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This information is current as of August 9, 2022.

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# CCL2 Shapes Macrophage Polarization by GM-CSF and M-CSF: Identification of CCL2/CCR2-Dependent Gene Expression Profile

Elena Sierra-Filardi,* Concha Nieto,* Ángeles Domínguez-Soto,* Rubén Barroso,[†] Paloma Sánchez-Mateos,[‡] Amaya Puig-Kroger,[‡] María López-Bravo,[†] Jorge Joven,[§] Carlos Ardavín,[†] José L. Rodríguez-Fernández,* Carmen Sánchez-Torres,[¶] Mario Mellado,[†] and Ángel L. Corbí*

The CCL2 chemokine mediates monocyte egress from bone marrow and recruitment into inflamed tissues through interaction with the CCR2 chemokine receptor, and its expression is upregulated by proinflammatory cytokines. Analysis of the gene expression profile in GM-CSF– and M-CSF–polarized macrophages revealed that a high CCL2 expression characterizes macrophages generated under the influence of M-CSF, whereas CCR2 is expressed only by GM-CSF–polarized macrophages. Analysis of the factors responsible for this differential expression identified activin A as a critical factor controlling the expression of the CCL2/CCR2 pair in macrophages, as activin A increased CCR2 expression but inhibited the acquisition of CCL2 expression by M-CSF–polarized macrophages. CCL2 and CCR2 were found to determine the extent of macrophage polarization because CCL2 enhances the LPS-induced production of IL-10, whereas CCL2 blockade leads to enhanced expression of M1 polarization-associated genes and cytokines, and diminished expression of M2-associated markers in human macrophages. Along the same line, *Ccr2*-deficient bone marrow–derived murine macrophages displayed an M1-skewed polarization profile at the transcriptomic level and exhibited a significantly higher expression of proinflammatory cytokines (TNF-α, IL-6) in response to LPS. Therefore, the CCL2-CCR2 axis regulates macrophage polarization by influencing the expression of functionally relevant and polarization-associated genes and downmodulating proinflammatory cytokine production.   *The Journal of Immunology*, 2014, 192: 3858–3867.

Macrophages originate from tissue-residing precursors or circulating monocytes that extravasate into tissues under the influence of cytokines and chemokines (1, 2). Upon damage or infection, inflammatory monocytes migrate into injured sites, whereas "patrolling" monocytes are later recruited to inflamed sites to resolve inflammatory processes (3). The heterogeneity and plasticity of macrophages has led to the identification of a plethora of functional polarization states, all of which are ultimately dependent on the macrophage extracellular environment. Thus, macrophages activated by LPS ± IFN-γ (M1 polarization) or Th2-derived cytokines (M2 polarization) constitute useful and representative examples of the large variety of macrophage polarization states (4). GM-CSF and M-CSF contribute to macrophage differentiation (5), and give rise to macrophages that differ in their gene expression profile and in the pattern of cytokine production upon stimulation. Whereas GM-CSF–polarized macrophages (GM-MØ) release large amounts of TNF-α and IL-6 upon TLR stimulation, M-CSF–polarized macrophages (M-MØ) produce low levels of proinflammatory cytokines and high levels of IL-10 (6–8). Consequently, macrophages generated under the influence of GM-CSF or M-CSF (sometimes also referred to as M1 or M2 macrophages) are usually considered as proinflammatory and anti-inflammatory macrophages, respectively.

Chemokines display a crucial role in immune and inflammatory reactions, and critically contribute to leukocyte migration under homeostatic and inflammatory conditions, as well as to polarization of immune responses (9). Monocyte and macrophages migrate toward inflamed tissues under the influence of CCL2 (monocyte chemotactic protein-1 [MCP-1]), a member of the MCP chemokine family that also includes CCL8/MCP-2, CCL7/MCP-3, and CCL13/MCP-4 (10–13). CCL2 is considered an inflammatory chemokine because it contributes to monocyte, memory T cell, and NK cell extravasation through vascular endothelium (14–16), and its expression is transcriptionally upregulated by TNF-α, IFN-γ, PDGF, VEGF, and LPS, but inhibited by retinoic, glucocorticoids, and estrogen (17). However, CCL2 is expressed by a wide array of cell types either constitutively or after stimulation (18–22), and promotes the expression of negative regulators of macrophage activation and proinflammatory

cytokine production (23–25). CCL2 acts as a potent factor for Th2 polarization (26) and contributes to pathologies like allergic asthma, rheumatoid arthritis, neurologic disorders, and insulin resistance (27). Tumor-derived CCL2 expression positively correlates with tumor-associated macrophage (TAM) infiltration, tumor vascularization, and angiogenesis (28). Along this line, CCL2 expression shifts human peripheral blood CD11b^{+} cells toward a CD206^{+} M2-polarized phenotype (29), whereas CCL2 or CCR2 deficiency results in murine dendritic cells and macrophages with higher TNF-α, but lower IL-10, expression than their wild type (WT) counterparts (30, 31). CCL2 mediates monocyte migration and infiltration via engagement of the chemokine receptor CCR2 (27), although it can also bind to CCR4 expressed on Th2 lymphocytes (32, 33). Two alternatively spliced isoforms of CCR2 exist in humans (CCR2A, CCR2B), which only differ in the C-terminal tail (34) required for receptor trafficking to the cell membrane. CCR2A and CCR2B can induce different signaling pathways, with CCR2B being the main isoform expressed by human monocytes (35).

Because M1 and M2 macrophages differ in their chemokine and chemokine receptor profiles, we have now analyzed the factors that control CCR2 and CCL2 expression along macrophage polarization by GM-CSF and M-CSF, and identified genes and effector functions controlled by the CCL2–CCR2 pair in macrophages. Our findings indicate that CCL2 and CCR2 expression is oppositely regulated along macrophage polarization, and that CCL2 favors the acquisition of an anti-inflammatory phenotype by modulating the profile of LPS-stimulated cytokines and the expression of functionally relevant and polarization-associated genes.

## Materials and Methods

### Generation of human monocyte- and murine bone marrow–derived macrophages in vitro

Human PBMCs were isolated from buffy coats from normal donors over a Lymphoprep (Nycomed Pharma, Oslo, Norway) gradient according to standard procedures. Monocytes were purified from PBMCs by magnetic cell sorting using CD14 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). Monocytes (>95% CD14^{+} cells) were cultured at $0.5 \times 10^6$ cells/ml for 7 d in RPMI 1640 supplemented with 10% inactivated FCS (complete medium), at 37°C in a humidified atmosphere with 5% $CO_2$, and containing 1000 U/ml GM-CSF (Immunotools GmbH, Friesoythe, Germany) or 10 ng/ml M-CSF (Immunotools GmbH), to generate GM-CSF–polarized (GM-MØ) or M-MØ, respectively. Cytokines were added every 2 d. When indicated, LPS (10–100 ng/ml), IL-4 (1000 U/ml), IFN-γ (500 U/ml), or recombinant human activin A (10 ng/ml; Miltenyi Biotech) was
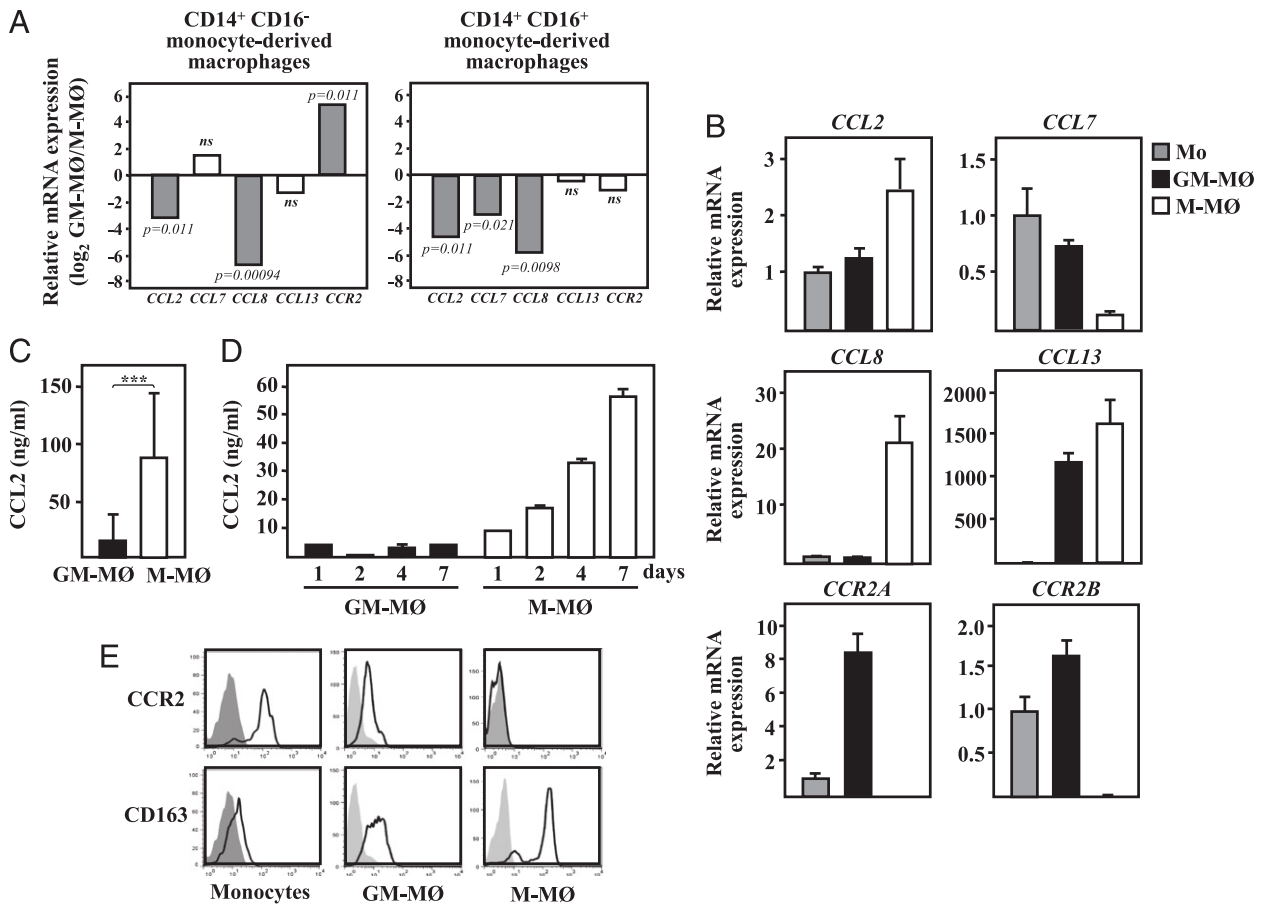


**FIGURE 1.** MCP1-4 and CCR2 expression in GM-MØ and M-MØ. (**A**) Relative expression of the indicated genes (expressed as $\log_2$ of the GM-MØ/M-MØ expression ratio) was determined by microarray DNA analysis (GEO GSE27792). Data are derived from the normalized fluorescence intensity of each gene. The adjusted $p$ value for the microarray data of each specific gene is indicated. (**B**) *CCL2, CCL7, CCL8, CCL13, CCR2A,* and *CCR2B* mRNA expression levels were determined by qRT-PCR on peripheral blood monocytes (Mo), GM-MØ, and M-MØ. Results are expressed as relative mRNA expression (relative to *GAPDH* RNA levels) and referred to the expression levels detected in Mo. (**C**) The levels of CCL2 in the supernatant of GM-MØ and M-MØ generated from 14 independent monocyte preparations were determined by ELISA. Means and SDs are shown (***$p < 0.001$). (**D**) The level of CCL2 was determined by ELISA on the supernatant of monocytes cultured in the presence of GM-CSF or M-CSF for 1, 2, 4, or 7 d. Each determination was performed in triplicate, and means and SDs are shown. One representative experiment (out of two) is shown. (**E**) Cell-surface expression of CCR2 and CD163 in monocytes, GM-MØ, and M-MØ, as determined by flow cytometry. Gray profiles indicate the immunofluorescence produced by isotype-matched control antibodies. *ns*, nonsignificant.
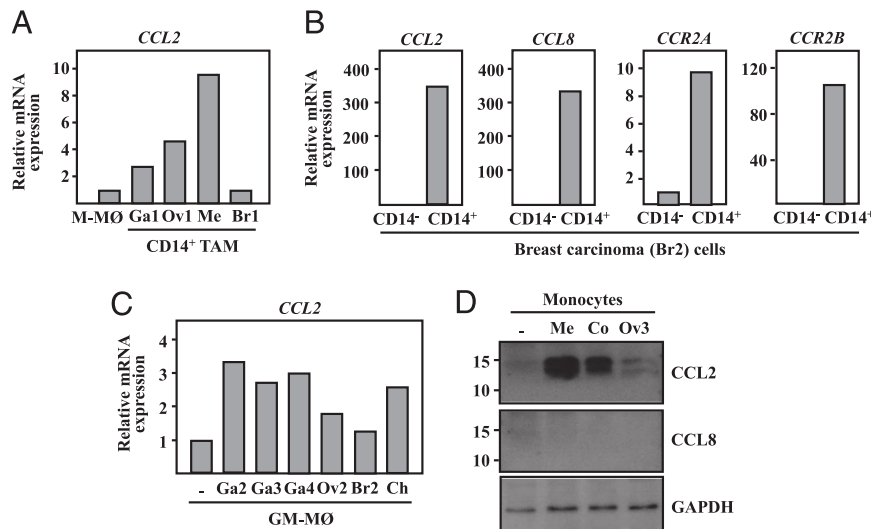
**FIGURE 2.** Expression of CCL2 and CCL8 in tumor-conditioned macrophages. (**A**) *CCL2* mRNA expression levels in M-MØ and CD14^{+} TAMs isolated from the ascitic fluid of gastric carcinoma (Ga1), ovarian carcinoma (Ov1), melanoma (Me), or breast carcinoma (Br1) samples, as determined by qRT-PCR. Results are expressed as relative mRNA expression (relative to *GAPDH* mRNA levels) and referred to the expression level of *CCL2* in in vitro–generated M-MØ. (**B**) *CCL2*, *CCL8*, *CCR2A*, and *CCR2B* mRNA expression levels in CD14^{+} and CD14^{−} cells isolated from the ascitic fluid of a breast carcinoma sample (Br2), as determined by qRT-PCR. Results are expressed as relative mRNA expression (relative to *GAPDH* mRNA levels). (**C**) *CCL2* mRNA expression levels in GM-MØ exposed for 48 h to ascitic fluids (dilution 1:1) derived from gastric carcinoma (Ga2, Ga3, Ga4), ovarian cancer (Ov2), breast cancer (Br2), and cholangiocarcinoma (Ch), as determined by qRT-PCR. Results are expressed as relative mRNA expression (relative to *GAPDH* mRNA levels) and referred to the expression level of *CCL2* in the presence of cirrhotic ascitic fluid (−). (**D**) Intracellular CCL2 and CCL8 levels in human peripheral blood monocytes either untreated (−) or exposed for 48 h to ascitic fluids (1:1) from melanoma (Me), colon cancer (Co), or ovarian cancer (Ov3), as determined by Western blot using specific polyclonal Abs against CCL2 or CCL8. As a loading control, the level of GAPDH was also determined using a specific mAb.

added together with the indicated cytokine. Blocking anti–activin A Ab (100 ng/ml; R&D Systems) or the inhibitor of ALK4, ALK5, and ALK7, SB431542 (10 μM), was added every 24 h.

Bone marrow–derived GM-MØ or M-MØ were obtained by flushing the femurs of 6- to 10-wk-old WT and *Ccr2*^{−/−} C57BL/6 mice, and culturing cells during 7 d in DMEM supplemented with 10% FCS and 50 μM 2-ME,
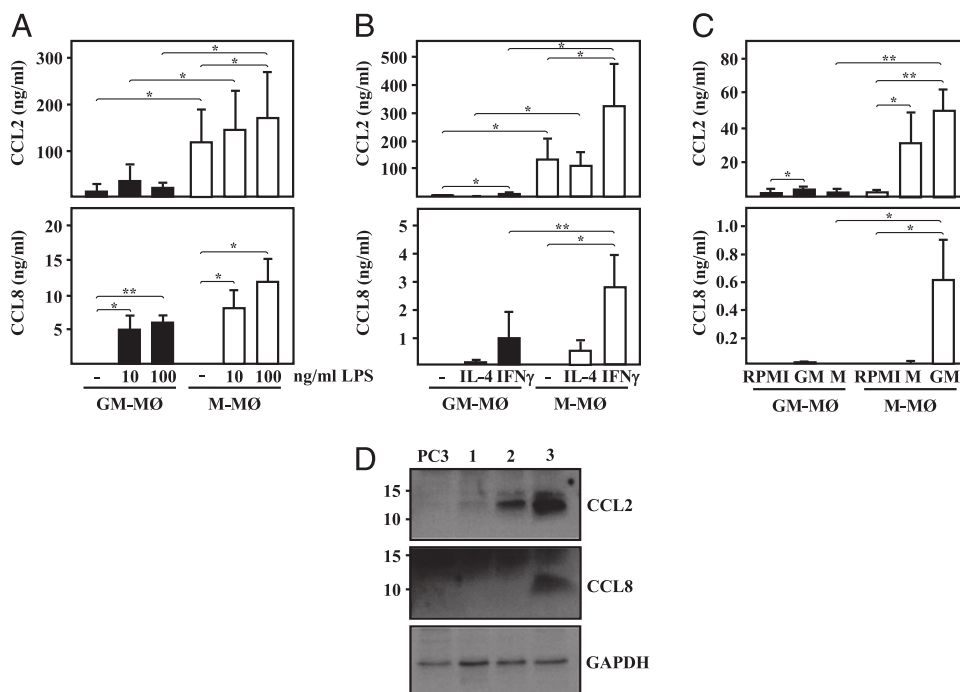


**FIGURE 3.** Regulation of CCL2 and CCL8 chemokine release from GM-MØ and M-MØ macrophages. (**A** and **B**) CCL2 and CCL8 release was determined by ELISA on culture supernatants from GM-MØ or M-MØ either untreated (−), stimulated with the indicated doses of LPS for 24 h (A), or treated with IL-4 (1000 U/ml) or IFN-γ (500 U/ml) for 48 h (B). (**C**) GM-MØ and M-MØ were extensively washed and cultured for 24 h in RPMI 1640, or RPMI 1640 containing either GM-CSF (1000 U/ml) or M-CSF (10 ng/ml). Culture supernatants were then collected, and the concentrations of CCL2 and CCL8 were determined by ELISA. Experiments were done in three independent donors, and means and SDs are shown (*$p < 0.05$, **$p < 0.01$). (**D**) Intracellular CCL2 and CCL8 levels in GM-MØ (*lane 1*), M-MØ (*lane 2*), and IFN-γ–treated M-MØ (*lane 3*) were determined by Western blot using specific polyclonal Abs against CCL2 and CCL8, and anti-GAPDH as loading control. As a control, cell lysates from bladder carcinoma PC3 cells were included.

containing murine GM-CSF (1000 U/ml; Peprotech) or human M-CSF (25 ng/ml; Immunotools GmbH), respectively (36, 37). Cytokines were added every 2 d. For activation, macrophages were treated with 100 ng/ml *E. coli* 055:B5 LPS for 24 h. Phenotypic analysis of monocytes and macrophages was carried out by flow cytometry using anti-CCR2 and anti-CD163 mAbs (Biolegend) and isotype-matched Abs as negative controls. All incubations were done in the presence of 50 μg/ml human IgG to prevent binding through the Fc portion of the Abs.

*Quantitative RT-PCR*

Total RNA was extracted using the RNeasy kit (Qiagen) and retrotranscribed, and individually amplified cDNA was quantified using the Universal Human Probe Roche library (Roche Diagnostics). Oligonucleotides for selected genes were designed according to the Roche software for quantitative real-time PCR. Alternatively, quantitative RT-PCR (qRT-PCR) was performed using custom-made panels (Roche Diagnostics, Germany) containing a wide array of genes differentially expressed between GM-MØ and M-MØ (38, 39). Assays were made in triplicates, and results were normalized according to the expression levels of *TBP* RNA or *GAPDH* mRNA. Results were expressed using the ΔΔCT (cycle threshold) method for quantification.

*Western blot and phosphorylated-protein array*

Cell lysates (40 μg) were subjected to SDS-PAGE and transferred onto an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking the unoccupied sites with 5% nonfat dry milk, protein detection was carried out with polyclonal Abs against CCL2 (ab9669; Abcam) or CCL8 (sc-1307; Santa Cruz Biotechnology, Santa Cruz, CA), or an mAb against GAPDH (sc-32233; Santa Cruz Biotechnology), and using the Supersignal West Pico Chemiluminescent system (Pierce, Rockford, IL). Screening of protein phosphorylation in response to CCL2 signaling was performed using Proteome Profiler protein array (R&D Systems), which detects the relative phosphorylation levels of 46 intracellular serine/threonine/tyrosine kinases.

*ELISA*

Macrophage supernatants were tested for the presence of cytokines and chemokines using commercially available ELISA for human CCL2, IL-12p40, TNF-α (all from BD Pharmingen), CCL8 and activin A (both from R&D Systems), IL-10 and IL-6 (both from Immunotools), and for murine IL-10, IL-6, CCL2, and TNF-α (from Biolegend), following the protocols supplied by the manufacturers.

*Migration assay*

Monocyte chemotaxis in response to chemokines or macrophage-conditioned medium (CM) was determined by measuring the number of migrated cells through a polycarbonate filter with 5-μm pore size in 24-well Transwell chambers. The upper chamber contained $1.5 \times 10^5$ monocytes in 100 μl RPMI 1640 medium containing 0.1% BSA. In control experiments, lower chambers contained 600 μl RPMI 1640 1% FCS with or without CCL2, CCL8, or CXCL12 (200 ng/ml; Peprotech). In the experiments where the chemotactic properties of macrophage-CM was assessed, the lower chamber included 600 μl macrophage-CM (diluted 1:10), in the absence or presence of anti-CCL2 blocking Ab (3 μg/ml; ab9669; Abcam), or an isotype control Ab. After incubation (2 h at 37°C), monocytes that migrated to the bottom chamber were counted, and the chemotactic index was calculated as the x-fold increase in migration observed over the negative control (RPMI 1640 1% FCS). In all cases, each experimental condition was analyzed in duplicate.

*Intracellular calcium determination*

Macrophages ($2.5 \times 10^6$ cells/ml) were suspended in RPMI 1640 medium containing 10% FCS and 10 mM HEPES, and incubated with 10 μl/$10^6$ cells of Fluo-3AM (300 μM in dimethyl sulfoxide; Calbiochem, San Diego, CA) for 30 min at 37°C. After incubation, cells were washed, placed in completed medium with 2 mM $CaCl_2$, and maintained at 4°C for 20 min before the addition of CCL2, CCL8 (20 nM; Peprotech), or CXCL12 (50 nM; Peprotech), to minimize membrane trafficking and to eliminate spontaneous $Ca^{2+}$ entry. $Ca^{2+}$ flux was measured in an EPICS XL
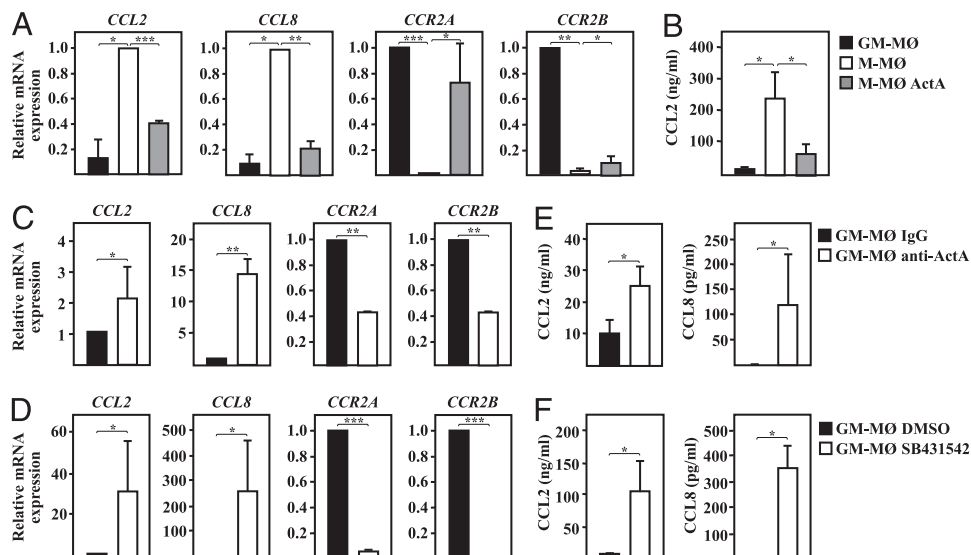
**FIGURE 4.** Activin A modulates the expression of CCL2, CCL8, and CCR2 in macrophages. (**A**) *CCL2*, *CCL8*, *CCR2A*, and *CCR2B* mRNA expression levels in macrophages polarized by GM-CSF (GM-MØ), M-CSF (M-MØ), or M-CSF plus activin A (M-MØ ActA; 10 ng/ml), as determined by qRT-PCR in three independent donors. Results are expressed as relative mRNA expression (relative to *GAPDH* mRNA levels) and referred to the expression level of each gene in M-MØ (*CCL2* and *CCL8*) or GM-MØ (*CCR2A* and *CCR2B*). (**B**) The concentration of CCL2 was determined by ELISA on the culture supernatants from GM-MØ, M-MØ, or M-MØ ActA. Three experiments were performed using monocytes from independent donors. (**C**) *CCL2*, *CCL8*, *CCR2A*, and *CCR2B* mRNA expression levels in GM-MØ generated in the presence of a blocking anti-ActA Ab (GM-MØ anti-ActA; 100 ng/ml) or an isotype-matched control Ab (GM-MØ IgG), as determined by qRT-PCR in three independent donors. Results are expressed as relative mRNA expression (relative to *GAPDH* mRNA levels) and referred to the expression level of each gene in the presence of the control Ab. (**D**) *CCL2*, *CCL8*, *CCR2A*, and *CCR2B* mRNA expression levels in GM-MØ generated in the presence of SB431542 (10 μM) (GM-MØ SB431542) or DMSO (GM-MØ DMSO), as determined by qRT-PCR in three independent donors. Results are expressed as relative mRNA expression (relative to *GAPDH* mRNA levels) and referred to the expression level of each gene in the presence of vehicle. (**E**) The concentration of CCL2 and CCL8 was determined by ELISA on the culture supernatants from GM-MØ anti-ActA or GM-MØ IgG. Three experiments were performed using monocytes from independent donors. (**F**) The concentration of CCL2 and CCL8 was determined by ELISA on the culture supernatants from GM-MØ SB431542 and GM-MØ DMSO. Three experiments were performed using monocytes from independent donors. (A–F) Mean and SD of triplicate determinations are shown (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

flow cytometer (Coulter). The level of probe loaded for each sample was assessed by determination of $Ca^{2+}$ mobilization induced by an ionophore (ionomycin, 5 μg/ml; Sigma Chemicals, St. Louis, MO).

*Statistical analysis*

Statistical analysis was performed using Student *t* test, and $p < 0.05$ was considered significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

# Results

### Differential and regulated expression of CCL2, CCL8, and CCR2 in macrophages

Human GM-MØ and M-MØ differ in their morphological, phenotypical, and functional features, and release distinct cytokines in response to pathogenic stimulation (6) (Supplemental Fig. 1). Expression profiling on GM-MØ and M-MØ (GEO GSE27792 http://www.ncbi.nlm.nih.gov/geo/) revealed that the expression of the *CCL2* and *CCL8* genes, which code for CCL2/MCP-1 and CCL8/MCP-2, respectively, is significantly higher in macrophages polarized by M-CSF (Fig. 1A). This differential expression was evident in macrophages derived from either CD14$^{++}$ CD16$^{-}$ monocytes (>8-fold, adjusted $p = 0.0116$ for *CCL2*, >64-fold, adjusted $p = 0.000945$ for *CCL8*) or CD14$^{+}$ CD16$^{+}$ monocytes (>16-fold, adjusted $p = 0.015$ for *CCL2*, >32-fold, adjusted $p = 0.0098$ for *CCL8*; Fig. 1A). Conversely, *CCL13* expression did not differ between both macrophage subtypes, whereas *CCL7* expression was higher in M-MØ derived from CD14$^{+}$ CD16$^{+}$ monocytes (Fig. 1A). Besides, and in agreement with previous findings (40, 41), the expression of the *CCR2* gene, which encodes a receptor for both CCL2 and CCL8 (42), was significantly higher in GM-MØ generated from CD14$^{++}$ CD16$^{-}$ monocytes (>32-fold, adjusted $p = 0.015$; Fig. 1A). These results were further substantiated on independent samples, which revealed that the expression of the CCR2A and CCR2B isoforms is higher in monocytes and GM-MØ (Fig. 1B). At the protein level, macrophages polarized by M-CSF also released significantly higher levels of CCL2 (Fig. 1C), a difference already observed 48 h after cytokine addition (Fig. 1D), whereas no CCL8 was detected in the supernatant of either macrophage type (data not shown). Moreover, and in agreement with RNA data, CCR2 cell-surface expression was detected only on macrophages polarized by GM-CSF (Fig. 1E). Therefore, M-MØ exhibit higher *CCL2* and *CCL8* gene expression than CCR2$^{+}$ GM-MØ.

Macrophage recruitment into the tumor stroma is essential for progression and metastasis of tumors. TAMs exert numerous protumoral functions, including enhancement of tumor cell proliferation, angiogenesis, matrix remodeling, and suppression of adaptive immunity (43). Consequently, the density of TAMs correlates with a worse outcome in several malignant tumor types. Because CCL2 expression correlates with TAM accumulation in vivo (44, 45), we next sought to confirm the in vivo relevance of the earlier findings. In agreement with the M2-like phenotype of TAM (39), CD14$^{+}$ TAMs isolated from the ascitic fluid of patients bearing tumors of different histological origins expressed *CCL2* to a similar extent than M-MØ generated in vitro (Fig. 2A). Although previous reports have found CCL2 expression in tumor cells from various origins (46), *CCL2* and *CCL8* mRNA levels were much higher in CD14$^{+}$ TAMs than in CD14$^{-}$ ascitic fluid cells (*CCL2*, 350 times higher; *CCL8*, 334 times higher; Fig. 2B), and a good correlation existed between *CCL2* mRNA levels in TAMs and CCL2 chemokine levels in ascitic fluids (data not shown), thus suggesting TAM as the primary source of both cytokines in tumor ascitic fluids. Regarding CCR2, and unlike the case of macrophages associated with human ovarian carcinoma (47), CD14$^{+}$ TAMs from breast carcinoma ascites expressed detectable levels of *CCR2* mRNA (Fig. 2B).

Next, ascitic fluids from a variety of human tumors were assayed for their ability to modulate CCL2 expression. Tumor-derived ascitic fluids increased *CCL2* mRNA levels in GM-MØ (Fig. 2C), as well as the intracellular level of CCL2, but not CCL8, protein in monocytes (Fig. 2D). Therefore, tumor-derived factors enhance the expression of *CCL2* in monocytes and macrophages, emphasizing the link between anti-inflammatory macrophage polarization and CCL2 expression.

The lack of CCL8 protein in ascitic fluid–treated monocytes and M-MØ (Fig. 2D and data not shown), which contain high *CCL8* mRNA levels (Fig. 1B), prompted us to analyze the factors that govern CCL2 and CCL8 production. M-MØ released CCL2 under basal conditions, and significantly higher levels upon stimulation by LPS, IFN-γ, or GM-CSF (Fig. 3A, 3B). Conversely, and even after stimulation, GM-MØ always produced lower levels of CCL2 (Fig. 3A–C). Regarding CCL8, the chemokine was produced by either macrophage type after stimulation by LPS (Fig. 3A) or IFN-γ (Fig. 3B), albeit the levels were always higher in macrophages polarized by M-CSF. In these macrophages, CCL8 expression was not affected by IL-4 (Fig. 3B) but was induced by GM-CSF (Fig. 3C). Western blot analysis also revealed the higher content of CCL2 in M-MØ and confirmed that CCL8 protein is only detected upon subsequent macrophage stimulation (Fig. 3D). Therefore, anti-inflammatory M-MØ consistently produce higher levels of CCL2 and CCL8 than macrophages polarized by GM-CSF. Interestingly, the CCL8 chemokine was released only after macrophage stimulation (Fig. 3A–C), thus indicating that its expression is subjected to posttranscriptional regulation.
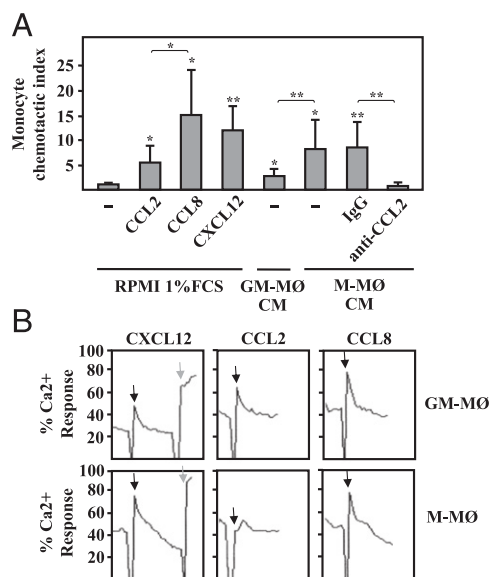


**FIGURE 5.** Functional role of the CCL2-CCR2 axis in GM-MØ and M-MØ. (**A**) GM-MØ and M-MØ were extensively washed and cultured in RPMI 1640 1% FCS. After 24 h, culture supernatants (CM) were collected and used for chemotaxis assays. Monocytes from healthy donors were assayed for chemotaxis toward GM-MØ CM or M-MØ CM. Recombinant human CCL2, CCL8, or CXCL12 chemokines, diluted in RPMI 1640 1% FCS, were assayed in parallel chemotactic assays. The CCL2 contribution to the monocyte recruitment ability of M-MØ CM was evaluated by determining monocyte migration toward M-MØ CM in the presence of either a blocking anti-CCL2 Ab (anti-CCL2) or an isotype-matched irrelevant Ab (IgG). Migration assays were done in four independent monocyte donors. Means and SDs are shown (*$p < 0.05$, **$p < 0.01$). Asterisks placed on the bars refer to SDs with respect to control monocytes. (**B**) CXCL12, CCL2-, or CCL8-induced $Ca^{2+}$ mobilization in GM-MØ and M-MØ, as measured by flow cytometry. Two independent experiments were done and one of them is shown.

## Activin A regulates the expression of CCL2, CCL8, and CCR2

We have previously shown that activin A promotes the acquisition of GM-MØ polarization markers and functions (38). Consistent with these results, the continuous presence of activin A (10 ng/ml) along M-MØ polarization significantly reduced the expression of *CCL2* and *CCL8* mRNA and protein, while concomitantly enhancing *CCR2A* and *CCR2B* mRNA expression (Fig. 4A, 4B). In GM-MØ, a blocking anti–activin A Ab (100 ng/ml), or an inhibitor of activin A–initiated signaling (SB431542, 10 μM) (48), enhanced *CCL2* and *CCL8* mRNA and protein expression, but reduced *CCR2A* and *CCR2B* mRNA levels (Fig. 4C–F). Therefore, the differential expression of *CCL2*, *CCL8*, and *CCR2* between GM-MØ and M-MØ is dependent on activin A, which enhances *CCR2* mRNA and inhibits *CCL2* and *CCL8* mRNA expression.

## Functional consequences of the differential expression of CCL2 and CCR2 in GM-MØ and M-MØ

To determine the relevance of CCL2 production by both types of macrophages, we assessed their CM for chemotactic activity on monocytes (12, 49). M-MØ CM exhibited a significantly higher ability for monocyte recruitment than GM-MØ CM (Fig. 5A). Importantly, the monocyte-recruitment capacity of M-MØ CM was almost abrogated by a blocking anti-CCL2 Ab (Fig. 5A). Regarding the exclusive expression of CCR2 in GM-MØ, and because CCL2 signals only through CCR2 (50, 51), we evaluated CCL2-initiated signals in both macrophages. As shown in Fig. 5B, CCL2 triggered a transient increase in intracellular $Ca^{2+}$ exclusively in CCR2$^+$ GM-MØ, whereas either CCL8 or CXCL12 increased intracellular $Ca^{2+}$ in both macrophage subtypes. Moreover, CCL2 stimulation of GM-MØ led to activation of p38, ERK1/2, MSK1/2, HSP27, JNK, STAT5a/b (Supplemental Fig. 2), some of which are required for the chemokine function (52, 53). Therefore, M-MØ exhibit higher CCL2-dependent monocyte recruitment ability than GM-MØ, whereas only the latter are capable of responding to CCL2.

## Determination of the CCL2-dependent gene expression profile in macrophages

We next evaluated the influence of CCL2 on the macrophage transcriptional and cytokine profile. To that end, CCR2$^+$ monocytes were polarized with M-CSF in the continuous presence of a blocking anti-CCL2 Ab. Subsequent analysis of a wide range of GM-MØ– and M-MØ–specific gene markers (38) revealed a significant increase in the expression of various GM-MØ–specific genes, including *INHBA*, *EGLN3*, and *SERPINE1*, and proinflammatory cytokines such as *IL12B*, *IL23A*, and *TNF* (Fig. 6A). Besides, the presence of the blocking anti-CCL2 Ab significantly diminished the expression of various M-MØ-specific markers such as *IGF1*, *FOLR2*, *HTR2B*, and *SLC40A1* (Fig. 6B), and modified the levels of IFN-γ–regulated genes like *IL8*, *SLC11A1*, and *NCF1* (Fig. 6C). In agreement with the transcriptomic results (Fig. 6D), blockade of CCL2 resulted in significantly higher levels of IL-6 secretion, as well as higher levels of activin A (Fig. 6E). Surprisingly, although the M-MØ–associated *MAF* gene was also reduced, expression of *ZC3H12A* and *ZC3H12D*, which encode CCL2-inducible ribonucleases (24, 54), were not modified upon CCL2 blockade (Supplemental Fig. 3). Therefore, neutralization of CCL2 results in impaired expression of M-CSF polarization-associated markers and enhanced expression of GM-CSF polarization–specific genes in human macrophages.

Because CCL2 binds to the CCR2 receptor, and to evaluate the extent of the earlier findings, we next assessed macrophage polarization in bone marrow–derived murine macrophages from WT and $Ccr2^{-/-}$ mice. Further supporting the opposite expression of *CCL2* and *CCR2* in human GM-MØ and M-MØ, *Ccl2* expression was significantly higher in murine M-MØ than in GM-MØ, whereas mouse GM-MØ exhibited higher levels of *Ccr2* (Supplemental Fig. 4A). In fact, the lack of *Ccr2* dramatically altered the M1/M2 ratio in mouse peritoneum, because CD11b$^{high}$ F4/80$^{int}$ M2 peritoneal macrophages (55) were virtually absent in $Ccr2^{-/-}$ mice (Supplemental Fig. 4B). The absence of *Ccr2* also affected the ex-
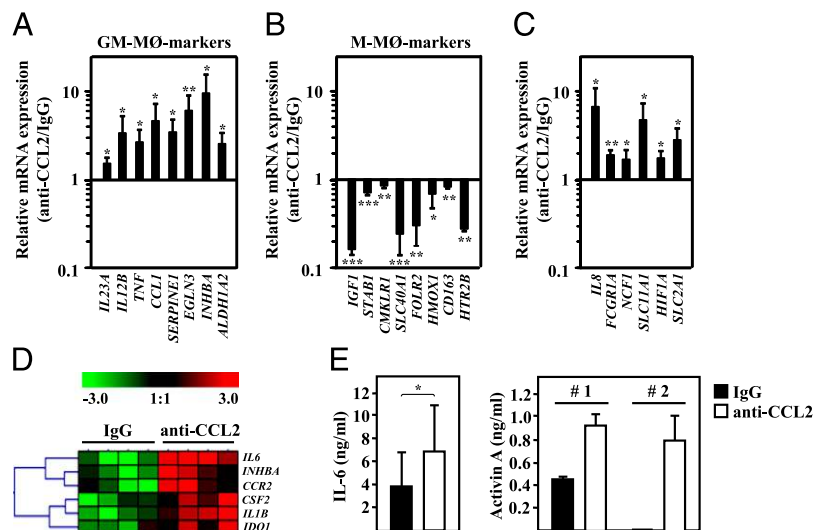


**FIGURE 6.** CCL2-dependent gene expression profile in human macrophages. (**A–C**) Relative mRNA expression of GM-MØ–associated genes (A), M-MØ–associated genes (B), and other genes (C) in M-MØ generated in the continuous presence of a neutralizing anti-CCL2 Ab, as determined by qRT-PCR in five independent donors. Results are expressed as relative mRNA expression (relative to *TBP* mRNA levels) and referred to the expression level of each gene in M-MØ generated in the presence of an isotype-matched control Ab (IgG). Means and SD are shown (*p < 0.05, **p < 0.01, ***p < 0.001). (**D**) Nonsupervised hierarchical clustering (Genesis software, http://genome.tugraz.at/genesisclient/genesisclient_description.shtml) on the mean expression level of each indicated gene under the indicated culture conditions. (**E**) *Left panel*, IL-6 levels released by M-MØ (from five independent donors) generated in the continuous presence of blocking anti-CCL2 Ab (α-CCL2) or an isotype-matched control Ab (IgG), as determined by ELISA. Mean and SD are shown (*p < 0.05). *Right panel*, Activin A levels released by M-MØ (from two independent donors) generated in the continuous presence of blocking anti-CCL2 Ab (α-CCL2) or an isotype-matched control Ab (IgG), as determined by ELISA.

pression of polarization markers in bone marrow–derived mouse macrophage. GM-MØ from *Ccr2* knockout (KO) mice exhibited significantly lower levels of the M-MØ polarization markers *Il10* and *Htr2b* (Fig. 7A), whereas M-MØ from *Ccr2* KO mice exhibited higher levels of *Tnf* mRNA and significantly reduced levels of *Folr2*, *Il10*, and *Htr2b* (Fig. 7B). Altogether, these results confirm that the CCL2 chemokine, preferentially produced by macrophages with anti-inflammatory activity, influences the transcriptomic polarization profile of human and murine macrophages.

*CCL2 and CCR2 modulate the LPS-induced cytokine profile in macrophages*

To find out whether the transcriptomic influence of the CCL2/CCR2 pair also extended to the stimulation-dependent cytokine profile in macrophages, we assessed the ability of CCL2 to modulate cytokine release in response to LPS. In CCR2$^+$ human GM-MØ, CCL2 prompted a significant increase in the LPS-stimulated IL-10 release, whereas the production of proinflammatory cytokines (TNF-α, IL-12p40, IL-6) was unaffected (Fig. 7C). Therefore, the effector functions of GM-CSF–polarized human macrophages are modulated by CCL2.

Next, the contribution of CCR2 to the LPS-stimulated production of proinflammatory and anti-inflammatory cytokines by murine macrophages was evaluated. To that end, bone marrow–derived GM-MØ and M-MØ from WT and *Ccr2*$^{-/-}$ mice were exposed to LPS. In agreement with the anti-inflammatory action

of CCL2 on human macrophages, *Ccr2*$^{-/-}$ GM-MØ produced significantly higher levels of IL-6, CCL2, and TNF-α than WT macrophages (Fig. 7D). Thus, our results support the anti-inflammatory nature of the CCL2 effects on both human and murine macrophages, because CCL2 enhances the LPS-induced IL-10 production from human GM-MØ, and because the defective expression of Ccr2 significantly enhances the LPS-induced IL-6 and TNF-α production from murine GM-MØ.

## Discussion

The CCL2 chemokine is expressed by hematopoietic and nonhematopoietic cells, and regulates the migration of monocytes, T lymphocytes, and NK cells under homeostatic and inflammatory conditions. As a consequence, modulation of CCL2 expression/function has attracted much attention as a potential therapy for multiple sclerosis (56), rheumatoid arthritis (57), atherosclerosis (58), and insulin-resistant diabetes (27, 59, 60), as well as for various types of cancer (61–63). In fact, CCL2 expression in tumor cells correlates with TAM infiltration, angiogenesis, and poor survival (64, 65). Because the CCL2/CCR2 axis governs both the egression from the bone marrow and recruitment to sites of infection of inflammatory monocytes (66), determination of the factors controlling the expression of CCL2 and CCR2 might identify novel potential intervention points for the treatment of inflammatory and autoimmune diseases. We now report the opposite expression of CCL2 and
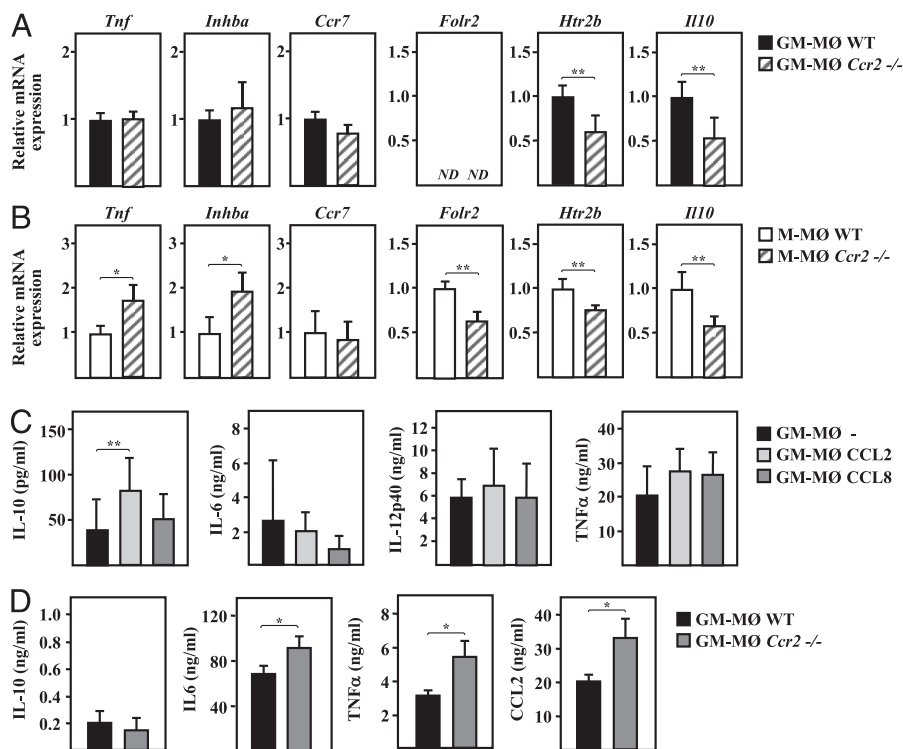
**FIGURE 7.** Gene expression profile and cytokine production is Ccr2 dependent in macrophages. (**A** and **B**) Relative mRNA expression levels of M1-associated (A) and M2-associated genes (B) in murine bone marrow–derived GM-MØ and M-MØ from WT and *Ccr2*$^{-/-}$ mice, as determined by qRT-PCR in three independent macrophage preparations. Results are expressed as relative mRNA expression (relative to *Tbp* RNA levels) and referred to the expression levels of each gene in GM-MØ WT (A) or M-MØ WT (B). Expression of *Folr2* mRNA was not detected (ND) in GM-MØ. Means and SDs are shown (*$p < 0.05$, **$p < 0.01$). (**C**) Human GM-MØ were extensively washed and cultured for 24 h in RPMI 1640 and 0.5% FCS containing either LPS alone (10 ng/ml; GM-MØ −), LPS plus CCL2 (GM-MØ CCL2), or LPS plus CCL8 (GM-MØ CCL8). Culture supernatants were collected after 24 h and their content of IL-10, IL-12p40, IL-6, and TNF-α was determined by ELISA. The experiment was performed on macrophages generated from three independent donors, and means and SDs are shown (**$p < 0.01$). (**D**) Bone marrow–derived mouse GM-MØ from WT and *Ccr2*$^{-/-}$ mice were collected and transferred onto tissue culture plates for 24 hours. Then LPS (100 ng/ml) was added, and the corresponding culture supernatants were collected after 24 h and used for determination of IL-10, IL-6, CCL2, and TNF-α levels by ELISA. Each determination was done on three independent macrophage preparations, and means and SDs are shown (*$p < 0.05$).

CCR2 in proinflammatory (CCR2$^+$) and anti-inflammatory (CCR2$^-$) monocyte-derived macrophages, demonstrate that activin A upregulates CCR2 and limits CCL2 expression in macrophages, and reveal that the CCL2–CCR2 pair skews macrophage polarization at the transcriptomic and functional level in human and murine macrophages.

Depending on the target cell and the duration and intensity of the responses, activin A displays proinflammatory and anti-inflammatory activities (67). The regulation of CCL2 and CCR2 expression by activin A is compatible with its dual effect on inflammation: activin A would increase CCL2 responsiveness by enhancing CCR2 expression while, concomitantly, it would reduce CCL2 levels within inflamed sites. Interestingly, such a dual way of action (proinflammatory and anti-inflammatory) has also been proposed for the CCL2-CCR2 pair, whose anti-inflammatory nature is illustrated by the enhanced susceptibility to collagen-induced arthritis of *Ccr2* KO mice (68) and the resistance toward *Leishmania major* infection seen in *Ccl2*$^{-/-}$ mice (69). Along the same line, CCL2 delimits tissue injury and facilitates tissue repair during myocardial stress response by virtue of its positive effect on the expression of endoplasmic reticulum chaperones like GRP78, GRP94, HSP25, HSP40, and HSP70 (60). Therefore, the plastic actions of CCL2 and activin A during inflammatory responses appear to be causally linked through the capacity of activin A to regulate the expression of both CCL2 and its receptor CCR2 in macrophages.

In addition to its role in chemotaxis, CCL2 has an impact on other leukocyte functions and influences gene expression in CCL2-responsive cells. CCL2 modulates angiogenesis via upregulation of HIF-1α and VEGF, and activation of the Ets-1 transcription factor (60), and also promotes matrix metalloproteinase expression in monocytes (70). CCL2 induces the expression of a set of proteins, collectively termed MCP-induced proteins (MCPIP1-4), that exhibit RNase activity and suppress microRNA activity and biogenesis (23–25). Specifically, MCPIP1 promotes mRNA decay of IL-6 (25) and upregulates c-Maf via downregulation of miR-155 (23). Because of the severity of the autoimmune phenotype of *Zc3h12a* (MCPIP1) KO mice, the existence of MCPIP1 targets distinct from IL-6 has been hypothesized (25). Our results are partially compatible with these previous findings because neutralization of CCL2 during macrophage polarization does not influence *Zc3h12a* mRNA levels (Supplemental Fig. 3), but results in enhanced expression of IL-6 and activin A, diminished levels of nine markers associated to GM-CSF–driven polarization, and increased expression of eight markers of M-MØ (38). Moreover, blockade of CCL2 during macrophage polarization also led to increased expression of proinflammatory cytokines like CCL8 (IL-8) and to upregulation of the M1-associated *HIF1A* gene (71). Therefore, the range of CCL2-dependent genes that we have identified clearly supports the anti-inflammatory nature of the intracellular signaling initiated upon CCR2 ligation by CCL2, and points to CCL2 as a factor that skews macrophage polarization toward the acquisition of an M2 profile. Together with the previously described functions of the MCPIP1/4 proteins (23–25), our results suggest that the CCL2-dependent recruitment of CCR2$^+$ macrophages toward an inflamed tissue might contribute to a transient limitation of the production of proinflammatory cytokines, thus avoiding their release before macrophages reach the inflammatory focus. Considering the involvement of CCL2 in monocyte egress from bone marrow (66), the CCL2/CCR2 axis could also prevent the release of proinflammatory cytokines in peripheral blood. In this regard, CCL2 could be considered as member of the set of genes whose expression is induced upon macrophage stimulation as a means of limiting/terminating macrophage activation to avoid excessive tissue damage (72).

In addition to CCL2, we have found that CCL8 mRNA expression characterizes anti-inflammatory M2 macrophages, and that CCL8 protein is only released upon cytokine activation. Like other chemokines, CCR2 ligands heterodimerize and form higher order oligomers that alter their functional activity (73). Recombinant CCL2 and CCL8 show strong heterodimer formation because of similar sequences in their N termini (73, 74), and synergistic effects are seen upon coexpression of MCP proteins and other chemokines (75). Specifically, CCL8 inhibits calcium flux induced by CCL2 in THP-1 cells (76) and increases the migratory capacity of monocytes toward CCL2. Accordingly, the functional activity of human macrophage-derived CCL2 would be modulated by CCL8 only after activation, because nonstimulated macrophages express CCL8 mRNA but do not release CCL8 chemokine in the absence of a stimulating cytokine. Future studies are necessary to determine the functional relevance of these associations in M2-polarized human macrophages, as we have observed that CCL2 and CCL8 become associated and are coprecipitated from the supernatant of IFN-γ–stimulated M-MØ (data not shown).

The ability of CCL2 to modulate macrophage polarization has further implications. On the one hand, the presence of altered levels of chemokines in various inflammatory diseases claims for the need to evaluate the contribution of chemokines to the polarization of macrophages in these pathological states (e.g., cancer, rheumatoid arthritis). On the other hand, the modification of chemokine expression might help to modulate not only leukocyte recruitment under inflammatory conditions, but the clinical evolution of pathologies derived from deregulated macrophage polarization (e.g., chronic inflammatory diseases). In this regard, modulation of CCL2 expression levels might represent a potential strategy to limit or potentiate macrophage activation in inflammatory pathologies.

## Disclosures

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