified into four families (C, CC, CXC, and CX3C) based on the number and spacing of cysteine residues and exert their biological effects interacting with specific cell surface receptors that belong to transmembrane G protein–coupled receptor family (7–9). Among the CC chemokines, CCL3/MIP-1α is chemoattractant for OCs and to induce RANKL in BM stromal cells (BMSC) and OC formation *in vitro* (10–12). Higher levels of CCL3/MIP-1α have been observed in BM plasma and isolated plasma cells of MM patients compared with normal subjects correlated with the presence of bone lesions (4, 12–14). Moreover, blocking anti-MIP-1α or anti-its receptor CCR5 antibodies as well as antisense RNA anti-MIP-1α reduced MM-induced *in vitro* OC formation (13–16). All these evidences suggest that CCL3/MIP-1α is a potential candidate as OC activation factor in MM. Actually, the potential involvement of other chemokines of CC family in OC formation, activation, and MM-induced osteoclastogenesis is not known.

CC-chemokine ligand 20 (CCL20)/MIP-3α and its selective receptor CC-chemokine receptor 6 (CCR6), known to be responsible for the chemoattraction of dendritic, T, and B cells in homeostatic conditions (17), have been recently involved in rheumatoid arthritis synovitis (17–19) and OC activation in rheumatoid arthritis patients being overexpressed in subchondral bone tissues (20). Moreover, it has been shown that the overexpression of CCR6 by Langerhans cells in malignant histiocytosis contributes to their accumulation in the bone together with CCL20/MIP-3α overproduction by osteoblasts in the involved bone (21).

Based on these evidences in this study, we have investigated the potential expression of CCL20/MIP-3α and CCR6 by MM and bone microenvironment cells, their role in the pathophysiology of OC formation induced by MM cells, and their potential relationship with the presence of osteolytic bone lesions in MM patients.

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Materials and Methods

Patients

We studied a total number of 64 patients with newly diagnosed MM in stages I to III. In addition, 16 subjects with monoclonal gammopathy of uncertain significance (MGUS) were included in the study. All MM patients underwent total body X-rays to identify the presence of osteolytic bone lesions. Bone disease was defined based on the presence of one or more lesions on X-rays. BM aspirates and bone biopsies were obtained from the iliac crest of all MM patients at diagnosis after obtaining informed consent according to the Declaration of Helsinki and our local ethical committee. The approval of the study was obtained from the University of Parma Institutional Review Board. BM plasma was obtained after centrifugation from BM aspirates treated with EDTA to prevent clotting.

Reagents and Cytokines

Recombinant human (rh) IL-6, IL-1β, TNFα, RANKL, and macrophage colony-stimulating factor (M-CSF) were obtained from Endogen. rhCCL20/MIP-3α and CCL3/MIP-1α were purchased from R&D Systems. Culture media RPMI 1640, DMEM, and α-MEM as well as glutamine, penicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Invitrogen Life Technologies.

Cells and Cell Culture Conditions

Cell lines. Human myeloma cell lines (HMCL) XG-6, XG-1, and JJN3 were obtained from Dr. Bataille (U892 INSERM, Nantes, France). U266 was obtained from the American Type Culture Collection. OPM2, RPMI-8226, and the EBV^+ cell line ARH-77 were purchased from the German Collection of Microorganisms and Cell Cultures. HMCLs were maintained for 48 h at the concentration of 10^6/mL to obtain conditioned medium (CM). Human trabecular SV40-transfected osteoblasts (HOBIT) were a generous gift from Dr. Riggs (Mayo Clinic, Rochester, MN). An immortalized mesenchymal/stromal cell line was a kind gift from Dr. Giuseppe Gaipa (Clinica Pediatrica Università Milano-Bicocca, Monza, Italy).

MM cell purification. Primary $CD138^+$ MM cells were isolated from BM mononuclear cells (BMMNC) of MM patients at diagnosis by immunomagnetic beads using anti-CD138 monoclonal antibody (mAb)-coated microbeads (MACS, Miltenyi Biotec). Only samples with purity >90%, checked by flow cytometry, were used immediately.

Human BMSCs and osteoprogenitor cells (PreOB). Primary BMSCs were obtained from BMMNCs after 2 to 4 wk of culture of the adherent cells in α-MEM supplemented with 15% FCS and 2 mmol/L glutamine. To induce the osteoblast phenotype (PreOB), BM cells recovered after the attachment period (3–5 d) were incubated in α-MEM with 15% FCS and 2 mmol/L glutamine in the presence of ascorbic acid (50 mg/mL) and dexamethasone (10^{-8} mol/L) for 2 to 4 wk, as previously described (22).

In some experiments, primary BMSC or immortalized stromal cells or PreOB were incubated in the presence or absence of CCL20/MIP-3α or CCL3/MIP-1α (0.1–10 ng/mL) or IL-1β (10 ng/mL) or TNFα (20 ng/mL) or IL-6 (40 ng/mL) or a combination of these cytokines for 24 to 72 h.

Osteoblast isolation. Human osteoblasts (hOB) were isolated from the iliac crest biopsies obtained from healthy or MGUS patients after collagenase digestion according to the method used by Robey and Termine, as previously published (20, 23). hOBs were fixed in 4% paraformaldehyde and analyzed using a FACStar Plus cytometer (Becton Dickinson) for the expression of osteoblast markers.

Cocultures. A series of cocultures with adherent confluent osteoblastic cells HOBIT or hOB or PreOB (2×10^6) and HMCLs or fresh purified MM cells (10×10^6) with or without a Transwell insert has been performed in the presence or absence of blocking anti-IL-1β (R&D Systems) or anti-TNFα antibodies (R&D Systems) or both or anti-IgG control antibodies for 24 to 48 h. At the end of culture period, CM were obtained and stored at −80°C. In addition, OCs obtained after 21 d, as further described, were cocultured with HMCLs (JJN3 or XG-1) in tissue culture chambers on glass slides (Falcon, Becton Dickinson) for 48 h. Then, OCs were fixed and stained for CCR6 expression by immunohistochemistry and immunofluorescence.

Osteoclastogenesis. CD14-positive cells were isolated from peripheral blood mononuclear cells by immunomagnetic method. Cells were resuspended in α-MEM with 10% FBS, glutamine, and antibiotics and then plated in eight-well slides (Nunc A/S) at the density of $7.5 \times 10^5/cm^2$ with RANKL (60 ng/mL) and M-CSF (25 ng/mL) in the presence or absence of CCL20/MIP-3α or CCL3/MIP-1α (0.02–10 ng/mL) with or without blocking anti-CCL20/MIP-3α mAbs (0.5 μg/mL; R&D Systems) or anti-CCR6 mAbs (0.5 μg/mL; R&D Systems) or anti-IgG control isotype antibodies.

In parallel condition, $CD14^+$ cells were incubated in α-MEM with 10% FBS or in CM/α-MEM with 10% FBS (ratio, 1:3) of MM cells cocultured with PreOB or with OCs in the presence or absence of blocking anti-CCL20/MIP-3α mAbs (0.5 μg/mL) or anti-CCR6 mAbs (0.5 μg/mL) or anti-IgG control isotype antibodies, replacing medium every 3 d. After 21 d, OCs were stained and counted.

OC evaluation assay. OC phenotype was assessed by cytochemical analysis of tartrate-resistant acid phosphatase (TRAP) by commercial kit (Sigma) and by immunocytochemical analysis of RANK (R&D Systems). TRAP-positive and RANK-positive multinucleated cells containing more than five nuclei (mature OCs) were counted under microscopic examination. Each sample was cultured in duplicate and 40 microscopy fields at high magnification (400×) were evaluated for each well. The results were expressed as mean number of OCs counted per well ± SD. OC activity has been evaluated using the pit assay method, as previously described (24).

Reverse Transcription-PCR Amplification

For reverse transcription-PCR (RT-PCR), 1 μg RNA was reverse transcribed with 400 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer's protocol. cDNAs were amplified by PCR using the following specific primer pairs: CCL20/MIP-3α, 5′-ATGTGCTGTACCAAGAGTTTGC-3′ (forward) and 5′-CCAATTCCATTCCAGAAAAGCC-3′ (reverse); CCR6, 5′-ATTTCAGCGA-TGTTTTCGACTC-3′ (forward) and 5′-GGAGAAGCCTGAGGACTTGTA-3′ (reverse); and β_2-microglobulin, 5′-CTCGCGCTACTCTCTTCTCTTTCTGG-3′ (forward) and 5′-GCTTACATGTCTCGATCCCACTTAA-3′ (reverse).

Annealing temperature was 53°C for both CCL20/MIP-3α and CCR6. Product size was 319 bp for CCL20/MIP-3α, 1,021 bp for CCR6, and 334 bp for β_2-microglobulin. PCRs were performed in a thermal cycler (MiniCycler, MyResearch) for 30 cycles.

Real-time Quantitative PCR

For RANKL real-time amplification, 2 μL cDNA prepared from 1 μg of total RNA was used in a total volume of 25 μL using Universal Master Mix (Applied Biosystems), 300 nmol/L RANKL-specific primer pair (sense: CGTTGGATCACAGCACATCAG; antisense: TGCTCCTCTTGGCCAGATCT), and 200 nmol/L Taqman probe (6-FAM-CAGAGAAAGCGATGGTG-GATGGCTCAT-MGB).

For CCL20/MIP-3α and CCR6 real-time PCR, the Taqman gene expression assays HS00171125_m1 and HS99999079_m1 were used, respectively, according to the manufacturer's protocols (Applied Biosystems). To normalize the difference in RNA quantity and quality, we applied the comparative C_t method using the endogenous reference gene *ABL*. The relative RANKL, CCL20/MIP-3α, and CCR6 mRNA quantification was performed by the comparative $\Delta\Delta C_t$ method as we previously published (25).

ELISA

The amount of CCL20/MIP-3α, CCL3/MIP-1α, IL-1β, TNFα, and IL-6 protein levels in cell culture medium and/or in BM plasma from MGUS and MM patients was evaluated by ELISA using commercially available assay kits (R&D Systems) and according to the manufacturer's procedures.

Immunofluorescence

OCs were fixed in 4% paraformaldehyde for 10 min, washed in PBS, and treated with 20% goat serum for 5 min. Slides were incubated with mouse anti-CCR6 primary antibody (working dilution, 1:1,000 in PBS 20% goat serum; R&D Systems) for 1 h at 37°C. Tetramethylrhodamine isothiocyanate–labeled anti-mouse IgG (working dilution, 1:20; Sigma) was used as secondary antibody and incubated for 1 h at 37°C. Nuclei were stained with 4′,6-diamidino-2-phenylindole for 10 min.

Immunohistochemistry

OCs were fixed in 4% buffered formalin (pH 7.2) for 10 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 in distilled water for 10 min. Slides were incubated with mouse anti-CCR6 primary antibody (working dilution, 1:2,000) for 30 min at room temperature and processed for immunohistochemical staining using the immunoperoxidase technique (Dako LSAB2 System; horseradish peroxidase, Dako).

BM biopsy samples were fixed in B5-formalin mixture, decalcified by EDTA, and embedded in paraffin. Serial sections of 3 μm thick were processed for immunohistochemical staining with anti-CCR6 or anti-CCR5 antibodies (working dilution, 1:500 and 1:600, respectively; R&D Systems) or anti-CCL20/MIP-3α or anti-CCL3/MIP-1α antibodies (working dilution, 1:20 and 1:40, respectively; R&D Systems) without antigen retrieval using the immunoperoxidase technique, as previously described (22). Anti-IgG isotype antibodies have been used as negative control of the reactions.

After the acquisition of bioptic images by Fotovix (Tamron), OCs and hOBs were identified using morphologic criteria, as previously described (22, 26). Immunostaining for the osteoblast marker osteocalcin using anti-osteocalcin mAb (working dilution, 1:20; R&D Systems) has been used to identify osteoblasts. Moreover, immunostaining for the OC marker TRAP with anti-human TRAP mAb (working dilution, 1:100; Novocastra) followed by the incubation with goat anti-mouse biotinylated and alkaline phosphatase–conjugated streptavidin antibodies (Kit BioGenex) at room temperature for 30 min was performed to identify OCs in bone biopsy.

Results

Expression of CCL20/MIP-3α and CCR6 by HMCLs and primary MM cells. First, we checked the potential expression of both CCL20/MIP-3α and its receptor CCR6 by HMCLs and primary purified $CD138^+$ MM cells.

Among the HMCLs tested, we found that only U266, JJN3, and the EBV-positive cell line ARH-77 expressed CCL20/MIP-3α mRNA by PCR with detectable levels of CCL20/MIP-3α in JJN3 (13 ± 2 pg/mL) and in ARH-77 (750 ± 30 pg/mL), as evaluated by ELISA. The presence of CCR6 at mRNA level was found in U266, OPM2, and ARH-77 (data not shown). CCL20/MIP-3α mRNA expression was investigated in purified $CD138^+$ MM cells, finding that 4 of 22 patients were positive for CCL20/MIP-3α mRNA. Similarly, we found that 5 of 22 MM patients expressed CCR6 mRNA (Table 1). CCL20/MIP-3α and CCR6 mRNA levels were further quantified by real-time PCR, as shown in Table 1 using appropriate positive and negative controls. The expression of CCR6 by MM cells was also confirmed at protein level by immunohistochemistry (data not shown).

Expression of CCL20/MIP-3α and CCR6 by osteoprogenitor cells, hOB, and OCs: effect of cytokines and modulation by MM cells in coculture. The expression of CCL20/MIP-3α and its receptor CCR6 on PreOB, hOB, and OCs and their modulation by MM cells were investigated. We found that in basal condition PreOB and OCs but not hOB expressed CCL20/MIP-3α mRNA, whereas the presence of its receptor CCR6 mRNA was found in OCs with a slight expression in hOB and PreOB (Fig. 1*A*). IL-1β treatment (10 ng/mL) stimulated CCL20/MIP-3α production by both PreOB and hOB; TNFα (20 ng/mL) increased CCL20/MIP-3α in PreOB, inducing a slight stimulatory effect in hOB, whereas no effect was observed on HMCLs, as shown for JJN3 (Fig. 1*B*). CCL20/MIP-3α secretion was up-regulated by a synergistic stimulatory effect of IL-1β plus TNFα treatment on both PreOB and hOB (Fig. 1*B*). On the contrary, IL-6 (40 ng/mL) alone did not increase the secretion of CCL20/MIP-3α by both PreOB and hOB, inducing a slight stimulatory effect in combination with IL-1β and TNFα that did not reach a statistical significance (Fig. 1*B*).

Table 1. Expression of CCL20/MIP-3α and CCR6 by purified $CD138^+$ MM cells in a subgroup of MM patients at diagnosis

	CCL20/MIP-3α	ΔC_t*	CCR6	ΔC_t †
Negative control ‡		ND		ND
Positive control§		5.4		3.6
MM1	+	7.5	+	5.1
MM2	−	ND	−	11.7
MM3	−	ND	−	9.6
MM4	−	11.45	−	11.5
MM5	−	10.2	−	11.6
MM6	+	7	+	4
MM7	−	10.1	−	10.85
MM8	−	ND	−	ND
MM9	−	ND	−	ND
MM10	−	ND	−	ND
MM11	+	7.2	+	5.1
MM12	−	ND	−	ND
MM13	−	ND	−	ND
MM14	−	ND	−	ND
MM15	−	11	+	6.8
MM16	−	ND	−	10.4
MM17	−	ND	−	9.15
MM18	+	7.5	+	4
MM19	−	ND	−	11.7
MM20	−	ND	−	ND
MM21	−	ND	−	10.4
MM22	−	ND	−	ND

Abbreviation: ND, not detectable.
*ΔC_t = mean $C_{t\ CCL20}$ − mean $C_{t\ ABL}$.
† ΔC_t = mean $C_{t\ CCR6}$ − mean $C_{t\ ABL}$.
‡ Negative control: $CD19^+$ B cells for CCL20 and BMSC for CCR6.
§Positive control: PreOB for CCL20 and $CD3^+$ T cells for CCR6.

Then, we checked the potential effect of myeloma cells in coculture on CCL20/MIP-3α production and CCR6 expression. Purified $CD138^+$ MM cells and HMCL (XG-1) significantly induced CCL20/MIP-3α secretion in coculture with both PreOB and hOB (Fig. 1*C*). No significant difference in the stimulatory effect was observed between MM cells obtained from osteolytic and non-osteolytic patients, as shown for two representative patients (MM1 and MM2; Fig. 1*C*). Blocking both IL-1β and TNFα significantly reduced CCL20/MIP-3α level in coculture between PreOB and MM cells (Fig. 1*C*). A similar inhibitory effect was observed with hOB (data not shown).

Moreover, we found that CCL20/MIP-3α secretion was significantly stimulated when OCs were cocultured with either purified $CD138^+$ cells or HMCLs, as shown for XG-1 (Fig. 1*D*). MM cells also up-regulated CCR6 expression by OCs, as shown for JJN3 by immunohistochemistry (Fig. 1*D, i*) and immunofluorescence (Fig. 1*D, ii*). Both the number of CCR6-positive OC cells (Fig. 1*D, iii*) and the intensity of CCR6 expression in the CCR6-positive OC cells (Fig. 1*D, iv*) were increased by HMCLs with a maximal effect with the CCL20/MIP-3α–positive JJN3 cells compared with the other HMCLs.

The osteoclastogenic effect of CCL20/MIP-3α. To check the potential role of CCL20/MIP-3α in MM-induced OC formation, first the effect of CCL20/MIP-3α on *in vitro* OC formation by peripheral

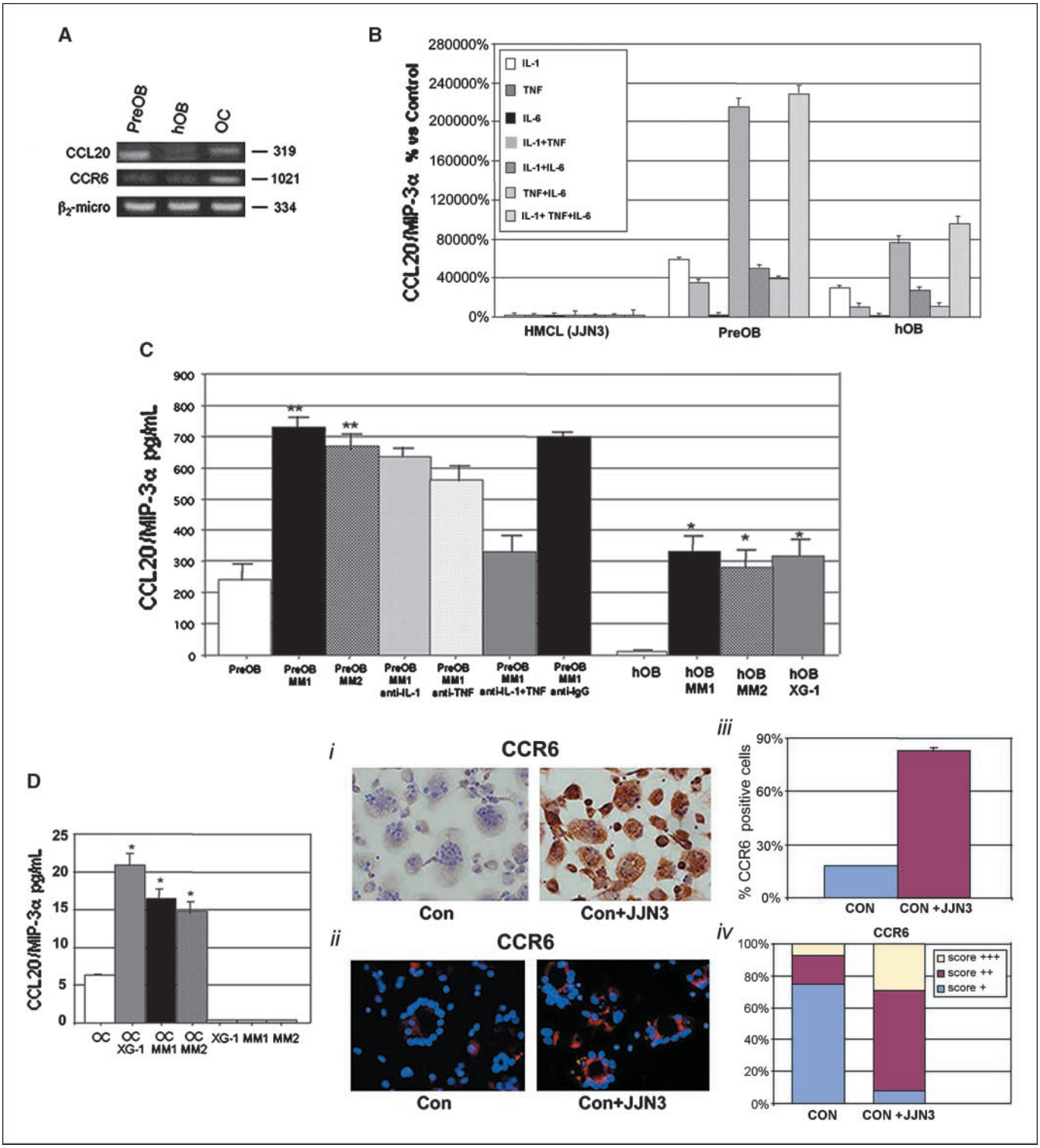

Figure 1. CCL20/MIP-3α and CCR6 expression by osteoprogenitor/osteoblastic cells and OCs: effects of cytokines and myeloma cells in coculture. *A,* the expression of CCL20/MIP-3α and CCR6 mRNA was evaluated by RT-PCR in PreOB, hOB, and OCs. *B,* confluent PreOB or hOB or the HMCL JJN3 was incubated either in the presence or absence of IL-1β (20 ng/mL), TNFα (10 ng/mL), IL-6 (40 ng/mL), or IL-1β plus TNFα, IL-1β plus IL-6, TNFα plus IL-6, and TNFα plus IL-1β plus IL-6. *C,* confluent PreOB or hOBs were cocultured in a Transwell insert with XG-1 or $CD138^+$ MM cells (MM1: osteolytic patient; MM2: nonosteolytic patient) for 48 h with or without blocking anti-IL-1β or anti-TNFα antibodies or both or anti-IgG control antibody. After the culture period, CCL20/MIP-3α levels were checked by ELISA in aliquots of CM. *B,* mean % variation of CCL20/MIP-3α levels compared with control. *C, columns,* mean CCL20/MIP-3α levels of three independent experiments measured twice; *bars,* SD. *, $P < 0.05$; **, $P < 0.01$. Human OCs were cocultured with HMCLs XG-1 and JJN3 or with purified $CD138^+$ MM cells from either osteolytic patient (MM1) or nonosteolytic one (MM2) placed in a Transwell insert in tissue culture chambers with glass slide for 48 h. After the culture period, aliquots of the CM were tested for CCL20/MIP-3α by ELISA. *D, columns,* mean CCL20/MIP-3α levels of three independent experiments measured twice; *bars,* SD. OCs were fixed and stained for CCR6 expression by immunohistochemistry (*i*) and by immunofluorescence (*ii*), as described in Materials and Methods. Original magnification, ×400. *iii, columns,* % mean of CCR6-positive OC cells evaluated by immunohistochemistry; *bars,* SD. *iv,* immunohistochemical score distribution of CCR6-positive OCs cells cocultured with or without JJN3.

monocytes was evaluated. We found that rhCCL20/MIP-3α significantly increased both the number of multinucleated TRAP$^+$ OCs and RANK$^+$ OC progenitor cells in the presence of RANKL and M-CSF with a maximal effect at 0.3 ng/mL (Fig. 2*A* and *B*). Consistently blocking anti-CCL20/MIP-3α or anti-CCR6 antibodies completely blunted the pro-osteoclastogenic effect of CCL20/MIP-3α (Fig. 2*A*). The *in vitro* pro-osteoclastogenic effect of CCL20/MIP-3α was compared with that induced by CCL3/MIP-1α. A similar effect on OC formation and activation was observed with the two chemokines (Fig. 2*C*). The potential effect of CCL20/MIP-3α on RANKL expression by BMSC was also evaluated. By quantitative real-time PCR, we found that CCL20/MIP-3α increased RANKL mRNA expression by PreOB with a mean fold expression change of 2.5 (data not shown).

Role of CCL20/MIP-3α and CCR6 system in myeloma-induced osteoclastogenesis. The role of CCL20/MIP-3α and CCR6 system in MM-induced osteoclastogenesis was further investigated. As expected, we found that 48-h CM of fresh purified MM cells cocultured with PreOB stimulated OC formation *in vitro*. In this system, a significant inhibitory effect on OC number was observed in the presence of both blocking anti-CCL20/MIP-3α and anti-CCR6 antibodies but not anti-IgG control antibodies (Fig. 2*D*).

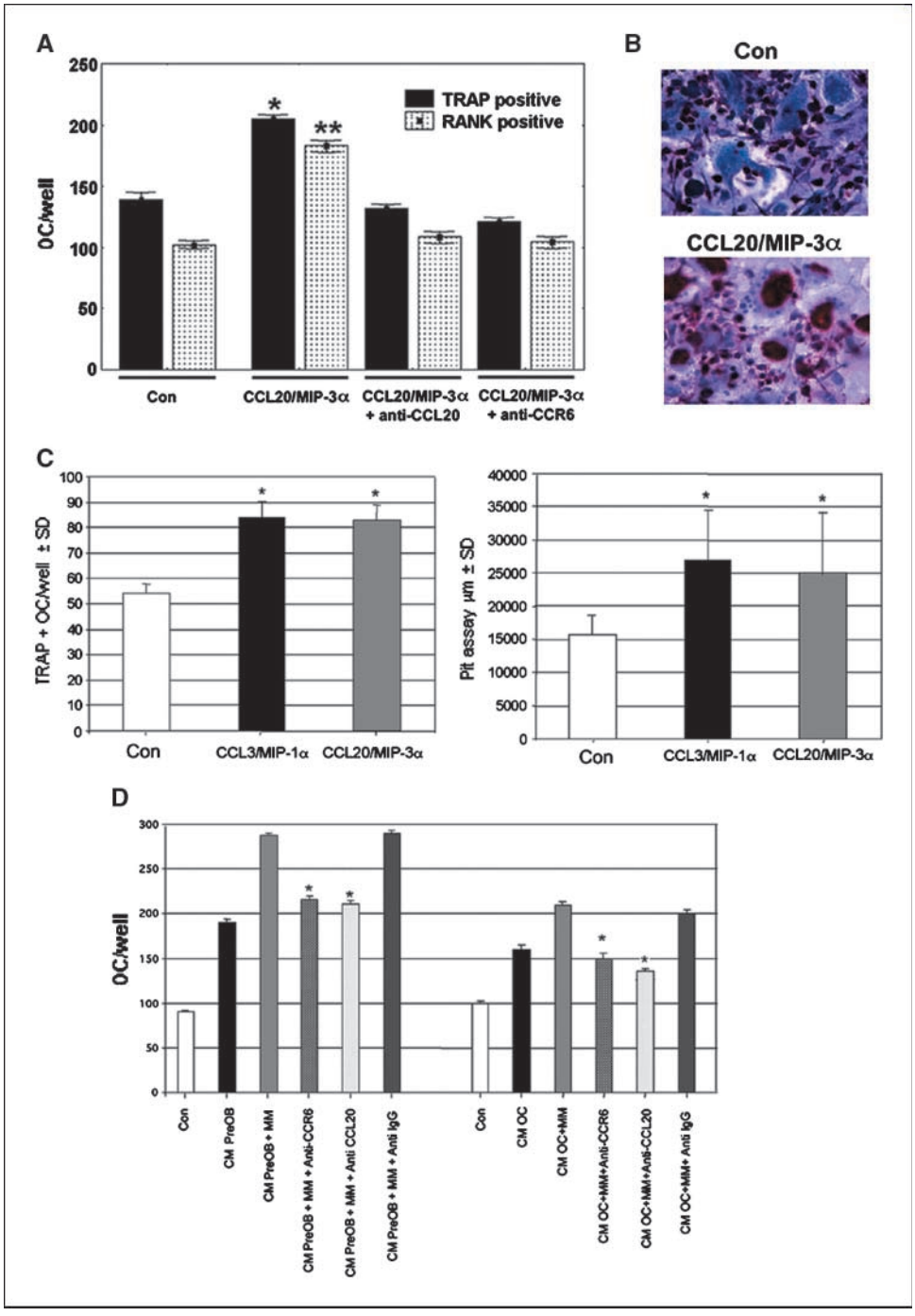

Figure 2. Effect of CCL20/MIP-3α on OC formation *in vitro* and role of CCL20/MIP-3α and CCR6 system in MM-induced osteoclastogenesis. OCs were generated from peripheral mononuclear cells in the presence or absence of rhCCL20/MIP-3α (0.3 ng/mL) with or without blocking anti-CCL20/MIP-3α or anti-CCR6 antibodies. OC formation was evaluated by TRAP and RANK expression by cytochemical and immunohistochemical analysis, respectively. *Columns,* mean of the OCs counted per well; *bars,* SD. *A,* statistical analysis: *, $P < 0.05$, control versus CCL20/MIP-3α, TRAP positive; **, $P < 0.01$, RANK positive. *B,* cytochemical analysis of TRAP-positive cells treated with or without CCL20/MIP-3α. Original magnification, ×400. In some experiments, OCs were generated from peripheral mononuclear cells in the presence or absence of rhCCL20/MIP-3α (0.3 ng/mL) or rhCCL3/MIP-1α (0.2 ng/mL). After 21 d, human OCs were identified as multinucleated cells positive for TRAP by cytochemical analysis and Pit assay was evaluated as marker of OC activity. *C, left, columns,* mean of the OCs counted per well; *bars,* SD; *right, columns,* mean of pit assay area (μm); *bars,* SD. *, $P < 0.01$. CM (ratio, 1:3) of myeloma cells cocultured with PreOB or OCs were checked in an osteoclastogenesis assay in the presence or absence of blocking anti-CCL20/MIP-3α or anti-CCR6 antibodies, as described in Materials and Methods. OCs were evaluated after 21 d as multinucleated cells positive for TRAP or RANKL expression. *D, columns,* mean of the OCs counted per well; *bars,* SD. *, $P < 0.05$.

Interestingly, the block of either CCL20/MIP-3α or CCR6 inhibited the osteoclastogenic effect of the CM of fresh purified MM cells cocultured with OC, as shown for a representative MM patient (Fig. 2*D*).

High CCL20/MIP-3α BM plasma levels in MM patients: relationship with the osteolytic lesions. CCL20/MIP-3α and CCR6 system was further studied *in vivo* in MM patients and compared with CCL3/MIP-1α, IL-1β, TNFα, and IL-6 levels. Cytokine levels were measured in the BM plasma of MGUS subjects (n = 16) and MM (n = 52) patients at the diagnosis in relationship with the presence of bone lesions (osteolytic, n = 32; nonosteolytic, n = 20). Significantly higher CCL20/MIP-3α levels were detected in MM patients versus MGUS (mean ± SD: 51.9 ± 2 versus 21 ± 3 pg/mL; median level: 14 versus 0.1 pg/mL; P = 0.01; Fig. 3*A*) and in MM osteolytic patients versus nonosteolytic ones (mean ± SD: 70.8 ± 5.9 versus 13.8 ± 1.1 pg/mL; median level: 24 versus 3.5 pg/mL; P = 0.001; Fig. 3*B*). Interestingly, no significant difference of CCL20/MIP-3α was observed between MGUS and nonosteolytic MM patients [P = 0.6, not significant (NS)].

Higher CCL3/MIP-1α BM levels were also observed in MM patients compared with MGUS subjects (mean ± SD: 36.63 ± 10.6 versus 13.2 ± 2 pg/mL; median level: 7.2 versus 2.6 pg/mL; P = 0.05; Fig. 3*A*) and in osteolytic versus nonosteolytic MM patients even if this difference did not reach a statistical significance (mean ± SD: 52.8 ± 20 versus 14.2 ± 19 pg/mL; median level: 9.12 versus 5.44 pg/mL; P = 0.09; Fig. 3*B*).

Significantly higher BM levels of IL-6, IL-1β, and TNFα were observed in MM patients compared with MGUS (P = 0.012, 0.017, and 0.024, respectively; Fig. 3*A*), whereas TNFα but not IL-6 and IL-1β levels were significantly higher in MM osteolytic patients compared with nonosteolytic ones (P = 0.05, 0.22, and 0.25, respectively; Fig. 3*B*). Finally, a significant correlation was observed in MM patients between BM levels of CCL20/MIP-3α and TNFα (Spearman's correlation coefficient, 0.44; two-tailed P = 0.02) and between CCL3/MIP-1α and TNFα (Spearman's correlation coefficient, 0.43; two-tailed P = 0.02).

CCL20/MIP-3α and CCR6 immunostaining in bone cells of MGUS and MM patients: relationship with the bone status. The expression of CCL20/MIP-3α, CCR6, CCL3/MIP-1α, and CCR5 was evaluated by immunohistochemistry in BM biopsies of MM patients (n = 64) and MGUS subjects (n = 16). First, we have found that plasma cells rarely express CCL20/MIP-3α (% mean ± SE: 7 ± 0.21%) and are negative for CCR6.

A significant number of CCL20/MIP-3α–positive hOBs were observed in MM patients compared with MGUS subjects where hOBs were negative (% mean ± SE/field: 14.8 ± 0.37% versus 0.7 ± 0.086%; median: 10.2% versus 0.5%; P < 0.03; Fig. 4*A*). Similarly, a significant number of CCR6-positive hOBs were found in MM patients compared with MGUS (% mean ± SE/field: 15.2 ± 0.06% versus 0.2 ± 0.086%; median: 5% versus 0.1%; P = 0.05; Fig. 4*B*).

Thereafter, we analyzed CCL20/MIP-3α and CCR6 expression in relationship with the bone status of MM patients. We found that osteolytic MM patients showed an overexpression of CCL20/MIP-3α compared with nonosteolytic ones (% mean ± SE/field: 22.3 ± 0.6% versus 10 ± 0.4%; median: 14.6% versus 2.8%; P = 0.01; Fig. 4*A*). On the other hand, no statistically significant difference was observed in the number of CCR6-positive hOBs between osteolytic and nonosteolytic ones even if osteolytic MM patients showed a higher number of CCR6-positive hOBs (% mean ± SE/field: 4.1 ± 0.09% versus 2.8 ± 0.08%: median: 3.3% versus 2.1%; P = 0.14; Fig. 4*B*).

The pattern of CCL20/MIP-3α expression by hOBs in MM patients was cytoplasmatic with a strong positive immunostaining in osteolytic versus nonosteolytic ones, as shown for two representative MM patients (Fig. 5*A, i, ii, iii*, and *iv*, respectively). A similar pattern of expression by hOBs was observed for CCR6 with a weaker CCR6 immunostaining in nonosteolytic MM patients versus osteolytic ones (Fig. 5*B, v* and *vi*).

CCL20/MIP-3α and CCR6 expression by OCs on bone biopsies was also investigated. A cytoplasmatic pattern of CCL20/MIP-3α expression by OCs as well as of CCR6 was observed in MM patients as shown for two representative MM patients with high and low number of OCs, respectively (Fig. 5*B, i–iv*). However, the difference in the number of OCs between osteolytic and nonosteolytic MM patients did not reach a statistical significance. On the other hand, an increased number of OCs was observed, as expected, in MM patients compared with MGUS; the % mean ± SE of CCL20/MIP-3α and CCR6 expression by OCs was 71 ± 0.7%/field and 92 ± 0.6%/field, respectively, in MM patients, whereas no CCL20/MIP-3–positive and CCR6-positive OC was counted in MGUS subjects.

To confirm the specificity of CCL20/MIP-3α and CCR6 immunostaining in hOBs and OCs, first an anti-IgG isotype-negative control was used (Fig. 5*C*), and second, an immunostaining for either the osteoblast marker osteocalcin (Fig. 5*D, i*) or the OC marker TRAP (Fig. 5*D, ii*) in relationship with CCL20/MIP-3α immunostaining has been performed.

The expression of CCL3/MIP-1α and CCR5 by bone cells was also investigated and compared with that of CCL20/MIP-3α and CCR6. A significantly higher number of CCR5-positive hOBs were found in MM patients compared with MGUS (% mean ± SE/field: 32 ± 2% versus 22 ± 4.8%: median: 34% versus 26%; P = 0.05), whereas the difference in the number of CCL3/MIP-1α–positive hOBs between MM and MGUS did not reach a statistical significance (% mean ± SE/field: 27 ± 13% versus 19 ± 4.%; median: 23% versus 18%; P = NS; Fig. 4*C* and *D*). On the contrary, any significant difference was not observed between osteolytic and nonosteolytic patients in CCL3/MIP-1α and CCR5 expression by hOBs (P = NS; Fig. 4*C* and *D*). Interestingly, a significant correlation was observed between the % of CCL20/MIP-3α– and CCL3/MIP-1α–positive hOBs (Spearman's correlation coefficient, 0.63; two-tailed P = 0.003) but not between CCR6- and CCR5-positive hOBs (P = NS). Finally, we found that CCL3/MIP-1α was rarely expressed by OCs, whereas CCR5 showed a strong immunostaining by OCs similarly to CCR6 (data not shown).

Discussion

In this study, we have evaluated the expression of the chemokine system of CCL20/MIP-3α and its receptor CCR6 in MM cells and bone microenvironment cells evaluating its role in OC formation and development of bone lesions in MM patients.

We found that CCL20/MIP-3α increases OC differentiation from $CD14^+$ cells *in vitro* in the presence of RANKL and M-CSF. In addition, the presence of the receptor CCR6 on OCs suggests that CCL20/MIP-3α may also act as activator of mature OCs.

A direct osteoclastogenic effect of CCL20/MIP-3α has been also recently reported in other studies (20), showing that this chemokine increases the differentiation of $CD11b^+$ precursors in an early phase by inducing cell fusion, cluster formation, and matrix metalloproteinase-9 secretion (20). Interestingly, the capability of CCL20/MIP-3α to induce OC differentiation was observed in our and other studies (20) at concentrations that can

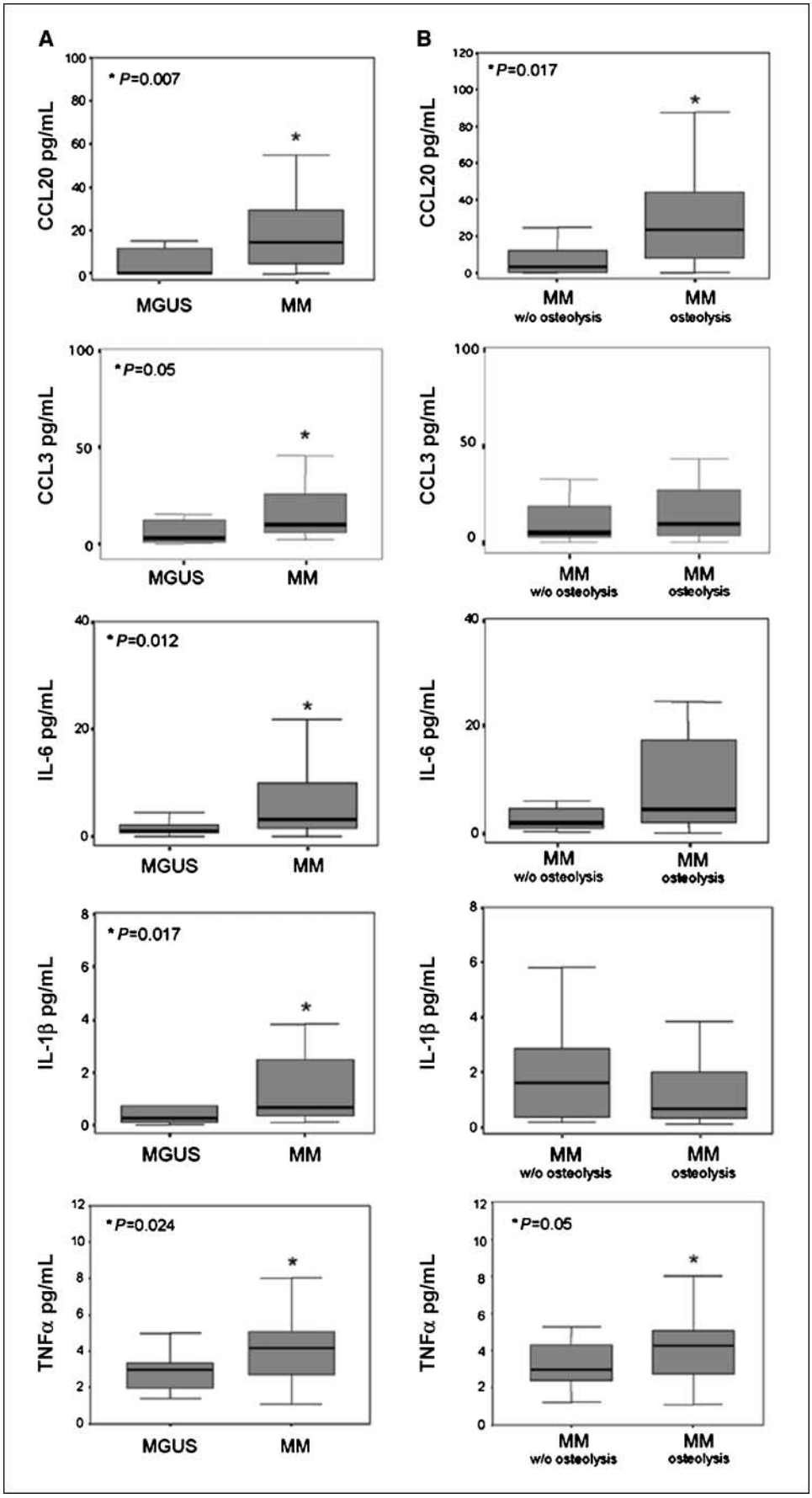

Figure 3. BM plasma levels of CCL20/MIP-3α, CCL3/MIP-1α, IL-6, IL-1β, and TNFα in MGUS and MM patients: relationship with bone status. CCL20/MIP-3α, CCL3/MIP-1α, IL-6, IL-1β, and TNFα levels were detected in the BM plasma of a cohort of MGUS (n = 16) and MM (n = 52) patients at the diagnosis (*A*) and in MM patients in relationship with the presence of bone lesions (osteolytic, n = 32; nonosteolytic, n = 20; *B*). Box plots represent the median level of the different cytokines and the 25th to 75th percentiles.

be reached in the BM plasma of MM patients, as reported in the cohort of patients analyzed. A stimulatory effect of CCL20/MIP-3α on the critical osteoclastogenic factor RANKL (27, 28) by human osteoprogenitor cells has been also shown, indicating that CCL20/MIP-3α may contribute to the osteoclastogenic process both directly and indirectly through the increase of RANKL expression in the bone microenvironment. Similarly to CCL20/MIP-3α, other chemokines belonging to CC family as CCL3/MIP-1α (10, 11) or CCL9/MIP-1γ (29, 30) show a stimulatory effect on OC precursors and RANKL expression by BM stromal and osteoblastic cells (11, 31). In particular, in this study, we show that the pro-osteoclastogenic effect of CCL20/MIP-3α is similar to that observed with CCL3/MIP-1α that it is an osteoclastic activator factor in MM (10–15).

Our data indicate that human MM cells rarely produce CCL20/MIP-3α. This observation was confirmed in a larger database of

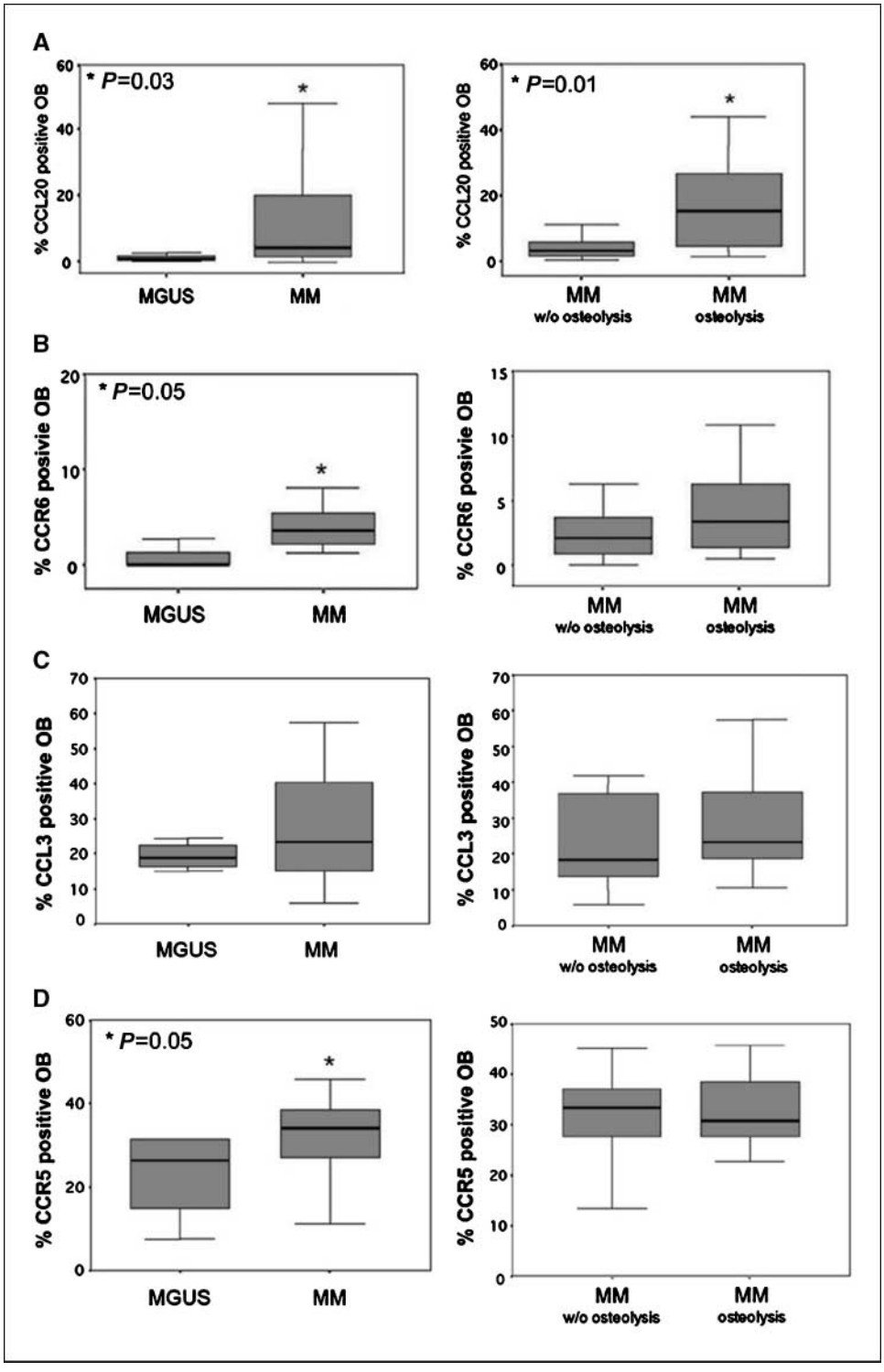

Figure 4. CCL20/MIP-3α, CCR6, CCL3/MIP-1α, and CCR5 expression by osteoblasts (*OB*) in MM and MGUS patients: relationship with the bone status. CCL20/MIP-3α, CCR6, CCL3/MIP-1α, and CCR5 expression was evaluated by immunohistochemistry in bone biopsy section obtained from MGUS (n = 16) and MM patients (n = 64). Bone biopsy sections were fixed in B5-formalin mixture, decalcified by EDTA, and embedded in paraffin. Serial sections of 3 μm thick were processed for immunohistochemical staining with primary antibodies and quantified, as described in Materials and Methods. Graphs represent the median % and the 25th to 75th percentiles of CCL20/MIP-3α–positive (*A*), CCR6-positive (*B*), CCL3/MIP-1α–positive (*C*), and CCR5-positive (*D*) osteoblast for field.

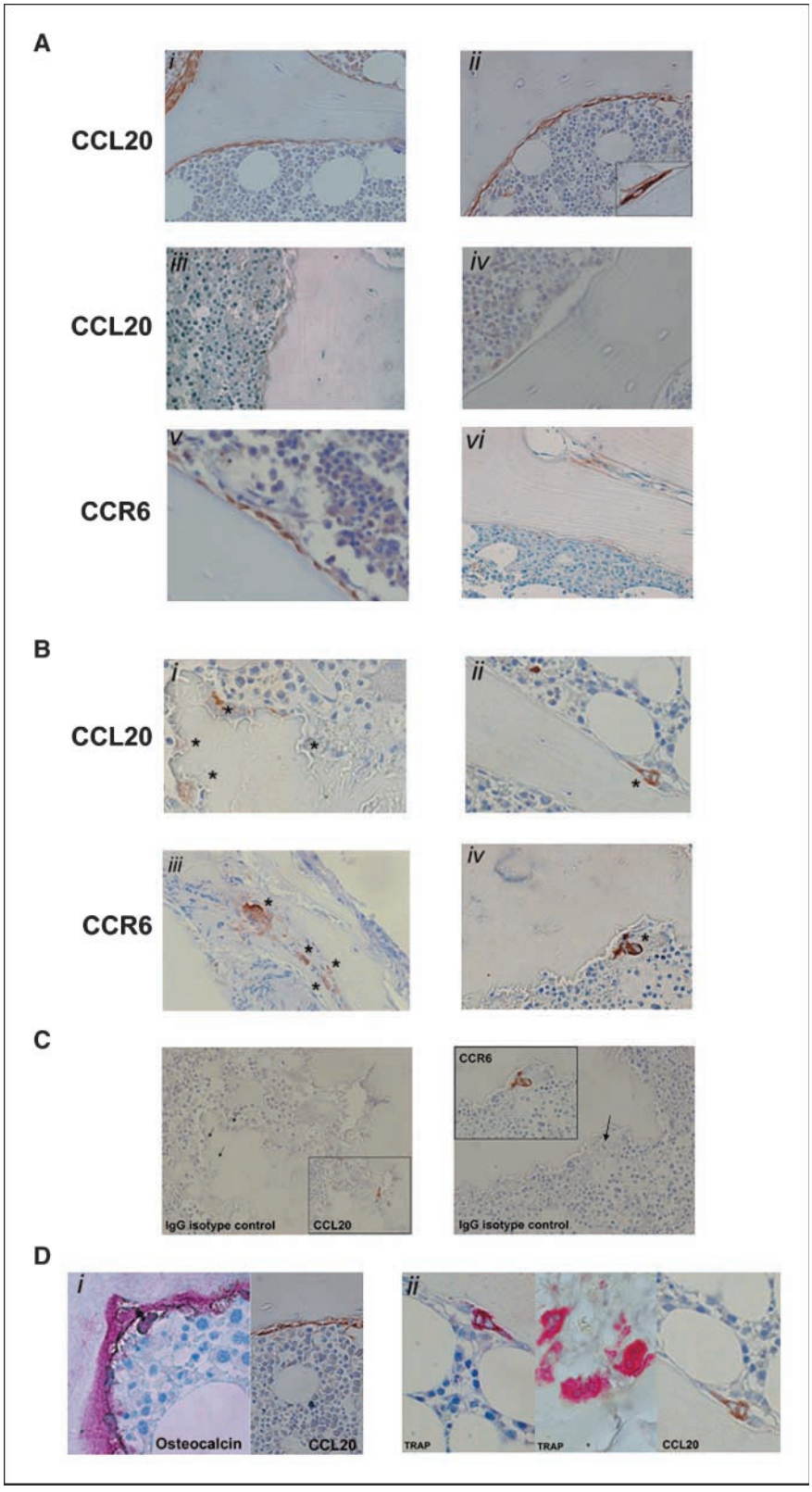

Figure 5. CCL20/MIP-3α and CCR6 immunostaining in osteoblasts and OC in MM patients. CCL20/MIP-3α and CCR6 immunostaining has been performed on fixed bone biopsy sections as described in Materials and Methods. *A,* CCL20/MIP-3α overexpression in hOB lining cells of two representative MM patients with osteolytic bone lesions (*i* and *ii*) compared with two nonosteolytic ones (*iii* and *iv*). Original magnifications, ×200 and ×400 (*ii*). CCR6 expression in hOB lining cells in a representative MM patient with bone lesions (*v*; original magnification, ×400) compared with nonosteolytic one (*vi*; original magnification, ×200). *B,* CCL20/MIP-3α immunostaining in OCs of two representative patients: one with osteolytic lesions and high number of OCs (*i*) and the other one without bone lesions and with low number of OCs (*ii*), respectively. *Asterisks,* OCs. Original magnification, ×200. CCR6 expression in OCs of a representative MM patient with bone lesions and high number of OCs (*iii*) compared with the other one without bone lesions and with low number of OCs (*iv*). *Asterisks,* OCs. Original magnification, ×200. *C,* CCL20/MIP-3α and CCR6 immunostaining with the relative IgG isotype-negative controls. Original magnification, ×200. *D,* immunostaining of the osteoblast marker osteocalcin (*i*) and OC maker TRAP (*ii*) in relationship with CCL20/MIP-3α immunostaining. Original magnifications, ×200 (*i*) and ×400 (*ii*).

MM patients by microarray analysis.[5] Similarly, the mRNA expression of the receptor CCR6 was observed in U266 and OPM2 and in few CD138$^+$ MM cells purified from MM patients, as also reported by other investigators (32).

Based on these evidences, we can suppose that a direct production of CCL20/MIP-3α as well as an autocrine loop rarely occurs in MM cells. On the contrary, an overexpression of CCR6 has

[5] S. Colla et al., unpublished data.

been shown in B-cell malignancy (33). Consistently, we found that the EBV^+ lymphoplasmacytoid cell line ARH-77 overexpresses CCR6 and produces CCL20/MIP-3α.

The presence of CCL20/MIP-3α was shown in mesenchymal/osteoprogenitor cells but not in mature hOB, whereas both cells were negative for CCR6 expression in basal condition. Among the potential soluble pro-osteoclastogenic factors, we found that IL-1β and TNFα have a synergistic effect up-regulating CCL20/MIP-3α secretion in both osteoprogenitor and osteoblastic cells, whereas IL-6 seems not to be involved in CCL20/MIP-3α secretion.

Interestingly, we show that CCL20/MIP-3α was up-regulated by MM cells in coculture systems with hOB or BM osteoprogenitor cells. This effect was blunted by the presence of both blocking anti-IL-1β and TNFα antibodies, suggesting that these cytokines are involved in CCL20/MIP-3α induction in the coculture systems. Consistently, a direct production of TNFα by MM cells (34, 35) as well as of IL-1β by MM cells or by microenvironment cells (35–38) in the presence of MM cells has been previously shown. Our evidences and those of the literature suggest that MM cells up-regulate CCL20/MIP-3α secretion in BM stromal/osteoblastic cells and that the high levels of both IL-1β and TNFα present in the BM microenvironment contribute to CCL20/MIP-3α up-regulation. The potential role of CCL20/MIP-3α in OC formation induced by MM cells was further confirmed by the finding that the block of either CCL20/MIP-3α or its receptor CCR6 is able to inhibit the OC formation induced by the CM of MM cells cocultured with osteoprogenitor and osteoblastic cells.

An autocrine effect of CCL20/MIP-3α on OCs and OC precursors can be postulated by our data. In fact, we found that OCs produce CCL20/MIP-3α themselves and express its receptor CCR6. In line with this observation, it has been reported that OCs express several chemokine receptors and produce the respective agonist chemokines that regulate OC chemotaxis, formation, and activity in autocrine manner (23, 24, 39). Interestingly, we found that MM cells induce the production of CCL20/MIP-3α by OCs in coculture and that blocking anti-CCL20/MIP-3α or anti-CCR6 antibodies significantly reduced the pro-osteoclastogenic properties of MM cells cocultured with OCs. These evidences suggest that an autocrine production of CCL20/MIP-3α induced by MM cells contributes to OC formation. Finally, we show that MM cells may induce the expression of CCR6 by OCs in coculture that could contribute to increase OC sensitivity to CCL20/MIP-3α.

Our *in vitro* evidences were further confirmed and expanded *in vivo* in MM patients. Higher CCL20/MIP-3α levels were detected in the BM plasma of MM patients compared with MGUS subjects; interestingly, MM patients with osteolytic bone lesions have higher CCL20/MIP-3α levels in comparison with nonosteolytic ones, whereas no significant difference was observed between MGUS subjects and nonosteolytic MM patients. Similarly to CCL20/MIP-3α, we found that BM levels of CCL3/MIP-1α were higher in MM patients compared with MGUS but they did not reach a statistical difference between osteolytic and nonosteolytic MM. Among the other cytokines tested, we found that IL-6, IL-1β, and TNFα levels were higher in MM compared with MGUS, whereas only the TNFα level was significantly different between osteolytic and nonosteolytic MM patients. Consistently, a significant correlation was found between CCL20/MIP-3α and TNFα levels.

Our observations suggest that the increase of OC formation that occurs in MM patients with osteolytic bone lesions (2, 3) is associated to high CCL20/MIP-3α BM levels and reflects the up-regulation of CCL20/MIP-3α in both hOB and OCs by MM cells even if other cells could be responsible for the high BM CCL20/MIP-3α levels as monocytes and macrophages, which are known to produce CCL20/MIP-3α (17).

In vitro evidences suggest that the high CCL20/MIP-3α BM levels contribute to the increased osteoclastogenesis that occurs in MM either directly by the capability of this chemokine to induce OC formation or indirectly through the stimulation of RANKL expression by osteoprogenitor cells contributing to the high RANKL levels present in the MM microenvironment (3). Other mechanisms could participate in the pro-osteoclastogenic effect of CCL20/MIP-3α in MM patients as the recruitment of Th17 lymphocytes. In rheumatoid arthritis, it has been shown that CCL20/MIP-3α recruits CCR6-positive Th17 lymphocytes (19) that are involved in OC activation and formation through IL-17, a potent stimulator of RANKL expression and OC formation (40). Interestingly, preliminary data indicate that MM patients have an increased number of Th17 into the BM compared with healthy or MGUS subjects (41) that it is correlated with the degree of lytic bone lesions and involved in OC activation through IL-17 (42).

The high CCL20/MIP-3α BM levels observed in osteolytic MM patients suggest that the measure of this chemokine could be useful as a marker of OC activation in MM patients. Clearly, further study will be necessary to compare CCL20/MIP-3α blood levels with those of markers of OC activity previously measured in MM patients (43).

The expression of CCL20/MIP-3α and CCR6 was also evaluated by immunohistochemistry on bone biopsies in a large cohort of MM patients. CCL20/MIP-3α and CCR6 were negative in hOBs from MGUS subject, whereas CCL20/MIP-3α was expressed by a significant number of hOBs in MM patients with osteolytic bone lesions, confirming that the overexpression of CCL20/MIP-3α induced by MM in hOBs can be involved in the OC formation. In addition, OCs expressed both CCL20/MIP-3α and CCR6 in MM patients compared with MGUS subjects. Interestingly, we observed that CCL20/MIP-3α and CCR6 were overexpressed within BM-infiltrated MM cells in line with their capability to induce CCL20/MIP-3α and CCR6 expression in osteoblastic and osteoclastic cells. All these evidences suggest that CCL20/MIP-3α system is up-regulated *in vivo* by MM cells in the bone microenvironment in relationship with OC activation and the presence of osteolytic bone lesions in MM patients.

Interestingly, a similar pattern of expression has been shown in malignant Langerhans cell histiocytosis with bone involvement (21) characterized by the presence of multiple lytic bone lesions and increased OC-like cells (44, 45). Immunohistochemistry on BM sections showed that hOB overexpressed CCL20/MIP-3α in the area of bone infiltration of Langerhans cells and OC-like cell formation (21). Similarly, increased expression of CCL20/MIP-3α and CCR6, involved in OC formation, has been shown in hOB of arthritis rheumatoid subchondral bone tissue (20).

The increased expression of CCL20/MIP-3α and CCR6 in the bone microenvironment in MM patients and its involvement in OC formation suggest that this system could be a potential future target for anti-osteoclastic therapy using CCR6 inhibitors or blocking mAbs given that CCR6 is selectively activated by CCL20/MIP-3α (17). Recently, inhibitors of CCL3/MIP-1α receptors CCR1 and CCR5 have been developed, showing an anti-osteoclastogenic effect both *in vitro* and *in vivo* in MM mouse models (46, 47), suggesting that the block of chemokine receptors can be a useful anti-osteoclastogenic approach.

In conclusion, our data indicate that MIP-3α/CCL20 and its receptor CCR6 are up-regulated in the bone microenvironment

cells by MM cells and involved in OC formation and potentially in the development of bone lesions in MM patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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