CCL2 and Interleukin-6 Promote Survival of Human CD11b⁺ Peripheral Blood Mononuclear Cells and Induce M2-type Macrophage Polarization*§

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CCL2 and interleukin (IL)-6 are among the most prevalent cytokines in the tumor microenvironment, with expression generally correlating with tumor progression and metastasis. CCL2 and IL-6 induced expression of each other in CD11b⁺ cells isolated from human peripheral blood. It was demonstrated that both cytokines induce up-regulation of the antiapoptotic protein cFLIP, (cellular caspase-8 (FLICE)-like inhibitory protein), Bcl-2, and Bcl-X₁ and inhibit the cleavage of caspase-8 and subsequent activation of the caspase-cascade, thus protecting cells from apoptosis under serum deprivation stress. Furthermore, both cytokines induced hyperactivation of autophagy in these cells. Upon CCL2 or IL-6 stimulation, CD11b⁺ cells demonstrated a significant increase in the mannose receptor (CD206) and the CD14⁺/CD206⁺ double-positive cells, suggesting a polarization of macrophages toward the CD206⁺ M2-type phenotype. Caspase-8 inhibitors mimicked the cyto-kine-induced up-regulation of autophagy and M2 polarization. Furthermore, E64D and leupeptin, which are able to function as inhibitors of autophagic degradation, reversed the effect of caspase-8 inhibitors in the M2-macrophage polarization, indicating a role of autophagy in this mechanism. Additionally, in patients with advanced castrate-resistant prostate cancer, metastatic lesions exhibited an increased CD14⁺/CD206⁺ double-positive cell population compared with normal tissues. Altogether, these findings suggest a role for CCL2 and IL-6 in the survival of myeloid monocytes recruited to the tumor microenviroment and their differentiation toward tumor-promoting M2-type macrophages via inhibition of caspase-8 cleavage and enhanced autophagy.

The recruitment of migratory hematopoietic cells, including tumor-infiltrating leukocytes, to the supporting stroma is essential in the progression and metastasis of tumors (1, 2). Resident macrophages are derived from monocytic precursor cells, which are released from the bone marrow and circulate in the blood stream until signaled to differentiate within target tissues, typically through interactions with cytokines and colony-stimulating factors (1, 3). Tumor-associated macrophages (TAMs)² perform a variety of functions essential for inflammation and tissue/matrix remodeling (3). Most malignant solid tumors contain a large number of TAMs that operate to attract additional macrophages to the developing cancer and also produce numerous growth factors, proteases, and cytokines that promote a more favorable tumor microenvironment (2–4).

An increase in TAM density characteristically potentiates tumor progression (3). However, macrophage responses are diverse and multifaceted and therefore also exhibit anti-tumor-igenic capacity (1, 3–5). The malleability of macrophage function is vastly dependant upon pro- and anti-tumorigenic signals from the local tumor microenvironment, and the resulting macrophage-mediated tissue morphogenesis is therefore relevant not only upon TAM abundance but also upon the specific subset of macrophages expressed (2, 4, 6).

Within metastatic lesions, infiltrate macrophages are typically skewed toward the protumorigenic M2-type macrophage phenotype (1). However, the TAM phenotype varies with the progression of tumor development. Although the proinflammatory, “classically activated,” M1-type macrophage phenotype predominates at sites of inflammation, tumor progression promotes a phenotypic switch in which TAMs exhibit a more immunosuppressive M2-type phenotype, which promotes tumor growth, survival, and metastasis (7, 8). “Alternatively activated” M2 macrophages are typically induced by interaction with IL-4, IL-13, and macrophage colony-stimulating factor and result in the production and recruitment of angiogenic cytokines and chemokines (7, 9).

The mannose receptor CD206 was initially identified as part of a tissue-protective mechanism against inflammatory damage through the removal of mannosal glycoconjugate ligands, including lysosomal hydrolases, both in vitro and in vivo (10, 11). Additionally, in accordance with the M2-type phenotype, mannose receptor activity is increased in response to IL-4 and IL-13 and is inhibited by interferon-γ (10, 12). Consequently, mannose receptor, which has been shown to be

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² The abbreviations used are: TAM, tumor-associated macrophage; IL, interleukin; PBS, phosphate-buffered saline; PI, propidium iodide; FSC, forward scatter; SSC, side scatter; mAb, monoclonal antibody; PARP, poly(ADP-ribose) polymerase; FITC, fluorescein isothiocyanate; PE, phycoerythin; cFLIP, cellular caspase-8 (FLICE)-like inhibitory protein.
highly expressed by TAMs, is a key marker for the differential activation of the protumorigenic M2-type macrophage phenotype (9).

The CC chemokine CCL2 (monocyte chemotactant protein-1) is one of the most frequently observed chemokines in a wide range of tumors and has been suggested to be one of the principle determinants of human tumor macrophage content (9, 13). CCL2 is abundantly expressed by macrophages and fibroblasts and by both endothelial and tumor cells (3, 13). In certain carcinomas, CCL2 has also shown strong correlations to TAM accumulation and has been suggested to be involved in Th2 polarization (14) while increasing levels of vascular endothelial growth factor, tumor necrosis factor α, and IL-8, indicating its close involvement in tumorigenic progression (3, 5, 9). Previously, it has been demonstrated that CCL2 is one of the most prevalent cytokines in the microenvironment of prostate cancer bone metastases and has a significant impact on the promotion of survival mechanisms in prostate cancer, PC3 cells in vitro (15–17). Consequently, an understanding of the cytokine/chemokine-mediated cellular responses that promote cell survival and macrophage polarization in the tumor microenvironment has broad therapeutic implications. Here, the survival and activation of CD11b+ myeloid monocytes, isolated from human peripheral blood, was investigated.

**EXPERIMENTAL PROCEDURES**

**University of Michigan Rapid Autopsy Series and Tissue Procurement**—Patients with advanced castrate-resistant prostate cancer were provided with detailed information regarding experimental aims and procedures of tissue donation. Patients or next of kin provided consent to participate in this Institutional Review Board-approved tissue donor program. Shortly after death, the body of the patient was transferred to the University of Michigan Morgue, and a gross analysis of tumor-bearing tissues was performed by a staff genitourinary pathologist and a medical oncologist. Tissue procurement was then performed by the medical oncologist, post-doctorate researchers, and laboratory assistants. Normal and tumor tissues were collected from three patients, corresponding to rapid autopsies 53–55. Tissue specimens were placed into RPMI medium (Invitrogen; catalog number 61870) plus 1% Antibiotic-Antimyotic (Invitrogen; catalog number 1004C) or IL-6 (100 ng/ml; Roche Applied Science) was used to assess cell viability of human CD11b+ cells. CD11b+ cells were isolated as described above and plated at 5 × 10^6 cells/well into 96-well tissue culture plates (Costar; #3596) in serum-free RPMI 1640 plus 1% Antibiotic-Antimycotic and then incubated at 37 °C for 48 h. Following incubation, the cells were treated or not with CCL2 (100 ng/ml) or IL-6 (100 ng/ml). WST-1 reagent was added in 24 h increments up to 72 h, following the manufacturer’s instructions, and the cells were returned to 37 °C for 105 min. Dye conversion was ascertained at 440 nm by a VERSAmax microplate reader and analyzed using Softmax Pro 3.12 software. The data for survival curves (n = 10) were normalized to average absorbance at t = 0 h (0 h time point corresponds to plated cells, immediately preceding treatment with chemokines). Standard errors were determined for each set of data points and represented by error bars.

**Cell Cycle Analysis**—Human CD11b+ cells were isolated as described above and plated at a density of 1.5 × 10^6 cells/ml in serum-free RPMI 1640 plus 1% Antibiotic-Antimycotic and then treated or not with CCL2 (100 ng/ml) or IL-6 (100 ng/ml). 48 and 96 h post-treatment, the cells were washed twice with cold PBS and then collected by gently scraping cells into 3 ml of cold PBS. The collected cells were pelleted by centrifugation at 1500 rpm in an Eppendorf 5810 centrifuge, then resuspended in 500 μl of PBS (Invitrogen), and fixed by adding 500 μl of 100% EtOH (equilibrated to −20 °C). Fixed cells were stored for a minimum of 20 min at 4 °C until samples from all aforementioned time points were collected. Following fixation, the cells were pelleted, EtOH/PBS was decanted, and cells were resuspended in 500 μl of propidium iodide (PI)-RNase solution (50 μg/ml propidium iodide...
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(Invitrogen), 100 µg/ml RNase A (Qiagen, diluted in PBS) and then incubated at room temperature for 20 min. PI-stained samples were analyzed on a FACSCalibur (Becton Dickinson) with Cell Quest Pro (Becton Dickinson) software. The viable cells were gated based upon forward scatter (FSC) and side scatter (SSC) parameters. PI fluorescence was detected in the FL2 channel on a logarithmic scale and in the FL3 channel on a linear scale. The voltage adjustments were performed for each sample to place the G0/G1 peak at a standard of 400 on the linear scale. For each sample, 2–3 × 10^5 events were collected for analysis as the Boolean intersection of viable cells, based upon the FSC/SSC gate. Single cell analysis was based upon FL3 width v. FL3 area doublet discrimination gate. The relative percentages of cells found within each phase of the cell cycle were determined using the Bradford Assay (Bio-Rad; catalog number 130-091-376)) and then detached by gentle scrapping into 3 ml of buffer. The cell concentrations were adjusted to 10^6 cells/ml and then incubated for 20 min at 4 °C with or without CD14-FITC-conjugated antibody (BD Biosciences; catalog number 555397), CD206-PE-conjugated antibody (BD Biosciences; catalog number 555954), or a combination of the two. The cells were then washed twice with 4 ml of flow buffer, then centrifuged, and resuspended in 0.5 ml of flow buffer for analysis. Flow cytometry was performed using a two-laser (488 and 635 nm) FACSCalibur flow cytometer (BD Biosciences, Palo Alto, CA). The cell populations were gated based upon FSC and SSC parameters and then evaluated for FITC (lower right quadrant) and/or PE (upper left quadrant) fluorescence and normalized to cells alone (without antibody) to adjust for cell-specific autofluorescence. All of the experiments were repeated at least three times.

Statistical Analysis—All of the average values are presented as the means ± S.D. The data were analyzed using GraphPad Prism software and the two-tailed unpaired t test. A probability level of less than 0.05 was considered significant.

RESULTS

CCL2 and IL-6 Induce Each Other in Human CD11b⁺ Cells—Tumor cells produce numerous chemokines that attract inflammatory cells, which are capable of producing an assorted array of cytokines, angiogenic growth factors, and proteases, all of which dictate the fate of a developing tumor. CCL2 is one of the most prevalent cytokines expressed in the tumor microenvironment and is a major chemoattractant of monocytes/macrophages to the sites of inflammation (9, 17, 18). Because CD11b⁺ cells are a significant component of prostate cancer metastatic tumors, comprising, in most cases, more than 60% of total isolated cells (supplemental Fig. S1), the cytokine expression profile of these cells was investigated. Additionally, the effect of CCL2 on this cytokine profile was evaluated by using human recombinant CCL2. CD11b⁺ cells were isolated from human peripheral blood mononuclear cells using CD11b MicroBeads. Following this isolation more than 80% of the cells were identified as monocytes, as determined by the Wright staining procedure (not shown). The cells were plated in serum-free conditions and treated for 48 h with CCL2, and the cytokine expression pattern in the media was analyzed using two human cytokine-antibody arrays. Untreated CD11b⁺ cells demonstrated positive CCL2 expression and low IL-6 levels (Fig. 1A). However, when the cells were further stimulated with CCL2, a striking up-regulation of the IL-6 cytokine was observed (Fig. 1, A and B). Similarly, CD11b⁻ cells stimulated with IL-6 showed a marked increase in the secreted CCL2 chemokine (Fig. 1B). A densitometric analysis of the cytokine arrays was performed to evaluate the induction of IL-6 by CCL2 and vice versa. The results demonstrate that the stimulation by CCL2 induced IL-6 more than 5-fold relative to control (untreated) cells. Similarly, IL-6 treatment stimulated the CCL2 chemokine levels ~2-fold (Fig. 1C). Although IL-8 expression was elevated in these cells, no significant changes were observed upon CCL2 or IL-6 treatment. Altogether, these
results suggest that CCL2 and IL-6 induce each other, which could result in amplification of the expression levels of these cytokines in the inflammatory microenvironment.

CCL2 and IL-6 Inhibit the Apoptotic Program Induced in CD11b+ Cells under Serum Deprivation Stress—The effect of CCL2 and IL-6 on CD11b+ cell survival was analyzed at increasing times (24, 48, and 72 h) by using the WST-1 assay, an indirect assessment of cell numbers based on mitochondrial dehydrogenase enzyme activity. As shown in Fig. 2A, independent stimulation with CCL2 or IL-6 promoted cell survival, as compared with untreated cells. A microscopic analysis of the cell morphology revealed differences between control and CCL2 or IL-6-stimulated cells (Fig. 2B). Control cells showed more rounded morphology 48 h post-treatment, suggesting a higher activation of cell death. Further analysis of these cell populations (control, CCL2, and IL-6) was focused on the identification of the activated mechanisms. Apoptosis was assessed by flow cytometry (48 and 96 h post-treatment) using propidium iodide staining. Sub-G1 cell populations were determined to be apoptotic and are depicted in the M2 region (Fig. 2C). These results demonstrate that a significantly higher percentage of untreated cells undergo apoptosis as compared with CCL2 or IL-6-stimulated cells and therefore correlates well with the cell viability results obtained previously (Fig. 2A).

Subsequent analysis was performed to identify the pathways activated in CD11b+ cells and to determine the antiapoptotic mechanisms elicited by CCL2 or IL-6. Immunoblot analysis of cleaved caspases revealed a significant activation of caspase-8, -3, -6, and -7 in control cells (Fig. 2D). In contrast, strong inhibition of caspase cleavage was observed upon CCL2 or IL-6 treatment. Consistent with the activated caspase cascade, cleaved lamin A was significantly higher in control, as compared with cytokine-stimulated cells (Fig. 2D). Con-

FIGURE 1. CCL2 and IL-6 exhibit mutual induction in human CD11b+ cells. A and B, isolated CD11b+ cells were plated in serum-free RPMI and allowed to attach at 37 °C prior to treatment. Following attachment, the cells were treated or not with 100 ng/ml recombinant CCL2 or IL-6 for 48 h. The chemokine expression profiles were evaluated in cell supernatants by using inflammation array 3 and cytokine array 5 (RayBiotech, Inc.) as indicated. The arrays displayed are representative examples of three assays. C, bar graph depicts densitometric analysis of all spots corresponding to CCL2 and IL-6, from both inflammation array 3 and cytokine array 5. The data presented represents average CCL2 and IL-6 spot density values (from n = 3 arrays) in response to treatment with IL-6 or CCL2, respectively. The data were normalized to array-specific positive controls and then compared with untreated cells to establish average fold changes.
versely, the activation of caspase-9 was not detected by Western blot at any time point (data not shown). These results correlate with the previous observation that CCL2 and IL-6 promote CD11b⁺ cell survival (Fig. 2, A–C) and suggest that the extrinsic apoptotic pathway, mediated by death receptor and caspase-8, is the predominant death mechanism activated in these cells.

To explore the possible mechanisms by which CCL2 and IL-6 oppose caspase-8 activation, the expression of cFLIP (19) was investigated. Western blot analysis revealed that both cytokines induce up-regulation of cFLIPL, an isoform that shares significant homology with caspase-8, but lacks the active site and does not have protease activity (Fig. 2E). It was previously demon-

FIGURE 2. CCL2 and IL-6 protect CD11b⁺ myeloid cells from apoptotic death. Isolated CD11b⁺ cells were plated in either 96- or 6-well plates at a density of 1.5 × 10⁶ cells/ml in serum-free RPMI. The cells were then treated or not with 100 ng/ml CCL2 or IL-6. A, CD11b⁺ cell survival in response to CCL2 or IL-6 treatment. Survival was evaluated by WST-1 dye conversion immediately preceding cytokine treatment and at 24-h increments, up to 72 h, following stimulation. The data for each treatment condition is representative of n = 10 samples and is normalized to average, 0-h absorbance. B, cell morphology analyzed 48 h post-treatment with the indicated cytokines. The images were obtained using an Olympus IX71 microscope (20× objective), fitted with an Olympus DP71 camera. C, total percentages of apoptotic cells 48 and 96 h post-chemokine treatment, as evaluated by flow cytometry. Sub-G₀/G₁ content (apoptotic cells, denoted as M2) was assessed by PI fluorescence and was determined by FSC/SSC gating. D, protein lysates were collected in 24-h increments to evaluate the expression of activated/cleaved caspases and lamin A, as well as anti-apoptotic proteins (Bcl-2 and Bcl-X₇) by Western blot. E, CCL2 and IL-6 effectively induce the caspase-8 inhibitor cFLIPL and prevent caspase-8 cleavage, as determined by Western blot 48 h post-treatment. The antibody against cleaved caspase-8 recognizes cleavage products p43/p41 and p18; all of the bands are shown here (separated by a single black line) from the same gel with the empty region in between the bands, corresponding to the cleavage products, removed. β-Actin was evaluated as a loading control for all blots.
strated that cFLIPL effectively inhibits the apoptosis induced by death receptors (19). Accordingly, up-regulation of cFLIP by CCL2 and IL-6 also correlated with the inhibition of caspase-8 cleavage in CD11b<sup>+</sup> cells (Fig. 2E), suggesting its role in this mechanism.

In addition to activating the extrinsic apoptotic pathway, caspase-8 can also cleave the BH3 domain-only protein, BID, thereby initiating the intrinsic (caspase 9-mediated) pathway, which is characterized by the loss of mitochondrial membrane potential and results in the release of cytochrome c. Both Bcl-2 and Bcl-X<sub>L</sub> inhibit this loss and oppose the activation of apoptosis (20). Furthermore, it has been reported that Bcl-2 can also regulate caspase activation and apoptosis independently of the cytochrome c/Apaf-1/caspase-9 apoptosome (21). Here, the up-regulation of the anti-apoptotic proteins Bcl-2 and Bcl-X<sub>L</sub> was observed in cells stimulated with CCL2 or IL-6 (Fig. 2D). Therefore, even though caspase-9 activation was not detected, these results suggest that CCL2 and IL-6 may oppose apoptosis via up-regulation of Bcl-2 and Bcl-X<sub>L</sub>.

CCL2 and IL-6 Hyperactivate Autophagy in CD11b<sup>+</sup> Cells—Because apoptosis and autophagy are functionally related (22) and autophagy is activated in response to starvation, this pathway was also analyzed. In some cellular settings, autophagy acts as a protective mechanism and prevents apoptotic cell death, whereas under different conditions it could constitute an alternative cell death pathway. Autophagy was monitored by analyzing the microtubule-associated protein LC3 (23). Activation of autophagy involves the cleavage of LC3-I and its conjugation with PE to form LC3-II, a process that is essential to autophagosome formation. The amount of LC3-II has been shown to correlate with autophagosome number (24). Accordingly, a time course analysis (24–96 h) by Western blot was performed with lysates from control, CCL2, and IL-6-treated cells using LC3B antibody (Fig. 3A). The observed results demonstrated a considerable increase in LC3-II protein in cells stimulated with CCL2 or IL-6, relative to control. Furthermore, the amount of LC3-II was initially lower at 24 h and significantly increased after 48 h, consistent with an escalation in autophagy under serum deprivation. This result suggested an increase in the autophagosome number in cells stimulated with CCL2 or IL-6; however, because LC3-II itself is degraded by autophagy, it was required to determine autophagic flux by also treating the cells with protease inhibitors E64D and leupeptin (23). As shown in Fig. 3B, a further increase in LC3-II was observed in response to treatment with protease inhibitors, indicating an autophagic degradation flux. A densitometric analysis was performed to calculate the ratio of the signals plus-minus protease inhibitors. The results illustrated in Fig. 3C demonstrate similar rates of autophagic flux (similar ratio) for all of the samples. These findings indicate an overall increase in the autophagic activity in the cells that were stimulated with CCL2 and IL-6, which correlated with the observed inhibition of apoptosis and suggests a protective role of autophagy in these cells.

The Protective, Antiapoptotic Role of IL-6 Is Partially Dependent upon the Induction of CCL2—Because it was clear that CCL2 and IL6 induce each other, the hierarchy of each
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CCL2 and IL-6 Induce M2-type Macrophage Polarization toward CD206⁺ M2-type Activation—Macrophage activation and function occurs in response to environmental influence, and cytokines play a key role in this activation (25, 26). An increasing number of reports show that TAMs contribute to tumor progression and represent a unique M2-polarized myeloid population (6, 8, 9). Furthermore, the expression of high levels of the mannose receptor (CD206) is a distinctive marker of M2-activated and tumor-associated macrophages (9, 12, 27). Consequently, the characterization of cell populations was performed by flow cytometry analysis using antibodies to detect the surface receptors CD14 and CD206. Approximately 84% of CD11b⁺ cells isolated from peripheral blood were identified as monocytes (CD14⁺), and as expected, the vast majority of these cells were CD206-negative (the mannose receptor is not expressed in monocytes (12)) (Fig. 5 A). The flow cytometry results of adherent cell populations corresponding to control (untreated) and CCL2- and IL-6-stimulated cells (48 h post-treatment) are presented in Fig. 5 (B–D). The cells were gated in R1, R2, and R3 populations. In contrast to peripheral blood CD11b, where most cells were collected in R2, over 50% of total adherent cells were collected in the gate R3, which could represent a transition to more differentiated cells. In both the cytokine with regards to inhibition of apoptosis was further investigated. The analysis was performed by using neutralizing antibodies against CCL2 or IL-6; cells stimulated with IL-6 were also treated with CCL2 neutralizing antibody, and vice versa CCL2-stimulated cells were additionally treated with IL-6 neutralizing antibody. Fig. 4 (A and B) demonstrated that treatment of IL-6-stimulated cells with neutralizing antibodies against CCL2 increased the cleavage of caspase-6 (Asp162), lamin A (Asp239), and PARP (Asp214) and simultaneously reduced the expression of Bcl-2, Bcl-XL, and LC3 (B) were evaluated by Western blot. β-Actin was assessed as a loading control for all blots. C, CD11b⁺ cell morphology, analyzed 48 h post-cytokine ± neutralizing antibody treatment. The images were obtained using an Olympus IX71 microscope (20× objective) fitted with an Olympus DP71 camera.

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CCL2- and IL-6-treated groups, the R3-gated cells showed a significant increase in CD206\(^+\) and double-positive CD14\(^+\)/CD206\(^+\) cells as compared with untreated control group (Fig. 5, B–D). Most cells in R2 (about 20% in control and CCL2 groups and 5–6% in the IL-6 group) were identified as CD14\(^+\), and CCL2 treatment significantly increased the CD206\(^+\) population in this gate as compared with control (Fig. 5, B and C). Finally, cells gated in R1 (about 20–25% of total cells) were characterized as double-negative for CD14 and CD206. The results summarized in Fig. 5E (all gates included) demonstrate a significant increase in CD206\(^+\) population with both CCL2 and IL-6. The double-positive CD14\(^+\)/CD206\(^+\) cell population substantially increased by CCL2 treatment (more than 2-fold), and a moderate increase was also induced by IL-6. Because CD14 is a surface marker expressed in macrophages, and CD206 is a marker of M2 polarized macrophages, these results suggest that CCL2 and IL-6 induce the differentiation of CD11b\(^+\) monocytes into M2-type macrophages.
Inhibition of Caspase-8 Induces Up-regulation of Autophagy and M2-type Macrophage Polarization—Given that autophagy and apoptosis have been shown to be linked in CCL2 and IL-6 signaling and that both mechanisms are responsible for the removal of damaged cells, the effect of caspase-8 inhibition on autophagy induction was investigated by using a cell-permeable, reversible caspase-8 inhibitor-I (IETD-CHO (2.5 μM)). CD14<sup>+</sup> monocytes isolated from human peripheral blood were treated or not with 2.5 and 5 μM caspase 8 inhibitor-I, IETD-CHO, or respective volumes of Me<sub>2</sub>SO for 48 h. A, the effect of the inhibition of caspase-8 cleavage on autophagy was evaluated by LC3 immunodetection. B, the increase in autophagy observed in Fig. 6A by caspase-8 inhibitor-I was validated by treatment with protease inhibitors E64D and leupeptin. Collected protein lysates were analyzed by Western blot to evaluate full-length caspase-8 (p55 and p53, the two predominant isoforms are indicated) and LC3 conversion. β-Actin was assessed as a loading control for all blots.

C–F, isolated CD14<sup>+</sup> monocytes were plated in the presence and absence of caspase-8 inhibitor-I (2.5 μM). The cells were incubated at 37 °C for 16 h and then treated or not with E64D and leupeptin (inhibitors of autophagic degradation, 10 μg/ml each). 48 h post-treatment, the cells were gently scraped and subjected to flow cytometry analysis with: CD14 FITC-conjugated antibodies, CD206 PE-conjugated antibodies, or a combination of both. Dot plots shown are representative of observed data from n = 2 replicate experiments. The cell populations were gated into regions R1, R2, and R3, based on FSC/SSC parameters. CD14<sup>+</sup> cells are shown in the lower-right quadrants, CD206<sup>+</sup> cells are shown in the upper left quadrants, and CD14<sup>+</sup>/CD206<sup>+</sup> double-positive cells are shown in the upper right quadrants. The percentages of cell populations, by region, are displayed in the tables (left side of each image) for all conditions.

G, table summarizing total percentage of cell populations (from all gates), as determined by flow cytometry.
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**A**

**B**

**FIGURE 7.** CD14+/CD206+ double-positive cell populations are enhanced in human prostate cancer metastatic lesions. The cells from tumor and respective normal tissues were collected from three patients, corresponding to University of Michigan rapid autopsy numbers 53–55. **A**, representative flow cytometry analysis showing the percentage of total cell populations that were CD14+/CD206+ double-positive using FITC-conjugated CD14 and PE-conjugated CD206 antibodies. The cells that express both CD14 and CD206 surface receptors are displayed in the upper right quadrants of the dot plots. The table summarizes the percentage of total double-positive cells for all tumor (right) and respective normal (left) tissue samples. **B**, graphic representation of a compiled data analysis from rapid autopsy tissues.

Studies of human caspase-8 deficiency demonstrated its essential role in the activation of T, B, and natural killer cells (28). Furthermore, as shown above, both CCL2 and IL-6 inhibit the activation of caspase-8, and induce autophagy and M2-type macrophage polarization. Thus, the roles of caspase-8 and autophagy in macrophage activation were examined by using the caspase-8 inhibitor-I (IETD-CHO; 2.5 μM) in combination with inhibitors of autophagic degradation (E64D and leupeptin). CD14+ monocytes treated or not with a caspase-8 inhibitor-I were further incubated with E64D and leupeptin, and the adherent cells were analyzed by flow cytometry (48 h post-treatment) using antibodies to detect the surface receptors CD14 and CD206. The results presented in Fig. 6 (C and D) show a significant increase in the CD206+ and double-positive CD14+/CD206+ cells upon treatment with caspase-8 inhibitor-I. Similar results were observed by using a cell-permeable, but irreversible caspase-8 inhibitor-II (Z-IETD-FMK; supplemental Fig. S2). However, when the cells treated with caspase-8 inhibitor-I were further incubated with E64D and leupeptin, a significant drop in the CD206+ and double-positive CD14+/CD206+ cells was observed, and the percentage of these populations was reduced to the levels similar to that observed in control cells (Fig. 6, C, F, and G). In contrast, the protease inhibitors caused only a minor change in the control cell populations (Fig. 6, C, E, and G). Altogether, these findings demonstrate that inhibition of caspase-8 mimics the effects of CCL2 and IL-6 by inducing autophagy and suggest a role for autophagy in the M2 macrophage polarization of these cells.

A Double-positive CD14+/CD206+ Cell Population Is Increased in the Metastatic Lesions as Compared with Normal Tissues in Prostate Cancer Patients—TAMs are derived from monocytes that are recruited to the tumor microenvironment by chemotactic factors, including CCL2. TAMs are a key component of the leukocyte infiltrate of tumors that promote tumor progression and metastasis (2, 4, 6). Furthermore, an increasing number of reports suggest that CCL2 plays an important role in prostate cancer tumor progression (15, 29), and as shown here, this chemokine promotes macrophage survival and stimulates the polarization of CD11b+ peripheral blood cells toward a double-positive CD14+/CD206+ population. Therefore the CD14+/CD206+ cell component was analyzed in metastatic prostate cancer tumors collected from three patients with advanced castrate-resistant prostate cancer in accordance with the University of Michigan rapid autopsy series. The lesions were identified by gross examination, and the tissues were isolated from: lymph node, dura mater, liver, adrenal, and bone marrow with the respective adjacent normal tissues. Following collagenase treatment samples were analyzed by flow cytometry using anti-human FITC-conjugated CD14 and PE-conjugated CD206 antibodies. Fig. 7 demonstrates an increase in the double-positive CD14+/CD206+ cell population in metastatic lesions relative to its adjacent normal tissues. These findings suggest that the CD14+/CD206+ cell population is activated in metastatic tumors, which is consistent with the view that TAMs are skewed toward an M2-polarized macrophages, which are characterized by high level expression of mannose receptor (CD206) (8, 9, 12, 27).
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DISCUSSION

In the tumor microenvironment, cross-talk between malignant cells and infiltrates of hematopoietic origin is critical in tumor progression and metastasis. By altering chemokine expression, tumor cells influence the recruitment of circulating inflammatory cells, which in turn secrete a wide array of cytokines and other factors that promote cancer growth and survival (2). Macrophages that derive from circulating mononuclear myeloid precursors are a major component of leukocyte infiltrate in tumors (6). It is increasingly appreciated that TAMs are vital constituents of solid tumors and have been shown to be generally correlated with tumor progression and metastasis as well as poor clinical outcomes (3). Additionally, an increasing number of reports implicate CCL2, a chemokine central in the recruitment monocytes/macrophages to sites of inflammation, in cancer progression and metastasis (29, 30). Previously, we discovered a protective role of CCL2 in prostate cancer cells (15); here we present the effect of this chemokine on the survival and activation of primary CD11b+ monocytes isolated from human peripheral blood.

CCL2 is widely expressed in a variety of tumors (sarcomas, gliomas, and cervical, ovarian, bladder, breast, and lung tumors) (3, 6), and more recently, it was found that it is also largely expressed in the tumors of prostate cancer bone metastasis (17). The data presented here demonstrate that CCL2 is also expressed by human CD11b+ cells isolated from peripheral blood, which are composed mainly of monocytes (more than 80%). In these cells, IL-6, which is typically expressed by many cancer cells including prostate (31), was only detected at very low levels; however, upon stimulation with CCL2 a 5-fold increase in the expression of IL-6 was observed. Similarly, a 2-fold induction of CCL2 was achieved when cells were treated with IL-6 (Fig. 1, A and B). These findings have important implications in the tumor microenvironment, where CCL2 could reach high concentrations, because multiple cells (macrophages, tumor, stromal, and endothelial) contribute to the expression of this chemokine (17, 30). On the other hand, IL-6 also has the potential to stimulate tumor cell proliferation and survival (32, 33). This amplification loop between CCL2 and IL-6 could have a direct effect not only in the CD11b+ infiltrates in the tumor microenvironment (including TAMs), but also in malignant cell proliferation and survival acting in an autocrine or paracrine fashion.

It was further demonstrated that both CCL2 and IL-6 inhibit apoptosis to promote CD11b+ cell survival under serum deprivation stress (Fig. 2, A–C). In fact, stimulation with either CCL2 or IL-6 inhibited the activation of the caspase cascade, detected as reduced cleavage product of caspase-8 (Asp391), caspase-6 (Asp165), caspase-3 (Asp175), caspase-7 (Asp198), and the subsequent lamin A (Asp230) (Fig. 2D). The activation of caspase-8 but not caspase-9 suggested that apoptosis was predominantly mediated by a death receptor (extrinsic) pathway, which typically results in the recruitment and activation of caspase-8 and involves the stimulation of members of tumor necrosis factor receptor superfamily (34). These results correlate with previous reports showing that human monocytes cultured in vitro undergo apoptosis mediated by Fas-Fas ligand interaction in a process that can be accelerated and enhanced by the removal of serum (35). It was further demonstrated that CCL2 and IL-6 induce the up-regulation of cFLIP (Fig. 2E). cFLIP was previously identified as an important regulator of apoptosis that is expressed as two alternative splice forms, cFLIPs (short) and cFLIP_L (long) (19). cFLIP_L was the only isoform detected by immunoblotting and was up-regulated by CCL2 and IL-6 in CD11b+ cells. It has been demonstrated that cFLIP_L can interact with the adaptor protein FADD and caspase-8 to inhibit apoptosis induced by death receptors (19, 36). In contrast to monocytes that express very low levels of cFLIP and are highly sensitive to CD95-induced death, macrophages express substantial amounts of cFLIP and are more resistant to cell death (37). It is therefore conceivable that the up-regulation of cFLIP_L induced by CCL2 or IL-6, could protect the monocytes recruited to the tumor microenvironment, allowing their differentiation into mature macrophages. On the other hand, the stimulation of CD11b+ cells with CCL2 or IL-6 does not involve the up-regulation of macrophage colony-stimulating factor (also known as CSF-1) (Fig. 1, A and B), which has been identified to promote survival of macrophages and the pro-tumor function of these cells (38, 39). Therefore, these results suggest that CCL2 and IL-6 could play a crucial role (independently of macrophage colony-stimulating factor) in the survival of TAMs in human tumors, where macrophage populations are generally not sustained by in situ proliferation (6). In support of these findings, it has been demonstrated that IL-6-deficient mice showed a defective recruitment of macrophages to the tumor site and a reduced incidence of myeloma (1). Moreover, IL-6 has been suggested to push myeloid precursors toward a macrophage-like phenotype and inhibit the maturation of dendritic cells, creating a microenvironment that potentiates the progression of metastatic tumors (40, 41).

The results presented here also demonstrate that CCL2 and IL-6 induce the activation of M2-type macrophages, characterized by the expression of the plasma membrane receptor CD14 and the increased expression of mannose receptor CD206 (Fig. 5, A–D). Previous studies suggest that macrophages use CD14 to bind apoptotic cells before engaging receptors that drive phagocytosis, and the dying cells are cleared without inducing the release of pro-inflammatory cytokines (42). Furthermore, there is strong evidence that mannose receptor can mediate phagocytosis in an actin-dependent manner, a process that plays a critical role in tissue remodeling (43, 44). The up-regulation of mannose receptor has also been associated with the removal of lysosomal hydrolases and neutrophil-derived myeloperoxidase that could reduce tissue damage during inflammation (45, 46). These functions of mannose receptor are associated with the function of polarized M2 macrophages. TAMs predominantly function similarly to M2 macrophages, express high levels of mannose receptor, and promote tumor progression and metastasis (9). Because prostate cancer patients display an increased polarized cell population of CD14+/CD206+ in the metastatic lesions, as compared with normal tissues (Fig. 7), these findings strongly support the idea that the progression of metastatic tumors requires the activation of infiltrated macrophages that develop M2-type function.
The dual role of CCL2 and IL-6 in the protection of monocytes from apoptosis and induction of M2-type macrophage activation suggests a possible molecular link between these two mechanisms. Considering that caspase-8 has been suggested to play a central role in lymphocyte activation (28), it is suspected that the inhibition of caspase-8 cleavage by these cytokines could be essential in macrophage polarization. In fact, the inhibition of caspase-8 cleavage in human monocytes by using caspase-8 inhibitors induced a significant increase in the CD206+ and the double-positive CD14+/CD206+ cell populations, mimicking the effect of CCL2 and IL-6 (Fig. 6, A and B). The significant increase in autophagosome formation and autophagic degradation induced by CCL2 and IL-6 (and mimicked by caspase-8 inhibitors), suggests that autophagy may play an active role in the activation of macrophages. This hypothesis was investigated by using E64D and leupeptin, which are able to function as inhibitors of autophagic phages. This hypothesis was investigated by using E64D and leupeptin, which are able to function as inhibitors of autophagic phages. Furthermore, it is tempting to speculate that cFLIPL, which is induced by both CCL2 and IL-6 (Fig. 2E), could also promote the activation of full-length caspase-8 but then restricts the degree of caspase-8 activation to a moderate nonapoptotic range as previously reported (47). Because it has been shown that a nonapoptotic molecular form of caspase-8 plays an important role in the regulation of other cellular mechanisms, we surmise that this molecule could also mediate macrophage activation; however, this hypothesis merits further investigation.

Because autophagy is activated as a direct response to starvation, the relationship between this mechanism, apoptosis, and macrophage activation was further analyzed. Surprisingly, CCL2 and IL-6 induced a marked up-regulation of autophagy (Fig. 3, A and B). The significant increase in autophagosome formation and autophagic degradation induced by CCL2 and IL-6 (and mimicked by caspase-8 inhibitors), suggests that autophagy may play an active role in the activation of macrophages. This hypothesis was investigated by using E64D and leupeptin, which are able to function as inhibitors of autophagic degradation (Fig. 6B). When these inhibitors were added in combination with caspase-8 inhibitors, they induced a marked decrease in the CD206+ cell populations to levels comparable with those of control cells (Fig. 6, C–G), suggesting that autophagy plays a significant role in M2 macrophage polarization. This process provides not only an additional source of nutrients and energy but also promotes the suppression of inflammation and supports tissue remodeling and repair, consistent with the function of M2 macrophages. Thus, CCL2 and IL-6 not only protect monocytes/macrophages from cell death but also facilitate the execution of the effective clearance of dying cells and other macromolecules. Additionally, the finding that caspase-8 inhibition substantially increases autophagy provides another example of a role that this molecule plays in the modulation of autophagic signaling (48, 49) and suggests that the mechanism by which CCL2 and IL-6 enhance autophagy could be mediated by a nonapoptotic molecular form of caspase-8. However, a more detailed elucidation of this mechanism requires further investigation.

Altogether these findings reveal how CCL2 and IL-6, by inducing each other, potentiate their tumor-promoting roles, not only by protecting tumor infiltrating monocytes, but also by inducing myeloid-monocyte activation toward M2 macrophages (depicted in Fig. 8). Furthermore, this cytokine-mediated hyperinduction of autophagy enhances the M2-type phagocytic functions that promote tumor growth: clearance of dying cells, tissue remodeling, and repair. Thus, a combined target of these cytokines may represent a valid strategy in cancer therapeutics.

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