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Mouse Monocyte Chemoattractant Protein 1 (MCP1) Functions as a Monomer

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Abstract

Monocyte chemoattractant protein 1 (MCP1) is an important chemoattractant for microglia. Rodent MCP1 carries a heavily glycosylated C-terminus, which has been predicted to increase local MCP1 concentration, promote MCP1 dimerization/oligomerization, and facilitate receptor engagement. Previous studies have shown that MCP1 mutant lacking the glycosylated C-terminus can't dimerize/oligomerize, but has higher chemotactic potency than the wild-type (full-length) MCP1, suggesting that rodent MCP1 may function as a monomer. Although many groups support this hypothesis, there is no direct evidence on whether rodent MCP1 dimer is functional. In this paper, using forced recombinant dimeric MCP1 proteins we show that the mouse MCP1 dimer is unable to activate Rac1, promote protrusion of lamellipodia, or induce microglial migration, although it can bind to CCR2 and mediate its internalization. These results support the idea that signaling events mediated by MCP1 require the presence of the monomeric form of this chemokine.

INTRODUCTION

Chemokines are small basic proteins that function as chemoattractants for certain types of cells, especially immune cells (Hulkower et al., 1993, Glabinski et al., 1996, Lahrtz et al., 1998). Based on the location of cysteine residues, chemokines are divided into 4 subtypes: CC, CXC, CX₃C, and C chemokines (Rollins, 1997, Yoshie et al., 1997). By binding to their seven-transmembrane G protein coupled receptors these chemokines exert important functions in physiological and pathological conditions. For example, monocyte chemoattractant protein-1 (MCP1, also known as CCL2), a CC-type chemokine, plays critical roles in many different diseases, such as asthma (Lamkhieoued et al., 2000, Tillie-Leblond et al., 2000), rheumatoid arthritis (Koch et al., 1992, Kunkel et al., 1996), and atherosclerosis (Chen et al., 1999, Kowala et al., 2000) (Sheehan et al., 2007). Due to its strong chemotactic potency for microglia (Van Der Voorn et al., 1999), the brain resident immune-competent cells, MCP1 also contributes to the pathogenesis and pathobiology of

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various CNS disorders, including stroke, trauma, infection, excitotoxic injury, and peripheral nerve axotomy (Mahad and Ransohoff, 2003, Dimitrijevic et al., 2006, Frangogiannis et al., 2007, Yan et al., 2007, Capoccia et al., 2008, Kim et al., 2008, Morimoto et al., 2008).

Although MCP1 is highly conserved in the N-terminus, the C-terminus varies significantly among different species (Yao and Tsirka, 2010, 2011). Compared to human MCP1, rodent MCP1 has an extra C-terminal tail, which is heavily glycosylated (Zhang et al., 1996). This highly glycosylated C-terminus can bind to glycosaminoglycans (GAGs) immobilized on cell surface, and thus enhance the local concentration of MCP1, promote its oligomerization, and facilitate MCP1 binding to its receptor CCR2 (Hoogewerf et al., 1997, Kuschert et al., 1999, Lau et al., 2004, Handel et al., 2005). Like many other chemokines, MCP1 can form homodimer and higher order oligomers. Although MCP1-CCR2 has been extensively studied, it has not been conclusively established whether MCP1 functions as a monomer or dimer/oligomer. On one hand, there is evidence suggesting that MCP1 binds to and activates CCR2 as a monomer. Paolini and colleagues (Paolini et al., 1994) have reported that human MCP1 has maximal chemotactic activity at nanomolar concentrations, at which MCP1 exists exclusively as monomer. In addition, the monomeric human MCP1 mutant (P8A) has been demonstrated to bind to and activate its receptor with wild-type potency and efficacy (Paavola et al., 1998). Furthermore, using a covalent MCP1 dimer (T10C), Tan and colleagues showed that human MCP1 dimer could not bind nor activate CCR2 (Tan et al., 2012). Consistent with these reports, using mouse MCP1 mutant lacking the C-terminus (K104Stop-MCP1), which is unable to dimerize/oligomerize (Yao and Tsirka, 2010), we have found that the monomeric K104Stop-MCP1 more efficiently binds to CCR2 on microglial cells, activates Rac1, polarizes and attracts microglia, compared to full length mouse MCP1 (FL-MCP1) (Yao and Tsirka, 2010). Moreover, addition of the heavily glycosylated mouse MCP1 C-terminus to the human MCP1 protein significantly decreases its affinity for CCR2 and reduces its chemotactic potency for microglia (Yao and Tsirka, 2011). These data suggest that human and mouse MCP1 may function as a monomer.

On the other hand, there is also literature support for the idea that human MCP1 works as a homodimer. Zhang and Rollins have shown that the activity of chemically cross-linked human MCP1 dimers in attracting monocytes is identical to that of non-cross-linked MCP1, and that the N-terminal deletion variant (7ND) of human MCP1 is able to inhibit chemotaxis of wild-type MCP1 but not that of cross-linked MCP1 (Zhang and Rollins, 1995, Zhang et al., 1996), suggesting that human MCP1 functions as a dimer. In addition, Proudfoot and colleagues reported that constitutively monomeric human MCP1 failed to recruit leukocytes *in vivo*, indicating that the dimerization of human MCP1 is indispensable for *in vivo* function.

Whether mouse MCP1 dimer can bind to CCR2 and activate intracellular signaling pathways remains elusive. To answer this question, we generated two recombinant forced dimers of mouse MCP1 and investigated their properties in CCR2 binding and intracellular signaling. We found that the mouse MCP1 dimers were able to bind to CCR2 and induce its endocytosis, but failed to activate Rac1/ERK, induce formation of lamellipodia, or provoke microglial migration, suggesting that these mouse MCP1 dimers cannot activate signaling cascades downstream of CCR2 in microglia.

EXPERIMENTAL PROCEDURES

Cell Culture

N9 cells were a generous gift from Dr. S. Barger at University of Arkansas, Fayetteville, AR and Dr. P. Ricciardi-Castagnoli at University of Milano-Bicocca, Milan, Italy. The cells were maintained in modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) at 37°C with 5% CO₂.

Primary microglia were prepared using mixed cortical cultures as described previously (Giulian and Baker, 1986, Yao and Tsirka, 2010). Briefly, P0-P2 brains from wild-type pups were collected, and the meninges and hippocampi were removed under dissection microscope. The cortical tissue was digested with 5× trypsin (0.25% in Hanks balanced saline solution, HBSS) for 15 min at 37°C, followed by mechanical dissociation using 1ml pipette tip. The mixture was filtered through a 70µm nylon mesh and the cell suspensions were plated onto poly-D-lysine-coated tissue culture dishes in Dulbecco's MEM (DMEM) supplemented with 10% FBS. After 3 days of plating, the medium was changed. By day 14, microglia were collected through addition of 15 mM lidocaine for 10 min at room temperature followed by centrifugation. For migration assay, these microglia were used immediately. For other experiments, microglia were maintained in DMEM with 1% FBS for 2 days.

Generation of recombinant MCP1 Proteins

Recombinant FL- and K104Stop-MCP1 proteins were expressed and purified as described previously (Sheehan et al., 2007). The forced dimeric FL- and K104Stop-MCP1 (FL-FL- and K104Stop-K104Stop-MCP1) were generated by connecting two FL or K104Stop-MCP1 sequences with a three-amino-acid linker (Gly-Thr-Met). Briefly, FL-, K104Stop-, FL-FL-, and K104Stop-K104Stop-MCP1 were subcloned without the signal peptide into the pET vector with an N-terminal 6xHis tag. BL21 cells were transformed with these constructs and 2mM isopropyl-beta-D-thiogalactopyranoside was used to induce the expression of target proteins for 5 hours. Recombinant proteins were purified using cobalt affinity resins (Clontech, Cambridge, UK) according to the manufacturer's instructions, followed by MonoS cation exchanger. The purified proteins were analyzed on 16% Tris-Tricine SDS-PAGE by coomassie blue staining and confirmed by immunoblotting using 1:1000 anti-MCP1 antibody (Serotec and Cell Sciences) or 1:1000 anti-6xHis antibody (Santa Cruz Biotechnology).

Rac Activation Assay

Rac Activation Assay was performed as described previously (Yao and Tsirka, 2010). Specifically, microglia were treated with 10nM MCP-1 proteins. At various time points, the cells were lysed in RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 1mM PMSF, 1× protease inhibitor cocktail, 1× Na₃VO₄). The activated Rac1 was pulled down using the GST-PBD beads (a generous gift from Dr. J. Prives, Stony Brook University, Stony Brook, USA) and detected by immunoblotting using anti-Rac1 antibody (1:1000, Millipore). Total Rac1 was determined by immunoblotting

using total cell lysates. Band intensities were quantified using Scion Image (Scion, Frederick, MD) or Odyssey Infrared Imaging system. The ratio of activated Rac1 intensity to total Rac1 intensity (A/T ratio) was used to indicate Rac1 activation. For FL- and K104Stop-MCP1, Rac1 activation (A/T ratio) was normalized to untreated controls at 0 time point. For FL-FL- and K104Stop-K104Stop-MCP1 Rac1 activation was normalized to their respective positive controls (FL Ctr and K104Stop Ctr), since the dimeric MCP1s failed to activate Rac1. Three independent experiments were performed for quantification.

Western Blot Analysis

Cells were treated with 10nM recombinant MCP1 proteins and lysed in RIPA buffer. DC (Bio-Rad) protein assay was used to determine total protein concentrations. Samples containing equal amounts of total protein were loaded and resolved in 10% or 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P transfer membranes (Millipore). After blocking, the membranes were incubated with anti-CCR2 (1:1000, Epitomics), anti- α -Tubulin (1:1000, Sigma), antiphosphorylated ERK (1:1000, Cell Signaling), and anti-ERK (1:1000, Cell Signaling) antibodies, followed by appropriate secondary antibodies. The bands were visualized and their intensity was quantified using the Odyssey infrared imaging system. The ratio of CCR2 intensity to α -tubulin intensity was used to indicate CCR2 expression level, and this ratio was normalized to untreated controls at the 0 time point. Similarly, the ratio of phosphorylated ERK (p-ERK) intensity to total ERK (t-ERK) intensity was used to indicate activation of ERK signaling pathway, and this ratio was normalized to untreated controls at 0 time point. Three independent experiments were performed for quantification.

Membrane Sheet Assay

This assay was performed as previously described (Robinson et al., 1992, Yao and Tsirka, 2010). Briefly, primary microglia were plated on coverslips and activated with 100ng/ml LPS overnight. These activated microglia were further treated with 10nM recombinant MCP1 for 1 hour at 37°C. Next, the cells were swelled in hypotonic buffer (25mM KCl, 10mM Hepes, 2mM MgCl₂, 1mM EGTA, 1mM PMSF, 1 $\times$ protease inhibitor cocktail, pH 7.5) for 20 min. After extensive wash, the attached cell membrane was fixed with 4% paraformaldehyde. Mac2 antibody (1:1000, BD Pharmingen) was used to label attached microglial membrane, and CCR2 antibody (1:500, Epitomics) was used to reveal CCR2 expression on microglial plasma membrane. Alexa-488 and Alexa-555 conjugated goat anti-rat and goat anti-rabbit antibodies (Invitrogen) were used to visualize Mac2 and CCR2 expression, respectively. Images were taken using Zeiss LSM510 confocal microscope. The fluorescent intensity of CCR2 and Mac2 was measured using the ImageJ software, and the relative CCR2 expression level on the membrane was quantified by normalizing the intensity of CCR2 to that of Mac2. Twenty fields from at least 3 independent experiments were used for quantification.

F-actin Cytoskeleton Imaging

This assay was performed as previously described (Yao and Tsirka, 2010). Briefly, heparin, which avidly binds MCP1, was spotted at the edge of 12-well plates, and recombinant MCP1 proteins were added directly onto the dried heparin spot to create an MCP1 point

source. Primary microglia attached to coverslips were then placed in the well. At different time points, the cells were washed and fixed with 4% paraformaldehyde. Next, the cells were stained with 1:20 Alexa-647 phalloidin (Invitrogen) overnight at room temperature. The formation of lamellipodia was examined under Zeiss LSM510 confocal microscope. The number of polarized and total cells per field was counted, and the percentage of polarized cells per field was quantified. Twenty fields from 3 independent experiments were used for quantification.

Migration Assay

This assay was performed as previously described (Yao and Tsirka, 2010). Briefly, recombinant MCP1 proteins suspended in MEM were added to the bottom wells of the chemotactic chambers (Boyden; NeuroProbe). Wild-type primary microglia suspended in MEM (5×10^5 /ml) were added to the top wells. A 5.0 μ m filter was inserted between the chemoattractants and microglial cells. The chamber was incubated at 37°C for 2 h. Cells that did not migrate into the membrane were wiped off, and cells that migrated into or through the membrane were fixed in -20°C methanol and stained in hematoxylin for 10 minutes. Images were taken at 100 $\times$ magnification. Cell migration responding to MEM only was used as a control. Total number of migrated cells per field was counted. For quantification, 6 random fields per experiment and 3 independent experiments were used. Additionally, the number of migrated cells in response to recombinant MCP1 proteins was normalized to that induced by MEM media (control).

Statistics

Statistics were performed using the two-tailed t test when groups of two were compared. In groups of 3 and more, one-way ANOVA was used. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Error bars indicate the SD.

RESULTS

MCP1 dimers reduce membrane-bound CCR2

To investigate how dimeric MCP1 proteins affect CCR2 expression in microglia, we first examined total cellular CCR2 level after MCP1 stimulation. Immunoblots revealed the presence of comparable levels of CCR2 over time after full length MCP1 (FL-MCP1) or active mutant lacking C-terminus (K104Stop-MCP1) treatment (Fig. 1A and B). Consistent with our previous findings (Yao and Tsirka, 2010), quantitative data showed that FL- and K104Stop-MCP1 did not affect the levels of total cellular CCR2 (Fig. 1E). Although full length dimeric MCP1 (FL-FL-MCP1) seemed to enhance CCR2 level after 30 minutes of treatment (Fig. 1C), this change was not statistically significant (Fig. 1E). Similarly, C-terminus truncated dimeric mutant (K104Stop-K104Stop-MCP1) did not alter total cellular CCR2 level in microglia over a period of 2 hours (Fig. 1D and E). These data suggest that the dimer MCP1-induced CCR2 degradation does not occur within 2 hours of MCP1 treatment.

MCP1 binds to CCR2 and induces its endocytosis, affecting CCR2 intracellular distribution (Jung et al., 2009, Yao and Tsirka, 2010). To further investigate whether the dimeric MCP1

proteins can bind to CCR2 and affect its intracellular distribution, we performed a membrane sheet assay. Mac2, a surface marker for microglia, was used to label plasma membrane in this assay. CCR2 expression (red) showed a punctate pattern on Mac2-positive (green) microglial plasma membrane in saline treated cells (Ctr, Fig. 2A). Consistent with our previous data (Yao and Tsirka, 2010), FL- and K104Stop-MCP1 significantly decreased CCR2 expression on the plasma membrane (Fig. 2B and C), presumably through ligand-induced receptor endocytosis. Like the monomeric MCP1s, FL-FL- and K104Stop-K104Stop-MCP1 reduced membrane-bound CCR2 on microglia (Fig. 2D–E). Secondary antibody only control showed no positive signals, suggesting the specificity of the staining (Fig. 2F). Quantification of fluorescence intensity revealed that FL-, K104Stop-, FL-FL-, or K104Stop-K104Stop-MCP1 significantly lowered membranebound CCR2 in microglia, compared to saline (Fig. 2G). Together, these data suggest that dimerization of MCP1 does not affect its receptor engagement.

Dimeric MCP1s have no chemotactic activity

Since dimeric MCP1 can interact with CCR2, we next asked whether these dimers are functional in inducing microglial migration. A chemotaxis Boyden chamber assay was used to examine the chemotactic activity of these dimeric MCP1s. As shown in Fig. 3A and 3F, the control (MEM medium only) attracted about 240 microglia across the porous membrane over the 2 hour timecourse. FL-MCP1, as expected, significantly increased the number of migrated microglia to approximately 390 (Fig. 3B and 3F). Consistent with our previous report that K104Stop-MCP1 has a higher chemotactic potency (Yao and Tsirka, 2010), more than 700 microglia migrated in response to K104Stop-MCP1 (Fig. 3C and F). The dimeric MCP1 proteins, on the contrary, induced microglial migration to a level comparable to the control (Fig. 3D–F). The quantifications of migrated microglia in absolute number (Fig. 3F) and fold change relative to the control (Fig. 3G) suggest that MCP1 dimers are unable to induce microglial migration, even though they can bind to the CCR2 receptor.

Dimeric MCP1s fail to activate Rac1

Rac1 is an important signaling molecule downstream of CCR2 in microglia (Maghazachi, 2000, Terashima et al., 2005, Yao and Tsirka, 2010). Upon activation of CCR2 by MCP1, Rac1 binds to GTP, which promotes the protrusion of lamellipodia and thus cell migration (Rickert et al., 2000, Ridley et al., 2003, Terashima et al., 2005). We investigated the activation of Rac1 in N9 microglial cells over 90 minutes after MCP1 treatment. Consistent with our previous report (Yao and Tsirka, 2010), FL-MCP1 treatment led to a long-lasting activation of Rac1 (Fig. 4A), whereas K104Stop-MCP1 treatment resulted in a rapid and transient activation of Rac1 (Fig. 4B). Quantification revealed that FL-MCP1 induced a moderate (2–3 fold) but significant activation of Rac1 starting at 15 minutes, which lasted until 90 minutes after treatment (Fig. 4E). K104Stop-MCP1, however, provoked a dramatic (about 12 fold) activation of Rac1 at 5 minutes, which dissipated at 15 minutes after treatment (Fig. 4E). The dimeric MCP1, however, failed to activate Rac1 over 90 minutes (Fig. 4C and 4D). Cells treated with FL-MCP1 for 15 minutes or K104Stop-MCP1 for 5 minutes were used as positive controls. Quantification (normalized to positive controls) showed none or negligible levels of Rac1 activation in microglial cells after FL-FL- or

K104Stop-K104Stop-MCP1 treatment (Fig. 4F). These data suggest that MCP1 dimers lose the ability to activate Rac1 in microglia.

Dimeric MCP1s fail to promote the formation of lamellipodia

To further investigate the effect of dimeric MCP1 in microglial migration, we examined the formation of lamellipodia, a cellular event downstream of Rac1 activation (Maghazachi, 2000, Pankov et al., 2005, Terashima et al., 2005). Exposure of primary microglia to a point source of FL-MCP1 and K104Stop-MCP1 significantly promoted the protrusion of lamellipodia (formation of cellular polarity, arrows in Fig. 5A). A high-magnification image of lamellipodia is shown in the insert in Fig. 5A. Quantitative data revealed that FL-MCP1 significantly elevated the percentage of polarized cells from 4–6% to 18–24% (Fig. 5B). K104Stop-MCP1, however, dramatically increased the percentage of polarized cells to 42–60% (Fig. 5B), indicating a higher chemotactic potency. The dimeric MCP1 proteins, on the contrary, failed to induce the formation of lamellipodia in microglia (Fig. 5A). Quantification of polarized cells demonstrated no significant difference between dimeric MCP1 and control treated microglia over time (Fig. 5B), suggesting that MCP1 dimers are unable to induce lamellipodia protrusions in microglia.

Dimeric MCP1s fail to activate MAPK cascade

Mitogen-activated protein kinase (MAPK) signaling pathway is a critical cascade that regulates many cellular processes including proliferation, differentiation and apoptosis (Jimenez-Sainz et al., 2003). We have shown in our previous studies that MCP1 monomers slightly activate extracellular signal-regulated Kinases 1 and 2 (ERK1/2) (Yao and Tsirka, 2010). Consistently, we detected a low level phosphorylation (activation) of ERK1/2 after FL- or K104Stop-MCP1 treatment (Fig. 6A and B). Quantification revealed significant differences at 15 minutes after FL-MCP1 and 5 minutes after K104Stop-MCP1 treatment (Fig. 6E). The dimeric MCP1, however, failed to induce activation of ERK1/2 in a time course of 2 hours (Fig. 6C and 6D). Interestingly, the level of activated ERK1/2 was significantly decreased 2 hours after K104Stop-K104Stop-MCP1 treatment (Fig. 6D and 6E), indicating an inhibitory effect of this dimeric MCP1 mutant in ERK activation. Altogether, these data suggest that the dimeric MCP1 proteins are unable to activate the MAPK signaling pathway in microglia.

DISCUSSION

Our results demonstrate that mouse MCP1 dimers with or without the heavily glycosylated C-terminus can induce CCR2 internalization, suggesting that they retain the ability to bind to CCR2. This is consistent with previous reports from both our lab and others that the MCP1 N-terminus is responsible for CCR2 engagement (Hemmerich et al., 1999, Yao and Tsirka, 2010). This result, however, is in disagreement with a recent study showing that covalent human MCP1 dimer (T10C) is unable to bind to CCR2 (Tan et al., 2012). This discrepancy may be due to different experimental setup. First the cells used were different: we used primary microglia that express CCR2 under normal conditions in our experiments, whereas inducible FlpIn TRex 293 cells over-expressing FLAG-CCR2 were used in the Tan et al. study. Next, the recombinant protein that we used was mouse MCP1, which contains

an extra heavily glycosylated C-terminus compared to the human MCP1 protein, which was used in the T10C MCP1 study. Our results showed that K104Stop-K104Stop-MCP1, a mouse MCP1 dimeric mutant that is highly homologous to human MCP1 dimer, was able to bind to CCR2 on microglia, suggesting that human and mouse MCP1 may use different mechanisms for CCR2 engagement.

Unlike monomers, mouse MCP1 dimers are unable to activate downstream signaling pathways (Rac1 activation and ERK phosphorylation) or induce microglial migration, although they can bind to CCR2. This finding is in agreement with the report by Tan and colleagues, which indicated that the T10C dimeric human MCP1 is unable to activate CCR2 (Tan et al., 2012), suggesting that dimerization of MCP1, independent of species, results in loss of the ability to activate CCR2. Altogether, these data support the idea that binding to CCR2 and activating CCR2 are two independent events. MCP1 N-terminus is sufficient for CCR2 binding, either in a monomeric or dimer form. MCP1 dimerization, however, potentially changes the conformation thus abrogating the ability of the dimer to activate CCR2. Therefore, we hypothesize that the second MCP1 sequence (FL or K104Stop) interferes with the accessibility of the CCR2-activating motif to CCR2, but does not affect the CCR2-binding motif, which has been localized to the N-terminus (Hemmerich et al., 1999). The CCR2-activating motif, however, remains elusive. Identifying and understanding the role of CCR2-activating motif will enrich our knowledge on MCP1 biology and provide important clues for the treatment of MCP1-related diseases.

It is worth noting that K104Stop-K104Stop-MCP1, but not FL-FL-MCP1, slowly inhibited ERK1/2 phosphorylation starting at 30 minutes after treatment and decreased ERK activation by more than 50% at 120 minutes, suggesting that the K104Stop-K104Stop-MCP1 has an ERK-inhibitory function. This effect of K104Stop-K104Stop-MCP1 is not likely due to its higher affinity for CCR2, given that no significant difference was observed in the membrane sheet assay between FL-FL- and K104Stop-K104Stop-MCP1 (Fig. 2G). Further study is needed to elucidate whether K104Stop-K104Stop-MCP1 has such an inhibitory role.

There is supporting evidence that MCP1 functions as a dimer, but not as a monomer. Zhang and Rollins showed that chemically cross-linked human MCP1 dimers were able to attract monocytes *in vitro*, and a dominant-negative mutant human MCP1 inhibited wildtype MCP1 activity but did not affect that of cross-linked MCP1 (Zhang and Rollins, 1995). This discrepancy could be explained by the use of different cross-linking reagent. Disuccinimidyl suberate, the cross-linking reagent used in this study, has a fixed spacer arm length of 11.4 Å. It may generate an abnormal MCP1 dimer, which behaves different from the MCP1 dimer formed in physiological conditions.

Proudfoot and colleagues reported that constitutively monomeric human MCP1 failed to recruit leukocytes *in vivo* after intraperitoneal injection (Proudfoot et al., 2003), suggesting that dimerization/oligomerization is necessary for MCP1's function *in vivo*. Altogether, we propose a model for MCP1-CCR2 interaction (Fig. 7). In rodents (Fig. 7A), astrocytes and neurons quickly secrete MCP1 at the injury site. By binding to cell surface GAGs via the heavily glycosylated C-terminal tail, MCP1 increases its local concentration and forms

dimers/oligomers. A concentration gradient is formed to attract microglia and/or monocytes. Locally released plasmin cleaves MCP1 at K104 (Sheehan et al., 2007, Yao and Tsirka, 2010), releasing the monomeric N-terminal fragment (K104Stop-MCP1), which binds to and activates CCR2 on microglia/monocytes, leading to migration of these cells towards the injury site. Although the dimeric MCP1 can bind to CCR2, it is unable to activate CCR2 and induce chemotaxis. In humans (Fig. 7B), locally released MCP1 interacts with other MCP1 molecules forming higher order oligomers. The monomeric and oligomeric forms of MCP1 maintain a delicate equilibrium with significantly more oligomers at the injury site due to high local concentration and relatively more monomer at distal areas due to low local MCP1 level. The monomeric MCP1 binds to and activates CCR2, leading to migration of microglia/monocytes to the injury site. Unlike rodent MCP1, human MCP1 dimers can not bind to CCR2 nor activate it.

Rodents use plasmin-mediated cleavage to regulate MCP1 activity (Sheehan et al., 2007, Yao and Tsirka, 2010). Since human MCP1 does not have the heavily glycosylated C-terminus, its activity is less likely to be regulated by plasmin. How human MCP1 activity is regulated remains unclear. Is it modulated at transcriptional, translational, or post-translational levels? Given that humans have two subtypes of CCR2, whereas rodents have only one, it is logical to speculate that CCR2 diversity may contribute to the regulation of MCP1-CCR2 interaction in humans. Future research is needed to answer these questions.

In summary, we have shown that rodent MCP1 dimers are unable to activate Rac1, promote phosphorylation of ERK, induce formation of lamellipodia, or provoke microglial migration, although they can bind to CCR2. These data suggest that the CCR2-binding motif and CCR2-activating motif are different, and dimerization alters/hides the CCR2-activating motif without affecting the CCR2-binding motif. Our results demonstrate that rodent MCP1 functions as a monomer, although its dimeric form can still bind to CCR2. Our data also provide evidence for the study of species-specific functions of MCP1. Furthermore, these data identified molecules that regulate MCP1 monomer-oligomer equilibrium as a novel target to interfere with MCP1-CCR2 signaling, and may contribute to the research and development of innovative therapies for many inflammatory diseases, including CNS injury and inflammatory bowel disease.

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Figure 1. Dimeric MCP1 proteins do not affect total CCR2 levels in primary microglia
Primary microglia were treated with 10nM FL- (A), K104Stop- (B), FL-FL- (C), and K104Stop-K104Stop-MCP1 (D) for 0, 5, 15, 30, 60, and 120 minutes. Total cell lysates were collected and assessed for CCR2 and α -tubulin by immunoblotting. (E) Relative CCR2 expression levels were quantified by normalizing the CCR2/ α -Tubulin ratio to untreated controls at 0 time point. Experiments were performed in triplicate. Data are expressed as Mean $\pm$ SD.

Figure 2. Dimeric MCP1 proteins can trigger CCR2 internalization

Primary microglia were treated with 100ng/ml LPS overnight, followed by saline (Ctr, A) or 10nM recombinant FL- (B), K104Stop- (C), FL-FL- (D), and K104Stop-K104Stop-MCP1 (E) for 1 hour. Then the cells were swelled in hypotonic buffer for 20 min and the plasma membrane that remained adhered to the coverslips was stained with Mac-2 (Green) and CCR2 (Red) antibodies. Microglia immunostained with secondary antibodies only were used as a control (F). Scale bar = 10 μ m. (G) Quantification of CCR2 expression levels on plasma membrane. Twenty cells per condition in 3 separate experiments were used for

quantification. The results were analyzed with one-Way ANOVA followed by Dunn's Test:
* $p < 0.05$, compared to Ctr.

Figure 3. Dimeric MCP1s are unable to induce microglial migration

Primary mouse microglia (5×10^4 cells) were plated in Boyden chamber and evaluated for chemotaxis in response to MEM only (Ctr, A), FL- (B), K104Stop- (C), FL-FL- (D), and K104Stop-K104Stop-MCP1 (E). White triangles indicate migrated microglia. Scale bar = 0.25 mm. (F and G) Quantification of migrated microglia in absolute numbers (F) and fold change relative to the control (G). Each experimental condition was assayed in triplicate. Data are expressed as Mean $\pm$ SD. ** $p < 0.01$ and *** $p < 0.001$ compared to Ctr.

Figure 4. Dimeric MCP1 proteins fail to activate Rac1

N9 microglia were treated with 10nM FL- (A), K104Stop- (B), FL-FL- (C), and K104Stop-K104Stop-MCP1 (D), and the activated Rac1 was immunoprecipitated using GST-PBD beads, followed by western blotting. Total Rac1 was detected using total cell lysates. (E) Quantification of activated Rac1 in A and B. The blots were normalized to untreated controls at 0 time point. * $p < 0.05$, compared to untreated controls. (F) Quantification of activated Rac1 in C and D. The blots were normalized to the positive controls. Each experimental condition was assayed in triplicate and the data were expressed as Mean $\pm$ SD.

Figure 5. Dimeric MCP1 proteins do not induce the formation of lamellipodia

Primary microglia were plated on coverslips and exposed to a point source of recombinant MCP1 proteins. At different time points, microglia were fixed and stained for F-actin using Alexa-647 Phalloidin. (A) Representative images of phalloidin staining after MCP1 treatment. Scale bar = 20 μ m. Arrows indicate lamellipodia. A high-magnification image of the lamellipodia is shown in the insert. (B) Quantification of the percentage of polarized cells. Data are expressed as Mean $\pm$ SD. * p <0.05 and ** p <0.01, compared to Ctr at each time point.

Figure 6. Dimeric MCP1s do not activate MAPK pathway

Microglia were incubated with 10nM FL- (A), K104Stop- (B), FL-FL- (C), and K104Stop-K104Stop-MCP1 (D) over time. At each time point, total cell lysates were analyzed for phosphorylated ERK (p-ERK) and total ERK (t-ERK) by western blotting. (E) The activation of ERK was quantified by normalizing the p-ERK/t-ERK ratio to untreated controls at 0 time point. Each experimental condition was assayed in triplicate and the data were expressed as Mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$, compared to untreated controls.

Figure 7. Working model for MCP1-CCR2 interaction

(A) In rodents MCP1 is quickly secreted and accumulates on the cell surface via the heavily glycosylated C-terminus after injury, which enhances local concentration and promotes the formation of dimers/oligomers. MCP1 forms a concentration gradient to attract CCR2-expressing microglia and/or monocytes. Plasmin activates MCP1 by removing its C-terminus, an event that releases the monomeric MCP1, which then binds to and activates CCR2, leading to chemotaxis. The dimeric MCP1, on the other hand, can not activate CCR2, although it can bind to CCR2. (B) Human secreted MCP1 forms dimers/oligomers and a delicate equilibrium is maintained between these forms. At the injury site, dimers/oligomers are the major form of MCP1 due to the extremely high concentration. Away from the injury site, however, MCP1 concentration decreases and monomeric MCP1 predominates. MCP1 monomer binds to and activates CCR2, resulting in chemotaxis. MCP1 dimer, on the contrary, fails to bind to, or activate CCR2.