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

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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 transcriptional 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.  

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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 isofrom 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 0.5–106 cells/ml for 7 d in RPMI 1640 supplemented with 10% inactivated FCS (complete medium), at 37˚C in a humidified atmosphere with 5% CO2, 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 log2 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.
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, and cultured for 24 h.

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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.

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 *GAPDH* and M-MØ (38, 39).

**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) containing an array of 46 intracellular serine/threonine tyrosine kinases.

**Intracellular calcium determination**

Macrophages (2.5 × 10^5 cells/ml) were suspended in RPMI 1640 medium containing 10% FCS and 10 mM HEPES, and incubated with 10 μM/10^5 cells of Fluoro-3 AM (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 CaCl₂, 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](http://www.jimmunol.org/) 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 vehicle. (D) 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. (E) 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 \(\mu\)g/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\(^{-}\) 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, antiinflammatory 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.
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). Importantly, the monocyte-recruitment capacity of M-MØ CM was almost abrogated by a blocking anti-CCL2 Ab (Fig. 5A). Regarding the differential expression of CCL2 and 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). Moreover, CCL2 stimulation of GM-MØ and CCR2 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 Ca2+ exclusively in CCR2+ 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+/− mice 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 CD11bhigh F4/80int 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...
CCL2 and CCR2 in macrophage polarization

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). Reconstituent 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

The authors have no financial conflicts of interest.

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