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Monocyte Chemoattractant Protein 1/CC Chemokine Ligand 2 enhances apoptotic cell removal by macrophages through Rac1 activation

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ABSTRACT

Apoptotic cell removal (efferocytosis) is an essential process in the regulation of inflammation and tissue repair. We have shown that monocyte-chemoattractant protein-1/CC chemokine ligand 2 (MCP-1/CCL2) enhances efferocytosis by alveolar macrophages in murine bacterial pneumonia. However, the mechanism by which MCP-1 exerts this effect remains to be determined. Here we explored that hypothesis that MCP-1 enhances efferocytosis through a Rac1/ phosphatidylinositol 3-kinase (PI3-kinase)-dependent mechanism.

We assessed phagocytosis of apoptotic cells by MCP-1 treated murine macrophages in vitro and in vivo. Rac activity in macrophages was measured using a Rac pull down assay and an ELISA based assay (GLISA). The downstream Rac1 activation pathway was studied using a specific Rac1 inhibitor and PI3-kinase inhibitor in in vitro assays.

MCP-1 enhanced efferocytosis of apoptotic cells by murine alveolar macrophages (AMs), peritoneal macrophages (PMs), the J774 macrophage cell line (J774s) in vitro, and murine AMs in vivo. Rac1 activation was demonstrated in these cell lines. The effect of MCP-1 on efferocytosis was completely negated by the Rac1 inhibitor and PI3-kinase inhibitor.

We demonstrated that MCP-1 enhances efferocytosis in a Rac1-PI3 kinase-dependent manner. Therefore MCP-1-Rac1-PI3K interaction plays a critical role in resolution of acute lung inflammation.

Word count: 192

Keywords: phagocytosis; apoptosis; MCP-1/CCL2; alveolar macrophage; lung inflammation;
Abbreviations used in this paper:

AM; alveolar macrophage
BI; Biding index
PM; peritoneal macrophage
J774; J774 macrophage cell line
PMN; polymorphonuclear neutrophil
BAL; Bronchoalveolar lavage
BALF; Bronchoalveolar lavage fluid
PI; Phagocytic index
PS; phosphatidylserine
PI3-kinase; phosphatidylinositol 3-kinase
MPO; myeloperoxidase
INTRODUCTION

Recognition and engulfment of apoptotic cells (termed efferocytosis) is an essential process in regulation of the inflammatory response and tissue repair through release of anti-inflammatory mediators[1, 2] and several key growth factors[2, 3]. Efferocytosis of apoptotic polymorphonuclear neutrophils (PMNs) is crucial for resolution of acute inflammation in the lung[4, 5]. Thus, a defect or inefficiency of this process and accumulation of secondarily necrotic cells leads to persistent inflammation and tissue damage in the lung[2, 5, 6]. Knowledge about the mechanism of efferocytosis has been advanced considerably during recent years. Exposed phosphatidylserine(PS) on the outer leaflet of apoptotic cells is a major ligand that is recognized by phagocytes and is known as an ‘eat me signal’[7]. Several bridge molecules and receptors for PS on phagocytes are reported to be essential molecules for recognition and removal of apoptotic cells[2, 8, 9]. On the other hand, it is not fully understood at molecular level how efferocytosis is facilitated at the beginning of resolution of acute inflammation in lung.

The importance of Rho-GTPases on efferocytosis is demonstrated in many studies and introduced in several reviews [10; 11]. Among so many known Rho-GTPases, classical Rho-GTPases such as Rac1, RhoA and Cdc42 are the most investigated. Rac1 and Cdc42 promote efferocytosis, whereas RhoA activation inhibits this process[8]. Rac1 activation is considered to be especially important in efferocytosis through cytoskeletal, morphological changes and consequent membrane ruffles that form the phagocytic cup[12]. However, factors that up-regulate Rac1 activity followed by membrane ruffling for efferocytosis during resolution of acute inflammation remain to be elucidated.
Monocyte chemoattractant protein-1/CC chemokine ligand 2 (MCP-1/CCL2) is a member of the CC subgroup of the chemokine superfamily and is a ligand for the seven transmembrane domain G-protein-coupled receptor CCR2. MCP-1 also acts as a potent chemoattractant and activator of monocyte/macrophages[13]. Several studies show its protective effect during infection of *Salmonella typhimurium, Pseudomonas aeruginosa,*[14], and *Streptococcus pneumoniae*[15] in the murine peritoneal cavity and lung respectively. These studies imply that MCP-1 enhances lung immunity by recruiting mononuclear cells to fight infection. We have previously reported that MCP-1 enhances efferocytosis, followed by hepatocyte growth factor (HGF) production and plays a crucial role in resolution of inflammation and tissue repair in murine acute bacterial pneumonia model[5]. However the molecular mechanism by which MCP-1 enhances efferocytosis still remains to be elucidated. Recently, the MCP-1-CCR2 axis was reported to activate Rac1 and have a pivotal function on monocyte chemotaxis[16]. This study evoked the hypothesis that MCP-1 enhances efferocytosis through Rac1 activation.

In the present study, we showed that MCP-1 enhances efferocytosis through Rac1-PI3K interaction using *in vitro, ex vivo* and *in vivo* approaches. These results indicate that MCP-1/Rac1/PI-3K pathway plays critical role in resolution of acute lung inflammation.
Material and Methods

Animals and experimental protocol

Specific pathogen-free, 4 week old and 8 week old ICR and Balb/c mice were obtained from Charles River Japan (Kanagawa, Japan). The mice were provided with sterile food and water ad libitum in an environmentally controlled room. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Nagasaki University School of Medicine.

Lipopolysaccharide (LPS) induced lung injury; 1mg/kg of LPS was instilled intratracheally, then bronchoalveolar lavage [17] was performed at the time indicated. Absolute macrophage and neutrophil counts were obtained by multiplying BAL leukocyte counts by differential cell counts. Bronchoalveolar lavage fluid (BALF) cytokines were measured using commercially available ELISA kits for murine MCP-1 (BIOSOURCE, Carlsbad, CA, USA), MIP-2 (RayBio, Norcross GA, USA) and TNFα (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

Reagents

Recombinant Mouse CCL2/JE/MCP-1 was purchased from R&D Systems (Minneapolis, MN, USA). Rac1 Inhibitor (NSC 23766) and PI3K inhibitor (LY 294002) were purchased from Calbiochem (San Diego, CA, USA). Lipopolysaccharide (E. Coli O111:B4) was purchased from SIGMA (St.Louis, MO, USA).

Isolation of Primary Cells

Human neutrophils were obtained from healthy donors and isolated via density gradient centrifugation as previously described[4]. Murine alveolar macrophage (AM) s were
isolated by whole lung lavage with PBS containing 100μM ethylenediaminetetraacetic acid (EDTA). Murine peritoneal macrophages (PM) s were isolated by lavage with PBS.

Murine thymocytes were isolated from the thymi of three to four week old, female ICR mice, by passing thymi through a 40-μm strainer (Fisher Scientific, Waltham, MA, USA) to separate individual cells.

**Cell culture**

Murine J774A.1 (J774)macrophages were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco’s modified Eagle media (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin in humidified 5% CO₂ at 37°C. Human leukemia Jurkat T cells were obtained from ATCC and cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin in humidified 5% CO₂ at 37°C.

**Induction of apoptosis**

Apoptosis was induced in human neutrophils, Jurkat T cells, and murine thymocytes by exposure to UV irradiation at 312 nm (Fotodyne) for 10min as previously described[18]. Human neutrophils were cultured in RPMI 1640 with 0.5%, low-endotoxin BSA (Sigma-Aldrich) for 2 h. Jurkat T cells and murine thymocytes were cultured in RPMI 1640 with 10% FBS at for 3 h in 5%CO₂ at 37°C.

**In vitro phagocytosis assays**

In vitro phagocytic assays were performed as previously described. Briefly,
macrophages were plated in 24-well plates at a concentration of $5 \times 10^5$ cells/well on glass coverslips (MATSUNAMI GLASS, Osaka, Japan). To test the effect of NSC23766 and LY294002, murine AMs were plated at $2 \times 10^5$ cells/well on plastic 8 well Lab-Tek (Nalge NUNC International, New York, USA). One or two days after, cells were treated with MCP-1 at 10 ng/ml for 4 hours, NSC 23766 at 50μM for overnight, LY294002 at 100μM for 30 minutes. Then, the cell culture supernatant was removed and apoptotic cells were added at a 10:1 ratio. The cells were cocultured for 60 minutes (murine PMs, J774A.1), 90 minutes (murine AMs), at $37^\circ C$ in 5% CO₂. Each well was washed with ice-cold PBS to remove uningested apoptotic cells and stained with Diff Quik (Sysmex, Kobe, Japan) staining. Phagocytosis was determined by visual inspection of samples by oil immersion light microscopy and was expressed as the phagocytic index (PI) as described [18]. Each condition was tested in duplicate and a minimum of 400 cells were counted per condition. In all cases, during analysis, the reader was blinded to the sample identification.

**In vivo phagocytosis assays**

The in vivo phagocytosis assay was performed using balb/c mice as previously described [18]. Mice were anesthetized with Nembutal (Dainippon Sumitomo Pharma, Osaka, Japan), following which $5 \times 10^6$ apoptotic human neutrophils, suspended in 50 μl of PBS, were instilled intratracheally. Three hours later, whole lung BAL was performed with a total of 5 ml of ice-cold PBS. Lavage cells were fixed and stained with Diff-Quik staining (Fisher Scientific, Pittsburgh, PA) and myeloperoxidase (MPO) staining using diaminobenzidine (DAB) substrate (Roche, Basel, Switzerland) following the instruction manuals. Because AMs are originally negative for MPO
staining, MPO positivity in the cytoplasm of AMs were identified as engulfment of apoptotic neutrophils. The phagocytic index was assessed as described previously[5].

The cell number in BALF was determined with the use of a hemocytometer. Cell differentials for BAL cells were assessed by Diff Quik stained samples.

**Rac activation assay**

The Rac activation assay was performed according to the manufacturer’s instructions (Millipore, Billerica, MA) (pull-down method). Briefly, after each indicated treatment, cells were lysed, then cell lysates were incubated with the agarose-immobilized GST-PAK1, and co-precipitates were subjected to anti-Rac1 Western blot analysis.

To evaluate relative Rac activity of murine alveolar macrophages, another commercially available Rac activation assay (G-LISA®: Cytoskeleton Inc. Denver, CO) was used. Overnight serum-starved murine AMs were treated same as above. The remainder of the protocol was followed as described in the kit manual. Briefly, snap frozen cell lysates were thawed and added to the Rho plate, binding Rho-GTP. The plate was washed, an antigen-presenting buffer was added, followed by primary and secondary antibodies. The reaction was detected using horseradish peroxidase detection reagent followed by the stop buffer. The plate was read immediately by measuring absorbance at 490 nm on a microplate spectrophotometer.

**Western Blotting**

Western blotting was performed as previously described[18].

**Statistics**
Data were expressed as mean ± SE. Statistical analysis was performed by two-tailed Student’s t test of unpaired samples. The Dunnett test was used for multiple comparisons. Significant differences were defined as p< 0.05.
RESULTS

The kinetics of MCP-1 differs from that of pro-inflammatory cytokines such as MIP-2 and TNF-α in acute lung inflammation (murine LPS induced lung injury model).

To show that MCP-1 plays an essential role in the resolution and repair processes during acute lung inflammation, we compared the kinetics of MCP-1 and some pro-inflammatory cytokines. In an LPS model of acute lung injury, the number of neutrophils in BALF increased rapidly within 48 hours after intratracheal instillation of LPS, reaching a maximal level at 48 hours. The neutrophil number declined thereafter and returned to a basal level by 7 days. A substantial number of AMs were detected in BALF of untreated mice. Intratracheal instillation of LPS induced a significant increase in macrophage numbers later than 96 hours, increasing gradually thereafter (Figure 1a). The number of macrophages ingesting neutrophils peaked at 48 hours and decreased thereafter, concomitantly with an increase in neutrophil number (Figure 1a and 1b).

Collectively, these results suggest that infiltrating neutrophils became apoptotic and were ingested by macrophages[19]. We then assessed MCP-1 levels in BALF. MCP-1 increased at 48 hours and reached a maximal level at 48-72 hours after LPS instillation, declining thereafter and returning to a basal level by 7 days (Figure 1c). In contrast to MCP-1, MIP-2 and TNF-α showed different kinetics in BALF, reaching a maximal level at 6 hours after LPS instillation which is much earlier than MCP-1 (Figure 1d).

Macrophages express CCR2 constitutively.

Before testing the effect of MCP-1 on efferocytosis, we confirmed that murine J774s (a murine macrophage cell line), peritoneal and alveolar macrophages express CCR2
MCP-1 stimulation enhances efferocytosis in vitro.

We extended our previous work[5] by showing that MCP-1 enhances efferocytosis by J774s, as well as murine peritoneal and alveolar macrophages. Based on dose-response experiments from our previous study, we stimulated macrophages with MCP-1 at 10ng/ml. As shown, MCP-1 increased efferocytosis by J774s (Figure 2c), murine PMs (Figure 2b, d) and murine AMs (Figure 2e). However, MCP-1 only increased binding of apoptotic cells to AMs but not J774s and PMs.

Efferocytosis in vivo was up-regulated with MCP-1

As with our in vitro experiments, MCP-1 also enhanced efferocytosis by AMs in vivo (Figure 3a,b). Considering that MCP-1 enhances monocyte chemotaxis and recruitment, we were concerned that MCP-1 may increase the number of AMs and thereby affect the phagocytic index. But the total number of AMs was similar in the presence or absence of MCP-1 (Figure 3c).

MCP-1 activates Rac1 activity in macrophages.

To examine our hypothesis that MCP-1 may enhance efferocytosis by increasing Rac activation, Rac activation assays were performed. Significant Rac activation by MCP-1 on murine AMs was confirmed using the G-LISA assay (Figure 4a). Rac pull-down assays also confirmed that MCP-1 increased Rac activation in J774 macrophages (Figure 4b) and murine PMs (Figure 4c).
MCP-1 upregulates efferocytosis by AMs through Rac1-PI3K (Phosphatidylinositol 3-kinase) pathway activation.

Rac activation is mediated by guanine nucleotide exchange factors (GEFs), like Trio and Tiam1 [20]. NSC 23766 (IC$_{50}$=50 μ M) specifically inhibits Trio and Tiam1, thereby inhibiting Rac1 activation. We used NSC 23766 to determine whether MCP-1 enhances efferocytosis through a Rac1-dependent mechanism. Pretreatment with NSC 23766 negated enhancement of efferocytosis by MCP-1, indicating that MCP-1 enhances efferocytosis via a Rac1-dependent mechanism (Figure 4d). We assessed the macrophage viability with this reagent with trypan blue. Compared to the control (without NSC 23766), the proportion of viable cells was similar (data not shown).

In the same way, the inhibition assay was performed with the pretreatment with LY 294002 (IC$_{50}$=1.4 μ M), an inhibitor of PI3 kinase. Pretreatment with LY 294002 following MCP-1 treatment also failed to enhance efferocytosis, indicating that MCP-1 enhanced efferocytosis through a PI3 kinase-dependent pathway (Figure 4e). We assessed the macrophage viability with this reagent with trypan blue. Compared to the control (without LY 294002), the proportion of viable cells was similar (data not shown).
DISCUSSION

The outcome of the acute inflammatory program is successful resolution and tissue repair. Removal of dead or dying neutrophils by phagocytes, called efferocytosis, is an essential step for resolution of inflammation[1, 21]. To date, the specific mediators that facilitate efferocytosis during the resolution phase of an acute inflammatory response have not been fully characterized. Data presented herein elucidate the molecular mechanisms by which MCP-1 enhances uptake of apoptotic cells by macrophages, mainly by AMs. While lipoxins, endogenous anti-inflammatory lipid mediators which are known to display diverse potent anti-inflammatory effects in various respiratory diseases, have been reported to enhance efferocytosis[22], details of their kinetics in acute inflammation have not been revealed. Thus, understanding the mechanism how MCP-1 regulates efferocytosis is one approach to investigate resolution of acute lung inflammation.

The process of apoptotic cell corpse removal by professional phagocytes is complex. It consists of some central elements: 1; expression of ‘eat-me signals’ on surface of apoptotic cells, 2; display ‘find-me signals’ such as lysophosphatidylcholine by apoptotic cells in order to induce the migration of phagocytes, 3; recognition and 4; subsequent engulfment of the apoptotic cell [2, 8, 9]. In this process, two different roles of Rac1 have been demonstrated[12]. The first is to mobilize plasma membranes and establish lamellipodia, and the second is to regulate the actin dynamics during engulfment. Thus, Rac1 activation ahead of recognition of dying cells is crucial in the process of efferocytosis. We speculate that MCP-1 may play an important role by
preactivating Rac1 in alveolar macrophages during the resolution phase of acute lung inflammation.

We measured Rac1 activation in murine AMs using the G-LISA® assay because cell collection through BAL was not sufficient for western blot analysis. Our data showed that MCP-1 activates Rac1 compared to the control; however the magnitude of the change was small. We consider two reasons. First, the character of naïve, resident AMs are heterogeneous and expression of CCR2 is lower than blood monocytes[23]. Second, RhoA is known to suppress Rac activity [10]. Therefore, the effect of MCP-1 on Rac1 activity in naïve AMs might be reduced due to simultaneous activation of RhoA by surfactant protein A (SP-A) and surfactant protein D (SP-D)[24]. MCP-1 increased binding indices in AMs in present study (Figure 2e). We have already shown that MCP-1 increases the expression of the molecule which was recognized by the mouse monoclonal antibody 217 (mAb217)[5]; receptors or bridging molecules for apoptotic cells might also contribute to enhanced binding and efferocytosis by AMs.

Focusing on respiratory medicine, MCP-1 has been recognized as an inflammatory chemokine that is present during acute and chronic inflammation. High expression of MCP-1 in chronic diseases, such as chronic obstructive pulmonary disease (COPD), pulmonary fibrosis and sarcoidosis[25, 26, 27] and the association between MCP-1-CCR2 and pathogenesis in bleomycin-induced mice pulmonary fibrosis has been reported[28]. The highly regulated kinetics of MCP-1 during acute inflammation (figure1c) caused us to speculate that MCP-1 may be crucial to control acute inflammation. By contrast, dysregulated and persistent MCP-1 secretion may be one of
causative factors of chronic inflammation. In fact, longer exposure (three days or more) to MCP-1 \textit{in vitro} did not enhance or inhibited efferocytosis by macrophages (unpublished data). Further examination on the mechanism of regulation of MCP-1 secretion in resolution phase of acute inflammation may contribute to elucidation of pathogenesis of chronic lung diseases.

To confirm the effect of Rac1 in the mechanism, we used NSC 23766 which blocks Rac function by binding to Trp56 and inhibits Trio and Tiam specifically. Noteworthy, enhancement of efferocytosis by MCP-1 was completely negated by the Rac1 inhibitor (Figure 4d). PI3-kinase is stimulated by a variety of hormones, growth factors and chemoattractants and Rac-GTP is involved in PI3-kinase activation\cite{29}. Similar to NSC 23766, the positive effect on efferocytosis by MCP-1 was largely suppressed by the PI3K inhibitor, LY 294002 (Figure 4e). Together, we concluded that MCP-1 regulates efferocytosis positively mainly through Rac1-PI3K dependent pathway.

In LPS-induced lung inflammation, resident alveolar macrophages are replaced by recruited alveolar macrophages in some proportion\cite{30}, and recruited alveolar macrophages have higher phagocytic activity than resident macrophages\cite{24}. As discussed above, we used naïve resident alveolar macrophages. This is our limitation and would be our next process for further analysis to see the effect of MCP-1/CCL2 on alveolar macrophages initiated by microorganisms or proinflammatory cytokines and recruited alveolar macrophages. The baseline Rac1 activity or Rac1/RhoA balance in alveolar macrophages might be involved in their phagocytic activity\cite{18}. Conceivably, baseline RhoGTPase activity in naïve alveolar macrophages might be at RhoA > Rac1
balance, and once inflammation begins to resolve resident macrophages and recruited macrophages might become Rac1 > RhoA so that effective engulfment takes place in the lung. We speculate that transient elevation of MCP-1 in resolution phase might work to switch from RhoA-balanced to Rac1-balanced state in the AM during acute lung inflammation.

Our findings in this study revealed the importance of Rac1-PI3K pathway in mechanism of positive regulation on efferocytosis by MCP-1 during the resolution of acute lung inflammation. Therefore, we consider that dysregulation of MCP-1-Rac1-PI3K axis in resolution phase might lead the transition to persistent, chronic inflammation.

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References


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Figure Legends

Figure 1.
(a) Kinetics of macrophages (○) and neutrophils (■) in bronchoalveolar lavage fluid (BALF) during LPS induced acute lung inflammation. (b) Phagocytic Index of alveolar macrophage (AM)s in BALF. The kinetics of (c) MCP-1, (d) MIP-2 (○), and TNF-α (□) in BALF. Each group consists of five animals. Mean ± SE were calculated.

Figure 2.
(a) The CCR2 receptor detected by immunoblotting using a goat polyclonal anti-mouse CCR2 antibody. A representative immunoblot is shown. (b) Photomicrographs (400x magnification) show typical images of unstimulated (left panel) and MCP-1 stimulated (right panel) murine Peritoneal macrophage (PM)s that have ingested apoptotic cells (arrows). Control sample (left), MCP-1 treated sample (right). Phagocytic index; PI (left) and Binding index; BI (right) of (c) J774 macrophages, (d) PMs, and (e) AMs. The mean PI as percent control ± SEM is shown for each group. The mean BI as percent control ± SEM is shown for each group. Control mean PI in J774 macrophages: 18.0 ± 2.8. Control mean PI in PMs: 70.5 ± 3.8. Control mean PI in AMs: 1.20 ± 0.19. *Significantly different from control (p<0.05).

Figure 3.
(a) Photomicrographs (1000x magnification) show typical images of MPO stained samples. Left: Control samples show AMs that have not ingested apoptotic neutrophils (arrow heads). Right: The MCP-1-treated sample shows AMs that have ingested
apoptotic neutrophils (arrows). (b) Phagocytic index (PI) was significantly higher in MCP-1 treated group than control group (p<0.01). (c) Absolute alveolar macrophage counts in BALF.

Figure 4.

(a) MCP-1/CCL2 activates Rac1 in murine alveolar macrophages. Rac1 activation was detected by the Rac G-LISA®. Significant difference from control (p<0.05). Rac activation assay of (b) J774 macrophages and (c) murine PMs. Results shown are representative of independent experiments. (d) Effect of a Rac1 inhibitor (NSC 23766) on MCP-1-induced efferocytosis by murine AMs. ( *p<0.05) The mean PI as percent control ± SEM is shown for each group. Control mean PI: 0.4± 0.42. (e) Effect of a PI3-kinase inhibitor (LY294002) on MCP-1-induced efferocytosis by murine AMs. ( *p<0.05) The mean PI as percent control ± SEM is shown for each group. Control mean PI: 0.3 ± 0.24.
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