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Original Paper

MCP-1 and MIP-3α Secreted from Necrotic Cell-Treated Glioblastoma Cells Promote Migration/Infiltration of Microglia

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Key Words
Necrosis • Glioblastoma • Microglia • Migration • Infiltration

Abstract
Background/Aims: Glioblastoma multiforme (GBM) is the most common primary brain tumor in adults. The defining characteristics of GBM are diffuse infiltration of tumor cells into normal brain parenchyma, rapid growth, a high degree of infiltration of microglia and macrophages, and the presence of necrosis. Microglia/macrophages are frequently found in gliomas and they extensively infiltrate GBM tissue, up to 30% of total tumor mass. However, little is known about the effect of necrotic cells (NCs) on microglia infiltration in GBM and the tumor-infiltrating microglia-induced factors in GBMs. ***Methods:*** In this study, to address whether necrosis or necrosis-exposed GBM cells affect the degree of microglia/macrophage infiltration, migration and invasion/infiltration assays were performed. Culture supernatants and nuclear extracts of CRT-MG cells treated or untreated with necrotic cells were analyzed using a chemokine array and electrophoretic mobility shift assay, respectively. ***Results:*** The presence of NCs promoted the migration/infiltration of microglia, and GBM cell line CRT-MG cells exposed to NCs further enhanced the migration and infiltration of HMO6 microglial cells. Treatment with NCs induced mRNA and protein expression of chemokines such as Monocyte Chemoattractant Protein-1 (CCL2/MCP-1) and Macrophage Inflammatory Protein-3α (CCL20/MIP-3α) in CRT-MG cells. In particular, CCL2/MCP-1 and CCL20/MIP-3α were significantly increased in NC-treated CRT-MG cells. NCs induced DNA binding of the transcription factors Nuclear Factor (NF)-κB and Activator Protein 1 (AP-1) to the CCL2/MCP-1 and CCL20/MIP-3α promoters, leading to increased CCL2/MCP-1 and CCL20/MIP-3α mRNA and protein expression in CRT-MG cells. ***Conclusion:*** These results provide evidence that NCs induce the expression of CCL2/MCP-1 and CCL20/MIP-3α in glioblastoma cells through activation of NF-κB and AP-1 and facilitate the infiltration of microglia into tumor tissues.

Yieun Jung and So-Hee Ahn contributed equally to this work

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Introduction

Astrocytoma is the most prevalent form of primary brain cancer, originating from star-shaped brain cells known as astrocytes [1]. Astrocytoma is divided into four pathologic grades by World Health Organization (WHO) classification; WHO grade IV glioblastoma multiforme (GBM) is the most malignant type of astrocytoma. GBM is a highly lethal cancer; patient survival with GBM is very poor, ranging from 12-15 months, despite aggressive treatment with surgery combined with chemotherapy and/or radiotherapy [2, 3]. GBM is characterized by the presence of necrosis, microglia/macrophage infiltration, vascular proliferation, and diffuse invasion [4-6]. The presence of necrosis has been considered a key diagnostic criterion for GBM and is correlated with poorer patient outcome [7]. Some studies have suggested a direct correlation between the grade of astrocytoma and the levels of resident tumor microglia/macrophage by demonstrating a large number of microglia/macrophages in the necrotic areas [8, 9]. Microglia occupy 5-20 % of the total glial cell population, serving as the resident macrophages in the central nervous system (CNS) [10]. Microglia are critical effector cells of the immune response in the CNS [11]. They constantly destroy pathogens or remove dead cells and debris and also promote tissue repair and neural regeneration [12, 13]. Recent evidence shows that the density of tumor-associated microglia/macrophage (TAM) in GBM is positively correlated with astrocytoma grade, proliferation, and invasiveness [9, 14, 15]. TAMs receive signals from diverse cells within the tumor microenvironment, which is regulated by several chemokines and chemokine receptors [16, 17].

Chemokines are a large group of small molecular weight proteins (8-12 kD) that interact with G protein-coupled transmembrane chemokine receptors. To date, more than 50 chemokines and 20 chemokine receptors have been identified [18]. Chemokines are classified into several structural groups (C, CC, CXC, and CX3C) based on the spacing of their cysteine residues [19]. Chemokines actively participate in inflammatory responses, such as allergies, autoimmune diseases, and viral infections, by attracting immune cells to the site of damaged and affected areas [20-22]. In addition, growing evidence indicates that chemokines play a crucial role in the development of cancer, promoting growth, angiogenesis, and metastasis. For example, Growth-regulated Oncogene α (CXCL1/GROα) expression in hepatocellular carcinoma (HCC) led to increased HCC cell proliferation, growth, and invasion [23]. In breast cancer, elevated levels of Monocyte Chemoattractant Protein-1 (CCL2/MCP-1) were associated with macrophage accumulation and angiogenesis [24]. The effects of chemokines and chemokine receptors on cell migration and invasion have been demonstrated in several studies. Macrophage Inflammatory Protein-3α (MIP-3α/CCL20) promotes the invasion and migration of thyroid cancer cells [25]. The interaction between CCL22 and CCR4 contributes to the migration of prostate cancer cells [26]. In our previous work, we showed that necrotic cells (NCs) induced secretion of the CXC motif chemokine ligand 8/interleukin-8 (CXCL8/IL-8) in glioblastoma, and this influenced migration and invasion of glioblastoma cells [6].

Several chemokines have been suggested as potential factors that recruit macrophage/microglia to tumors, including CCL2/MCP-1 and Regulated on Activation, Normal T cell Expressed and Secreted (CCL5/RANTES) [27]. Previous studies have shown that chemokines, such as CX3CL1/fractalkine and CCL2/MCP-1, and their receptors play crucial roles in the infiltration of microglia/macrophages in GBM [17, 28, 29]. To date, no study has characterized the effect of NCs or NC-treated GBM cells on tumor infiltration by microglia. In this study, to address whether necrosis or NC-treated GBM cells affect the degree of microglia/macrophage infiltration and migration, invasion/infiltration assays were performed, and chemokine expression was profiled using microarray and a chemokine array.

Materials and Methods

Cells

Human glioblastoma cell line CRT-MG and U87-MG [30] and the immortalized human microglial cell line HMO6, which has been previously established [31] and was kindly provided by Prof. S.U. Kim, were

maintained in Dulbecco's modified essential medium (DMEM, WelGENE Inc., Daegu, Korea) containing 10% fetal bovine serum (FBS, WelGENE Inc.), L-glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin. For preparation of necrotic cells, CRT-MG and U87-MG cells were frozen and thawed through five cycles of liquid nitrogen-water bath treatment as described previously [6].

Reagents and antibodies

Human recombinant CCL2/MCP-1, CCL20/MIP-3α and CXCL8/IL-8 proteins, and polyclonal anti-human CCL2/MCP-1, CCL20/MIP-3α and CXCL8/IL-8 antibodies were obtained from R&D Systems (Minneapolis, MN, USA). Control nonspecific IgG from goat serum was purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). The NF-κB inhibitor BAY 11-7092, c-Jun N-terminal Kinase (JNK) inhibitor SP600125, p38 inhibitor SB203580 and were purchased from Sigma-Aldrich, Co. Extracellular signal-regulated Kinase (ERK) inhibitor PD98059 was obtained from Merck (Darmstadt, Germany). Exo-mCherry (mChrry-loaded exosome) and Exo-srIκB (srIκB-loaded exosome) were obtained from Cellex Life Sciences Inc. (Daejeon, Korea) [32].

Cell migration assay

The ability of HMO6 cells to migrate through membrane was measured in a Boyden chamber. HMO6 cells (1.5×10^5) suspended in 1% FBS culture media placed in the top chamber of 8.0 μm polycarbonate membrane transwell inserts (Corning Inc., Corning, NY, USA). CRT-MG cells were either untreated or treated with necrotic CRT-MG cells for 24 h, and then supernatants were collected and centrifuged at 4 ˚C, 12, 000 rpm (13523*g*) for 20 min. The culture supernatants of CRT-MG either untreated or treated with necrotic cells and necrotic cell were placed in the lower chamber (Corning Inc.) as a chemoattractant. After 24 h, cells that migrated through the pores were collected and centrifuged, and then living cells were counted using a hemocytometer (Superior, Marienfeld, Germany) after trypan blue staining.

Cell invasion/infiltration assay

For invasion/infiltration assay, the upper surface membranes of transwell inserts (Corning Inc.) were coated with 200 μl matrigel plus cold serum-free DMEM allowed to solidify overnight at 37 ˚C. HMO6 cells were seeded onto the upper chambers. The lower chamber contained either necrotic cell lysates or the supernatants obtained from necrotic cells-treated or untreated CRT-MG cells. After 24 h incubation, non-invaded cells in the upper chambers were removed with a cotton swab, and invaded cells were fixed in 4% formaldehyde for 10 min and washed with phosphate-buffered saline (PBS). Fixed cells were stained with 0.1% crystal violet for 10 min and then washed with PBS. After being dried, the stained cells were observed using Eclipse TE-300 microscope (Nikon, Tokyo, Japan). For quantification, crystal violet staining was re-solubilized in 95% ethanol and the optical density (O.D.) at 540 nm was measured.

Microarray analysis

Microarray analysis was performed using Illumina Human HT-12 B4 Expression BeadChip (Illumina, Inc., San Diego, USA) according to the manufacturer's protocol at Macrogen (Macrogen, Seoul, Korea). The array detects the expression levels for more than 47, 000 probes derived from the National Center for Biotechnology Information Reference Sequence (NCBI) RefSeu Release 38. Total RNA was extracted from the cells using a RNA extract kit (easy-Blue™, Intron, Gyeonggido, Korea) and 500 ng of total RNA from each sample amplified and labelled using TargetAmp-Nano Labeling Kit for Illumina Expression BeadChip (Epicentre, Madison, USA). 750 ng of labeled cRNA were hybridized to the Human HT-12 V4.0 Expression Beadchip for 17 h at 58°C Arrays were scanned using the Illumina Bead Array Reader Confocal Scanner and data were extracted using GenomeStudio v2011.1 (Gene Expression Module v1.9.0).

Human chemokine array

Chemokines secreted by necrotic cells-treated CRT-MG were screened using commercially available protein array systems (Proteome profiler™ Arrays, #ARY017, R&D Systems) according to the manufacturer's instructions. The array consists of 31 different human chemokines spotted in duplicate onto a membrane. CRT-MG cells were either untreated or treated with necrotic cells for 24 h, and then 1 ml of the supernatant and detection antibody mixture were incubated at room temperature (RT) for 1 h. Then, the prepared mixtures were added on each membrane at 4°C for overnight, and streptavidin-HRP was treated into each

membrane for 30 min at RT on a rocking platform shaker. Membranes were exposed to blue X-ray film (AGFA, Mortsel, Belgium) by using chemiluminescent detection reagent (Amersham, Buckinghamshire, UK) for 3-5 min. The relative level of chemokine expression was measured by the changed spot intensity divided by control spot intensity, using Image J software (NIH, Bethesda, Maryland, USA; http://rsb. info.nih.gov/ij/).

Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR)

Total cellular RNA was extracted from isolated HMO6 cells using easy-BLUE™ (Intron) according to the manufacturer's protocol. cDNA was synthesized from 1 μg of total RNA with reverse transcription and PCR was performed using 3μl cDNA with a thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). Quantitative PCR was performed on an ABI StepOnePlus™ Real-Time PCR machine (Applied Biosystems, Foster City, CA, USA) using a Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. The cDNA abundance of each gene was calculated relative to the expression of a housekeeping gene, *GAPDH* (glyceraldehyde-3-phosphate-dehydrogenase) and presented as the fold-change in abundance compared to the appropriate controls. The primer sequences were as follows: *CXCL8/IL-8*, forward 5′-CTCCAAACCTTTCCACCCC-3′, reverse 5′-GATTCTTGGATACCACAGAGAATG-3′; *CCL2/MCP-1*, forward 5'-CCCCA GTCACCTGCTGTTAT-3', reverse 5'-TGGAATCCTGAACCCACTTC-3'; *CCL20/MIP-3α*, forward 5'-GCAAGCAACTTTGACTGCTG-3', reverse 5'-ATTTGCGCACACACAGACAA CT-3'; *CCR2*, forward 5'-AGAGGCATAGGGCAGTGAGA-3', reverse 5'-GCAGTGAGTC ATCCCAAGAG-3'; *CCR6*, forward 5'-CCATTCTGGGCAGTGAGTCA-3', reverse 5'-AG CAGCATCCCGCAGTTAA-3'; *GAPDH*, forward 5'-GGAGCCAAAAGGGTCATCAT-3', reverse 5'-GTGATGGCATGGACTGTGGT-3'.

Enzyme-linked immunosorbent assay (ELISA)

Chemokine levels were measured in the culture supernatants of CRT-MG plated in 60 mm culture plates at final concentration of 9×10^5 cells. After stabilization, cells were treated with either untreated or treated with necrotic cells for 24 h. Supernatants were obtained and centrifuged at 4°C, 12, 000 rpm (13523*g*) for 20 min and chemokine concentrations were determined by a sandwich ELISA method with human CCL2/MCP-1 and CCL20/MIP-3α (R&D Systems) according to the manufacturer's instructions. The concentration of CCL2/MCP-1 and CCL20/MIP-3α in each sample was determined with a standard curve generated by known amounts of CCL2/MCP-1 and CCL20/MIP-3α.

Electrophoretic mobility shift assays (EMSA)

EMSA was performed with 3 μg of nuclear extracts, as previously described [6]. Briefly, nuclear extracts from CRT-MG treated with or without necrotic cells were incubated with the CCL2/MCP-1 or CCL20/MIP-3α sequences including putative NF-κB or AP-1 binding element, which was end-labeled with [^{32}P] ATP using T4 polynucleotide kinase (New England Biolabs, Inc (NEB), Ipswich, MA, USA). Bound and free DNA were resolved by electrophoresis in 5% native polyacrylamide gels using 0.5X Tris-borate-EDTA (TBE) as running buffer. The gels were dried and then bound DNA was assessed by autography. Competition assays were performed with 100-fold molar excess of unlabeled oligonucleotide was added to the nuclear extracts for 10 min before addition of the labeled probe. For supershift experiments, binding reactions containing nuclear extract were incubated for 30 min at RT with anti-c-Fos, anti-p50 or anti-p65 antibody (1/100 dilution), (Santa Cruz, CA, USA). The oligonucleotide probe sequences and putative NF-κB or AP-1 binding sites are shown in Fig. 4.

Statistical analysis

Mean ± standard error of the mean (SEM) were showed in all bar graphs from at least three independent experiments, and statistical significance was analyzed by ANOVA and student t-test using the SPSS software version 23.0 (SPSS Inc., Chicago, IL, USA). A value of $p<0.05$ was regarded statistically significant.

Results

NCs and NC-treated glioma cells induce migration and infiltration of microglial cells

We first investigated the effect of 1) necrotic CRT-MG cells and 2) CRT-MG cells exposed to the necrotic CRT-MG cells (NC-treated CRT-MG) on the migration and infiltration of HMO6

human microglial cells. Necrotic CRT-MG cells were prepared by freezing/thawing CRT-MG cells for five cycles. Next, different ratios of necrotic CRT-MG cells were either diluted in media (NC-only) or used to treat pre-seeded CRT-MG cells for 24 h. Media were collected from the pre-seeded CRT-MG culture (NC-treated glioblastoma conditioned medium, NC-GCM) and the NC-only group. HMO6 cells were seeded at an equal density (1.5×10^5 cells/well) in the upper chamber, and the collected NC-GCM and NC-only were placed in the lower chambers (Fig. 1a). As shown in Fig. 1b, both NC-GCM and NC-only stimulation increased the migration of HMO6 cells. To further explore the effect of NC-only and NC-GCM on HMO6 cell infiltration, matrigel matrix-coated 24-well microchemotaxis chambers were used in the presence of the NC-GCM or NC-only culture supernatants in the lower chambers. As shown in Fig. 1c, NC-GCM and NC-only increased HMO6 cell infiltration in a ratio-dependent manner. Interestingly, the migration and infiltration activity of HMO6 cells showed a tendency toward a significant increase in NC-GCM compared to NC-only, suggesting that CRT-MG cells exposed to NCs enhance the migration and infiltration of HMO6 microglial cells.

Effect of NCs on gene and chemokine expression in CRT-MG glioma cells

We next performed a microarray to identify the changes in gene expression profiles between CRT-MG cells (Con) and NC-treated CRT-MG cells (NC) (Fig. 2a). For this analysis, CRT-MG cells were either treated with NCs or not treated for 24 h, and purified RNA was prepared for each group. Of 47, 323 genes detected by the array, 46, 580 genes were not significantly influenced by the exposure to NCs. In the treated cells, 439 genes were upregulated, and 304 genes were downregulated (a 2-fold change was used as the cut off). Compared with untreated cells, dysregulated expression levels with 2-fold change in NC-treated CRT-MG cells were tabulated (Fig. 2b). CCL20/MIP-3α, CXCL8/IL-8, CXCL2/MIP-2α, CXC motif chemokine ligand 5/Epithelial cell-derived Neutrophil-activating peptide 78 (CXCL5/ENA-78), CCL2/MCP-1, and CX3CL1/fractalkine were significantly increased in NC-treated CRT-MG cells. We also utilized reverse transcription–polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qRT-PCR) for direct validation of differential expression of mRNA in necrotic cell treated (NC) and untreated CRT-MG cells (Con) (Fig. 2c). To examine whether NCs induced chemokine secretion in CRT-MG cells, we performed a chemokine array with the culture media from CRT-MG that were either treated with NCs for 24 h or untreated. The chemokine array showed that the secretion of several chemokines was significantly increased in NC-treated CRT-MG cells compared to that in the control (Fig. 2d, solid box). These results clearly show that NCs induce the expression and secretion of several inflammatory chemokines such as CCL2/MCP-1 and CCL20/MIP-3α in CRT-MG glioma cells.

NCs induce protein and mRNA expression of CCL2/MCP-1 and CCL20/MIP-3α in CRT-MG glioma cells

Since we observed that the fold intensity changes for spots corresponding to CCL2/MCP-1 and CCL20/MIP-3α were increased over 100-fold by exposure to necrotic cells (Fig. 2d), we next performed ELISA to detect and quantify CCL2/MCP-1 and CCL20/MIP-3α levels secreted from NC-treated CRT-MG cells. MCP-1 and MIP-3α secretion was increased in both CRT-MG cells treated with NC and NC-only in a ratio-dependent manner; however, the degree of secretion of MCP-1 and MIP-3α was higher in CRT-MG cells treated with NC than in NC-only. (Figs. 3b and 3c). Similar to protein expression, expression of CCL2/MCP-1 and CCL20/MIP-3α mRNA was also increased in NC-treated CRT-MG cells (Figs. 3d and 3e). Similar effects of necrotic cells on CCL2/MCP-1 and CCL20/MIP-3α induction were also observed in U87-MG cells (Figs. 3f and 3g). These results demonstrate that NCs directly induce expression of CCL2/MCP-1 and CCL20/MIP-3α in glioma cells.

NCs induce NF-κB and AP-1 DNA binding to the CCL2/MCP-1 and CCL20/MIP-3α promoters in CRT-MG glioma cells

Recently we reported that NCs induce the activation of AP-1 and NF-κB signaling pathways, leading to enhanced DNA binding to the IL-8 promoter [6]. To investigate the

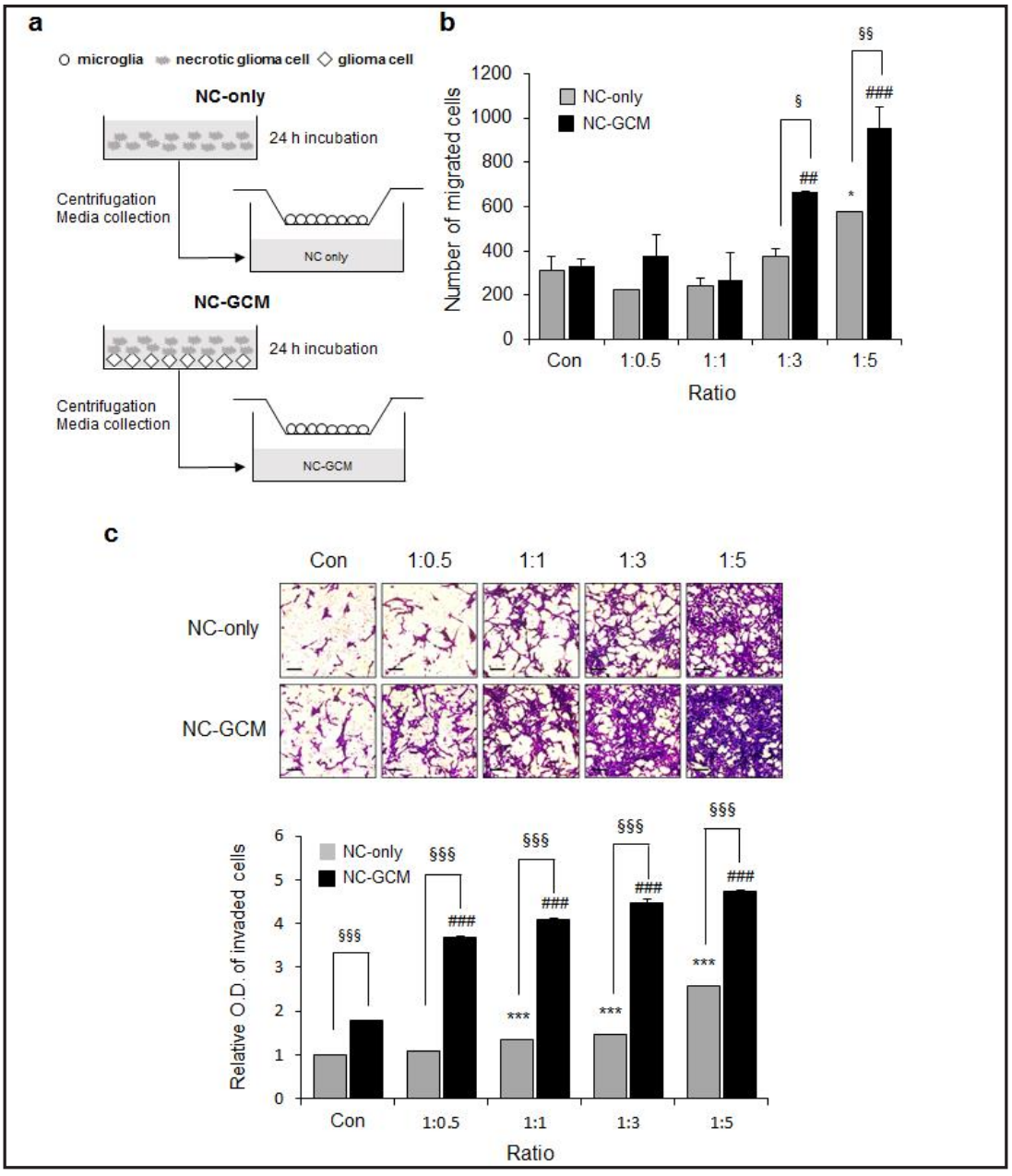

Fig. 1. Necrotic cell (NC)-treated glioma cells induce migration and infiltration of microglia. (a) Illustration of the transwell migration assay. CRT-MG cells treated with different ratios of necrotic cell for 24 h as indicated, and each NC-treated glioblastoma conditioned medium (NC-GCM) and necrotic cells conditioned medium (NC-only) were collected and centrifugation. HMO6 cells were seeded at equal density (1.5×10^5cells/well) in the upper chamber, and the collected NC-GCM and NC-only were placed in the lower chambers. The ratio of NC treatment is shown as CRT-MG cell number: necrotic CRT-MG cell number. (b) Migration of HMO6 cell was determined by counting cells that migrated through the membrane of transwell inserts. Data shown are representative of at least three experiments. *P<0.05, ##P<0.01, ###P<0.001 vs. each control, §P<0.05, §§P<0.01 vs NC-only. (c) Infiltration of HMO6 cell was assayed with a matrigel matrix-coated cell culture insert. Representative images of crystal violet staining of the bottom surface of cell culture insert are shown (upper panel, scale bar = 100 μm). Cell infiltration was measured by OD quantification by reading the eluted crystal violet (bottom panel). The extent of infiltration in the NC-only control was set 1 and data presented as the fold induction compared with NC-only control cells. Data shown are representative of at least three experiments. ***P<0.001, ###P<0.001 vs. each control (Con), §§§P<0.01 vs. NC-only.

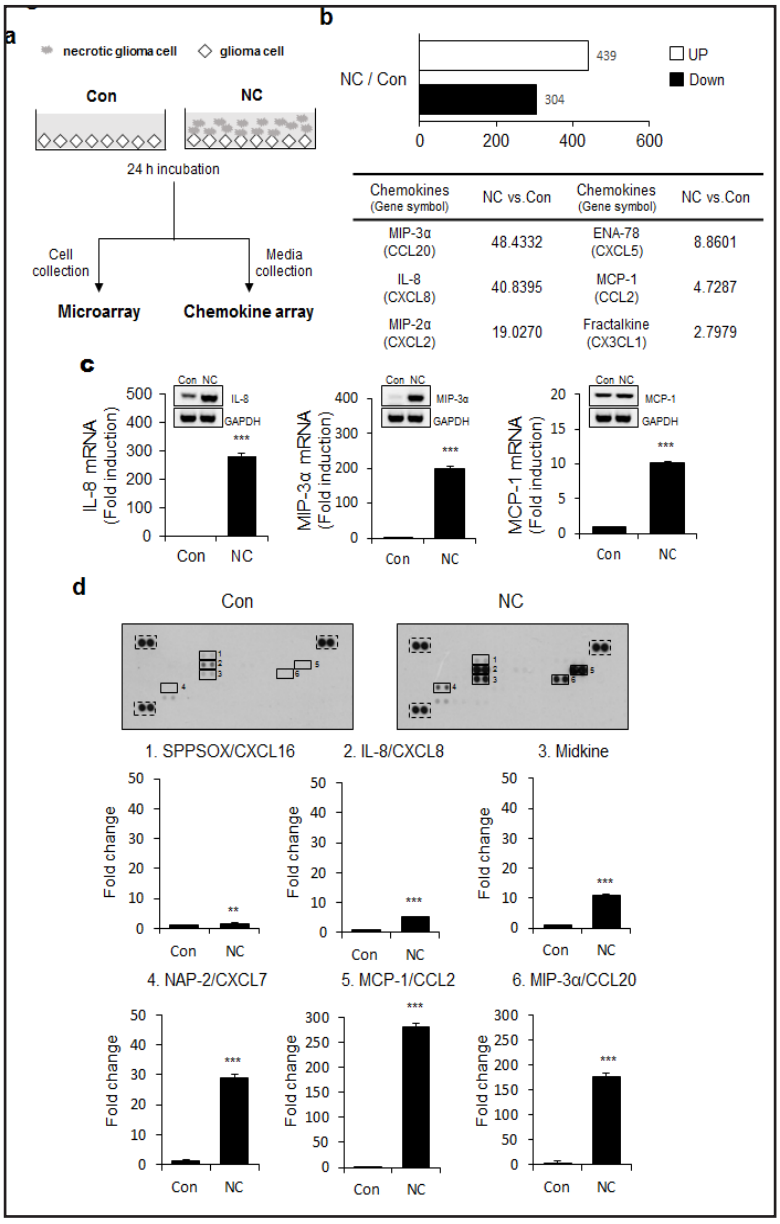

Fig. 2. Effect of necrotic cells on chemokine expression in CRT-MG glioma cells. (a) CRT-MG cells treated with necrotic cell and untreated cells for 24 h as indicated. After incubation, cells and supernatants from each condition were collected for microarray and chemokine array respectively. Con, control; NC, necrotic cells. (b) CRT-MG cells were incubated in the absence or presence of NCs for 24 h, and total RNA was extracted and subjected to microarray analysis. The proportion of genes that are up- or downregulated (2-fold change) between untreated and NC-treated cells is shown. Tabulation of upregulated chemokine genes demonstrating higher fold induction in NC-treated CRT-MG cells compared with that in untreated CRT-MG cells. (c) The expression level of three upregulated mRNA (CCL2/MCP-1, CCL20/MIP-3α and CXCL8/IL-8) were validated by RT-PCR and qRT-PCR. GAPDH gene was evaluated as external control. The expression of each mRNA in the untreated control was set as 1. Data shown are representative of at least three experiments. ***P<0.001 vs. Con (untreated control). (d) Culture supernatants of CRT-MG cells treated or untreated with NCs for 24 h were analyzed using a chemokine array. A solid box indicates the duplicate spots corresponding to six chemokines, and reference spots are indicated by a dashed line box (left). The intensity of spots corresponding to each chemokine and positive control was quantified using Image J software and subtracted from the background, then expressed as a ratio to the positive control (right). The value of each chemokine in the untreated control was set as 1. Data are representative of at least 3 experiments. **P<0.01, ***P<0.001 vs. Con (untreated control).

contribution of AP-1 and NF-κB to NC-induced CCL2/MCP-1 and CCL20/MIP-3α expression, we next performed an electrophoretic mobility shift assay (EMSA). We found putative NF-κB and AP-1 transcription factor-binding sites in the CCL2/MCP-1 and CCL20/MIP-3α promoters (Figs. 4a and 4b). Nuclear extracts (NEs) from untreated or different ratios of NCs-treated CRT-MG cells were analyzed with probes for putative binding sites within the CCL2/MCP-1 or CCL20/MIP-3α promoters (Figs. 4c and 4d). Utilizing a DNA probe containing the specific NF-κB binding site ② of the CCL2/MCP-1 promoter, binding was increased

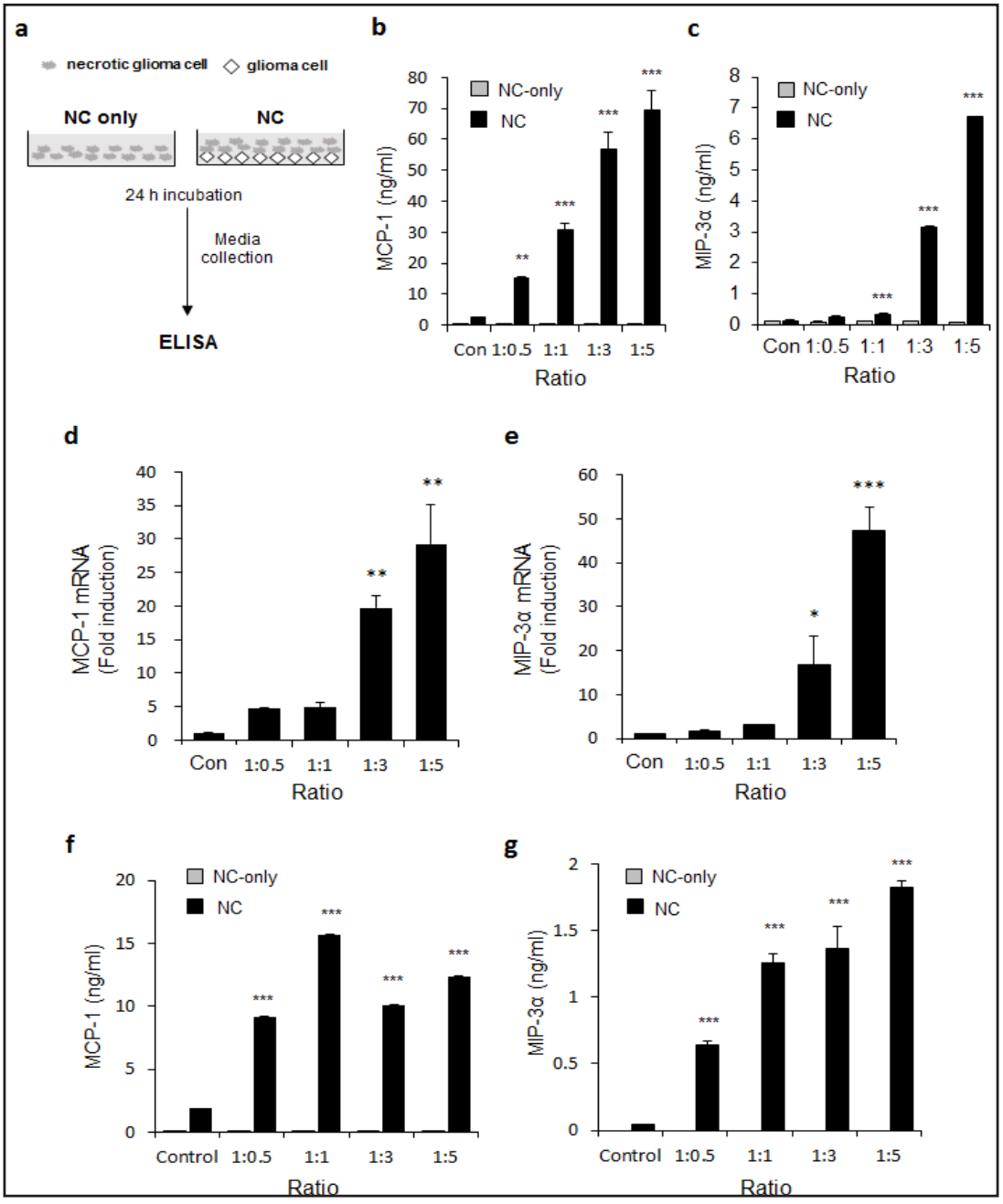

Fig. 3. Necrotic cells induce mRNA and protein expression of CCL2/MCP-1 and CCL20/MIP-3α in glioma cells. (a) CRT-MG cells treated with different ratios of necrotic cells (NC) for 24 h as indicated. After incubation, supernatants from each condition and NC-diluted medium (NC-only) were collected, and secretory protein levels were measured by ELISA. (b, c) The protein levels from the culture supernatants were analyzed via ELISA for CCL2/MCP-1 (b) and CCL20/MIP-3α (c). Data shown are representative of at least three experiments. **P<0.01, ***P<0.001 vs. Con (untreated control). (d, e) CRT-MG cells treated with different ratios of NCs for 24 h as indicated. After incubation, cells were collected, and total RNA was isolated using an RNA extraction kit. CCL2/MCP-1 and CCL20/MIP-3α mRNA expression was analyzed by quantitative real-time PCR (qRT-PCR). (f, g) U87-MG cells treated with different ratios of necrotic cells (NC) for 24 h. After incubation, supernatants from each condition and NC-diluted medium (NC-only) were collected and analyzed via ELISA for CCL2/MCP-1 (f) and CCL20/MIP-3α (g). Data were presented as fold induction compared with untreated control cells. Data shown are representative of at least three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. Con (untreated control).

in NC-treated CRT-MG cells in a ratio-dependent manner. In the CCL20/MIP-3α promoter, DNA probes containing the specific NF-κB binding site ② and AP-1 binding site ② showed enhanced binding to NE from NC-treated CRT-MG. Competition using a 100-molar excess unlabeled oligonucleotide abrogated complex formation; in addition, antibodies for p50 or c-fos supershifted and weakened the complex, confirming the specific interaction of NF-κB and AP-1 in both CCL2/MCP-1 and CCL20/MIP-3α promoters (Figs. 4e and 4f). These results indicate that NCs induce the activation of NF-κB and AP-1 transcription factors, leading to increased DNA binding activity to the CCL2/MCP-1 and CCL20/MIP-3α promoters.

Inhibition of NF-κB and AP-1 reduces CCL2/MCP-1 and CCL20/MIP-3α secretion in NC-treated CRT-MG cells

Since we observed that NF-κB and AP-1 were involved in regulation of CCL2/MCP-1 and CCL20/MIP-3α genes in response to NC exposure (Fig. 4), we next examined the effect of inhibition of NF-κB, c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK) on NC-induced CCL2/MCP-1 and CCL20/MIP-3α expression in glioma cells, using specific inhibitors for NF-κB: BAY 11-7082, JNK: SP600125, p38: SB203580 and ERK: PD98059. CRT-MG cells were pretreated with pharmacological inhibitors for 30 min and then exposed to NCs for 24 h. Both CCL2/MCP-1 and CCL20/MIP-3α expression/secretion in NC-treated CRT-MG cells were most strongly suppressed by NF- κB inhibitor BAY 11-7082 (Figs. 5a and 5b). These results showed that NF-κB was to be major transcription factor that regulated CCL2/MCP-1 and CCL20/MIP-3α expression. When CRT-MG cells were

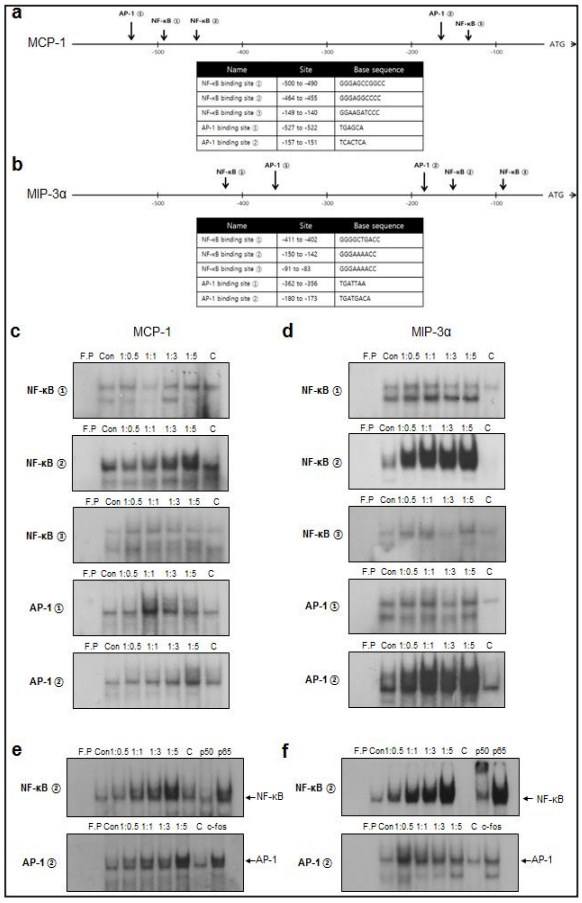

Fig. 4. Necrotic cells increase NF-κB and AP-1 DNA binding to CCL2/MCP-1 and CCL20/MIP-3α promoters in CRT-MG glioma cells. Schematic representations of the human CCL2/MCP-1 (a) and CCL20/MIP-3α (b) promoters. The transcription start site and putative binding sites for NF-κB and AP-1 are indicated by an arrow (upper), and details for each transcription factor are displayed in the table (lower). (c, d) The nuclear extracts (NEs) from CRT-MG cells treated with or without NCs were incubated with a radiolabeled DNA probe for the human CCL2/MCP-1 (c) and CCL20/MIP-3α (d) promoters and subjected to an electromobility shift assay (EMSA). The competition assay was performed by adding a 100-fold molar excess of cold probe. Data shown are representative of at least three experiments. (e,f) EMSA was performed using the labeled probes NF-κB ② and AP-1 ② for the CCL2/MCP-1 (e) or NF-κB ② and AP-1 ② for the CCL20/MIP-3α (f) promoter. Supershift assays were performed with specific antibodies against NF-κB subunits p50 and p65 or the AP-1 subunit c-fos. Anti-p50, anti-p65, or anti-c-fos antibodies were added to test the specificity of interaction. Data shown are representative of at least three experiments. FP, free probe; C, competition.

incubated with 5×10^8 particles/ml mCherry:EXPLORs or srIκB:EXPLORs, srIκB-loaded exosome as a specific inhibitor for NF-κB, for 16 h and either treated with NCs or untreated for an additional 24 h, we observed that treatment with srIκB:EXPLORs significantly reduced both CCL2/MCP-1 and CCL20/MIP-3α expression in NC-treated CRT-MG cells (Figs. 5c and 5d). These results collectively indicate that blocking NF-κB signaling pathways effectively reduces CCL2/MCP-1 and CCL20/MIP-3α expression/secretion in CRT-MG glioma cells stimulated by NCs.

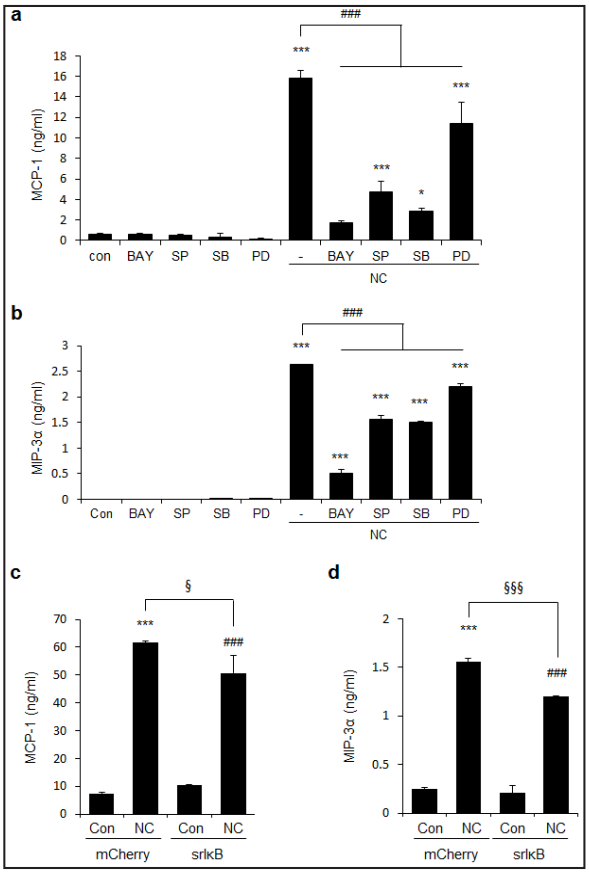

Fig. 5. Inhibition of NF-κB and AP-1 reduces CCL2/MCP-1 and CCL20/MIP-3α expression/secretion in CRT-MG cells. (a, b) CRT-MG cells were pretreated with NF-κB inhibitor (BAY 11-7082 [BAY]), a JNK inhibitor (SP600125 [SP]), a p38 inhibitor (SB203580 [SB]), or MAPK/ERK kinase inhibitor (PD98059 [PD]) 5μM for 30 min and then further incubated with necrotic cells (NC) for 24 h. Indicated supernatants from each condition were collected, and then CCL2/MCP-1 (a) or CCL20/MIP-3α (b) protein levels were measured by ELISA. Absorbance was measured at 450 nm. Data shown represent three independent experiments. ***P<0.001 vs. Con, ###P<0.001 vs. NC. Con, untreated control; NC, necrotic cells; BAY, BAY11-7082; SP, SB600125; SB, SB203580; PD, PD98059. (c,d) CRT-MG cells were incubated with 5×10^8 particles/ml mCherry:EXPLORs or srIκB:EXPLORs for 16 h, and then treated with NCs for an additional 24 h. Indicated supernatants from each condition were collected, and CCL2/MCP-1 (c) or CCL20/MIP-3α (d) protein levels were measured by ELISA. Data shown represent three independent experiments. ***P<0.001 vs. mCherry Con, ###P<0.001 vs. srIκB Con, §P<0.05, §§§P<0.05 vs. mCherry NC. Con, untreated control; NC, necrotic cells.

CCL2/MCP-1 and CCL20/MIP-3α induce migration and infiltration of microglial cells

CCL2/MCP-1 and CCL20/MIP-3α are generally considered important chemoattractants for monocytes [28]. Because NC-GCM induced both migration and infiltration of microglial cells (Fig. 1), and NC directly induced CCL2/MCP-1 and CCL20/MIP-3α secretion in glioma cells, we asked whether CCL2/MCP-1 and CCL20/MIP-3α secreted from NC-treated glioma cells directly mediated the migration and infiltratin of HMO6 microglial cells. First, we investigated whether the receptors for CCL2/MCP-1 (CCR2) and CCL20/MIP-3α (CCR6) are present in HMO6 microglial cells and whether NC-only and NC-GCM affect the expression of CCR2 and CCR6 on microglia. HMO6 cells were 1) not treated or incubated with 2) glioblastoma conditioned media (GCM), 3) NC-only, or 4) NC-GCM for 24 h (Fig. 6a), and total RNA was extracted and analyzed by RT-PCR. CCL2/MCP-1 receptor *CCR2* and CCL20/MIP-3α receptor *CCR6* mRNA was expressed in HMO6 cells (Fig. 6b), similar to previous reports [33]. Previously, we showed that NCs induced the secretion of CXCL8/IL-8, which influenced the migration and invasion of glioblastoma cells. To test whether CCL2/MCP-1, CCL20/MIP-3α, and CXCL8/IL-8 have effects on microglia migration/infiltration, HMO6 cells were seeded at equal density (1.5×10^5 cells/well) in the upper chamber, and control medium supplemented with 1 μg/ml of recombinant CCL2/MCP-1,

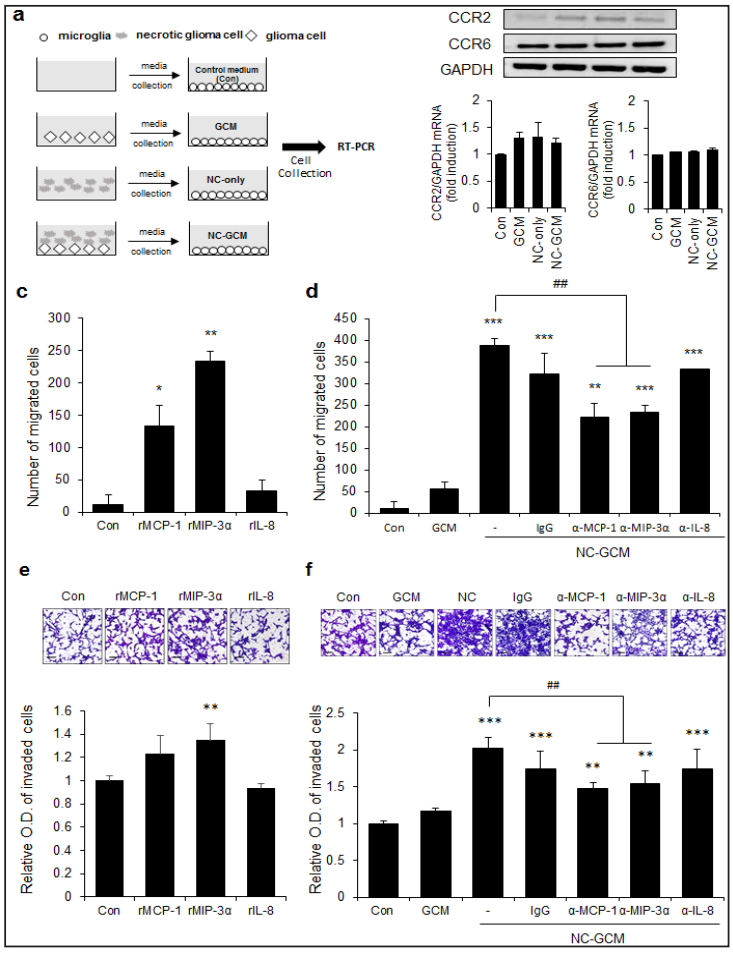

Fig. 6. Neutralizing antibodies to CCL2/MCP-1 and CCL20/MIP-3α block microglia infiltration-promoting effects of necrotic cell-treated glioblastoma conditioned medium. (a) Control medium (Con), glioblastoma conditioned medium (GCM), NC only, and NC-GCM were harvested from cultured supernatants for 24h and treated to microglia cultures for 24 h. (b) HMO6 microglia cells were either not treated or treated with GCM, NC-only, or NC-GCM for 24 h, and total RNA was extracted and analyzed by RT-PCR for CCR2 and CCR6 mRNA. (c-f) Transwell migration and invasion/infiltration assay showing the migration and infiltration of HMO6 cells toward control medium supplemented with recombinant protein and NC-GCM in the presence or absence neutralizing antibody. (c,d) The migration assay was performed using 1 μg/ml recombinant CCL2/MCP-1, CCL20/MIP-3α, or CXCL8/IL-8 protein (c). CRT-MG cells were either untreated or treated with NC for 24 h and collected. NC-GCM was placed in the lower chamber in the presence or absence of 2 μg/ml anti-CCL2/MCP-1, anti-CCL20/MIP-3α, or anti-CXCL8/IL-8 neutralizing antibody or nonspecific goat-IgG as a control (d). Data are representative of at least 3 experiments. *P<0.05, **P<0.01, ***P<0.001 vs. Con, ##P<0.01 vs. NC-GCM (-). (e, f) Invasion/infiltration of HMO6 cells was analyzed invasion/infiltration assay. Representative images of crystal violet staining of the bottom surface are shown (upper panel, scale bar = 100 μm). Cell infiltration was measured by OD quantification by reading the eluted crystal violet (bottom panel). The extent of infiltration in the control was set 1 and data presented as the fold induction compared with control. Data shown are representative of at least three experiments. *P<0.05, **P<0.01, ***P<0.001 vs. Con, ##P<0.01 vs. NC-GCM. Con, control medium; GCM, glioblastoma conditioned medium; NC, necrotic cells; NC-GCM, NC-treated glioblastoma conditioned medium; r, recombinant.

CCL20/MIP-3α, or CXCL8/IL-8 protein was placed in the lower chamber (Figs. 6c and 6e). In the presence of recombinant CCL2/MCP-1 and CCL20/MIP-3α, HMO6 migration and infiltration were increased, whereas the effect of CXCL8/IL-8 protein was not significant. Also, we investigated the migration and infiltration of HMO6 by incubating the NC-GCM with neutralizing antibodies for CCL2/MCP-1, CCL20/MIP-3α, or CXCL8/IL-8 (2 μg/ml). As shown in Figures 6d and 6f, anti-CCL2/MCP-1 and anti-CCL20/MIP-3α, but not anti-CXCL8/IL-8 and control non-specific IgG, significantly reduced the migration and infiltration of HMO6 cells. These results collectively indicate that CCL2/MCP-1 and CCL20/MIP-3α, which are secreted from NC-treated glioma cells, directly induce microglial cell migration and infiltration.

Discussion

To the best of our knowledge, this study is the first to report the effect of necrosis on chemokine production and microglia infiltration mediated by GBM cells. We showed that NCs stimulate microglia migration/infiltration into GBM by upregulating CCL2/MCP-1 and CCL20/MIP-3α expression in glioblastoma cells (Fig. 7). Using the human GBM cell line and human microglia cell line, we demonstrated that the presence of NCs promoted the migration/infiltration of microglia, and that GBM cells exposed to NCs further enhanced the migration and infiltration of HMO6 microglial cells. Treatment of NCs induced the mRNA and protein expression of chemokines such as CCL2/MCP-1 and CCL20/MIP-3α in CRT-MG cells. In particular, CCL2/MCP-1 and CCL20/MIP-3α were significantly increased in NC-treated CRT-MG and U87-MG cells. NCs induced DNA binding of the transcription factors NF-κB and AP-1 to the CCL2/MCP-1 and CCL20/MIP-3α promoters, leading to increased CCL2/MCP-1 and CCL20/MIP-3α mRNA and protein expression.

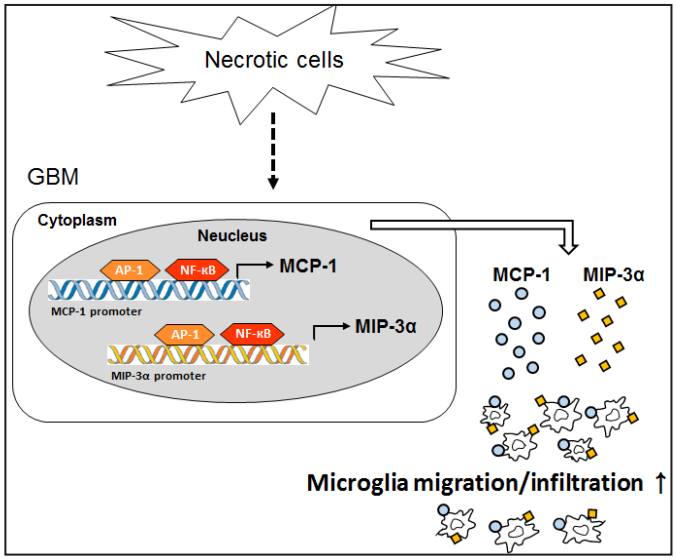

Fig. 7. A proposed model for glioblastoma microenvironment. Exposure to necrotic cells (NC) activates AP-1 and NF-κB transcription factors and their binding to the CCL2/MCP-1 and CCL20/MIP-3α promoters, leading to enhanced CCL2/MCP-1 and CCL20/MIP-3α production and secretion in glioblastoma cells. As a result, an increase in CCL2/MCP-1 and CCL20/MIP-3α secretion from NC-exposed glioblastoma promotes migration/infiltration of microglial cells within glioblastoma.

GBM is characterized by the presence of necrosis, which has been considered a key diagnostic criterion for GBM and is correlated with poorer patient outcome [7]. Several studies have shown that tumor necrosis is associated with a poorer prognosis, advanced malignant progression, and decreased survival rate. In endometrial cancer, tumor necrosis is related to enhanced angiogenesis via increased expression of angiogenic markers such as Vascular Endothelial Growth Factor A (VEGF-A), VEGF-C, VEGF-D, and Fibroglast Growth Factor (βFGF) [34]. In renal cell carcinoma, the extent of necrosis is correlated with higher grade and greater tumor size [35]. During necrosis, cell death leads to the release of cellular contents into the extracellular environment, which in turn promotes cell growth, cancer progression, and tumor-infiltrating leukocyte recruitment. For example, high-mobility group box 1 protein, a proinflammatory mediator that distinguishes necrosis from apoptosis, is known to promote the recruitment of inflammatory cells [36]. Our results demonstrate that NC-diluted medium (NC-only) could increase the migration/infiltration of microglial cells in a NC-number-dependent manner (Fig. 1). However, the degree of migration/infiltration of HMO6 cells was further enhanced when CRT-MG cells were exposed to and stimulated by NCs, suggesting that CRT-MG cells exposed to NCs enhance the migration and infiltration of HMO6 microglial cells. These results indicate that the interaction between necrosis and glioblastoma cells greatly contributes to microglia infiltration within GBM.

In this study, we showed several chemokines produced by NC-treated GBM by performing microarray and chemokine array, suggesting that these chemokines released from cancer cells could promote and facilitate the migration/infiltration of microglia cells. The inflammatory microenvironment of a tumor contains a significant number of host leukocytes, including macrophages, dendritic cells, and lymphocytes [37]. Of these, TAMs are a major component of the infiltrate in tumors, and accumulating evidence demonstrates that increased density

of macrophages is associated with tumor growth and metastasis in human malignancies, including breast cancer, gastric cancer, and GBM [9, 14, 15, 38]. TAMs are known to be recruited by chemokines, which are secreted from tumor tissues. The levels of CCL2/MCP-1, CCL3/MIP-1α, and CCL5/RANTES were higher in non-small cell lung cancer (NSCLC) tumor tissues than in normal lung tissues, correlated with the degree of infiltrated macrophages within tumor tissues [39]. In colorectal cancers, the infiltration of macrophages is positively correlated with the level of CCL2/MCP-1 expression [16]. Previous studies have shown that the chemokines, such as CX3CL1/fractalkine and CCL2/MCP-1, and their receptors play important roles in the infiltration of microglia/macrophages in GBM [17, 28, 29]. Our results demonstrate that both CCL2/MCP-1 and CCL20/MIP-3α increased in NC-treated glioma cells in a NC ratio-dependent manner (Figs. 3b and 3c) and addition of neutralizing CCL2/MCP-1 and CCL20/MIP-3α antibodies to NC-GCM significantly reduced migration/infiltration of microglial cells (Figs. 6d and 6f), indicating that these chemokines contribute to an NC-ratio-dependent increase in the migration/infiltration of microglial cells.

In human glomerular endothelial cells, TNF-α increased CCL2/MCP-1 expression via the cooperative action of NF-κB and AP-1 [40]. Interaction between CCL20 and CCR6 that promotes the invasion and migration of thyroid cancer cells is associated with NF-κB activation [25]. Prolactin induces CCL20/MIP-3α production through AP-1 activation in keratinocytes [41]. We found putative NF-κB and AP-1 transcription factor-binding sites in the CCL2/MCP-1 and CCL20/MIP-3α promoters (Figs. 4a and 4b). Our results also show that CCL2/MCP-1 and CCL20/MIP-3α production in NC-treated GBM is mediated by NF-κB and AP-1 activation. In the current study, it was observed that anti-p50 antibody supershifted or weakened the DNA-protein complex, whereas anti-p65 antibody did not. These results indicate the specific interaction between the p50 subunit and NF-κB element of CCL2/MCP-1 and CCL20/MIP-3α.

In summary, we showed that NCs stimulate chemokine production in GBM cells, leading to significant promotion of microglial infiltration into GBM. Remarkably, although NCs alone influenced the migration/infiltration of microglia cells, glioblastoma cells that were exposed to NCs stimulated enhanced microglia migration/infiltration. Our results suggest that chemokines produced by NC-exposed GBM cells promote and facilitate the migration and infiltration of microglia, thus providing a better understanding of the effect of necrosis on microglia infiltration in GBM.

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Disclosure Statement

The authors declare no conflict of interests.

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