Published in final edited form as:

Cell Immunol. 2017 April ; 314: 63–72. doi:10.1016/j.cellimm.2017.02.005.

Enhancement of macrophage inflammatory responses by CCL2 is correlated with increased miR-9 expression and downregulation of the ERK1/2 phosphatase Dusp6

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Abstract

Macrophage polarization plays a central role in both protective immunity and immunopathology. While the role of cytokines in driving macrophage polarization is well characterized, less is understood about the role of chemokines. The purpose of this study was to determine if C-C chemokine 2 (CCL2/MCP1) could influence macrophage polarization in response to subsequent activation with cytokines and microbial products. Treatment of bone marrow-derived macrophages with CCL2 alone did not result in increased expression of either classical or alternatively-activated macrophage genes as compared to standard skewing cytokines or Tolllike receptor agonists. However, subsequent stimulation of CCL2 pre-treated macrophages with classical activation stimuli resulted in enhanced expression of genes associated with classical activation. This enhancement correlated with increased phosphorylation of ERK1/2 kinases, a decrease in expression of the ERK phosphatase Dusp6 and enhanced expression of miR-9. These results indicate that CCL2 supports the classical activation of macrophages, with miR-9 mediated down-regulation of Dusp6 and enhanced ERK-mediated signal transduction possibly mediating this enhanced pro-inflammatory gene expression.

Graphical abstract

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Keywords

Chemokines; macrophages; signal transduction; gene regulation; micro-RNA

1. INTRODUCTION

Macrophage activation is tightly regulated by signal transduction events initiated by soluble mediators in the local inflammatory environment. Pathogen-associated molecular patterns (PAMPs) and leukocyte-derived cytokines synergize to drive the activation of macrophages along specific effector phenotypes. Classically activated/M1 macrophages upregulate the expression of genes involved with the clearance of pathogens, and are preferentially generated via exposure to microbe-derived products (e.g. lipopolysaccharide) and interferon gamma ($\text{IFN}\gamma$). In contrast, alternatively activated/M2 macrophages upregulate the expression of genes involved with wound healing and clearance of dead and dying cells and tissues, and are preferentially generated via exposure to interleukin-4 (IL4) [1]. Tight regulation of macrophage activation via cytokine stimulation is central to productive immunity, and improper activation of macrophages can lead to immunopathology. For example, chronic M1 activation can participate in fulminating inflammation characteristic of autoimmune diseases [2], while aberrant M2 activation can help perpetuate allergic responses [3] and support the growth of malignancies [4]. Therefore, a better understanding of the molecular mechanisms governing macrophage activation is central to the development of treatments aimed at modulating macrophage effector functions.

In contrast to the current understanding of the role of cytokines, the role of chemokines in guiding macrophage polarization is less characterized. Chemokine signaling is a critical component in guiding tissue inflammatory processes, primarily through guiding chemotaxis of leukocytes to sites of infection [5]. Chemokine signaling can also induce activation of leukocytes in a similar fashion to cytokine stimulus; for example, chemokines drive

upregulation of adhesion molecules essential for tethering of leukocytes to endothelial cells prior to extravasation from peripheral blood into interstitial tissues [6]. Chemokine signaling is relatively promiscuous, with multiple chemokines binding to multiple receptors.

Expression of specific chemokine receptors is often restricted to specific immune cell lineages, and differential chemokine receptor expression can often be used as a tool to delineate leukocyte subsets. Numerous studies have attempted to characterize chemokine expression into functional subsets, in a similar fashion to cytokine expression by polarized lymphocytes. However, these studies often ascribe similar chemokine expression patterns to disparate inflammatory processes, suggesting that chemokine signaling in leukocytes may not have as clear a role as polarizing cytokines. Interestingly, while specific chemokine expression patterns have been observed in polarized inflammatory responses (e.g. classical vs. alternatively activated macrophage environments), the role of these chemokines in directly polarizing macrophages remains unclear.

C-C chemokine 2 (CCL2), also known as monocyte chemoattractant protein 1 (MCP1), is an inflammatory chemokine produced by monocytic cells with specific chemotactic activity for innate immune cell monocytes and basophils [7]. Numerous other cell types can also produce CCL2, including stromal cells (such as fibroblasts) [8] and structural cells (such as epithelial cells) [9]. CCL2 is an important soluble factor in driving monocytic infiltration of tissues during inflammatory processes via preferential interactions with CCR2 [10]. Expression of CCL2 is observed in response to numerous inflammatory stimuli, including microbial infection and tissue damage, and CCL2-mediated chemotaxis is critical for monocyte recruitment to inflammatory foci. Inhibition of CCL2 signaling via genetic manipulation or biological inactivation (i.e. blocking antibody treatment) has drastic effects on immune cell responses in a wide variety of inflammatory disease models [11–13].

Despite the volume of previously published reports on the role of CCL2 in monocyte chemotaxis and inflammatory responses, the ability of CCL2 to act as a polarizing signal for macrophages remains unclear. CCL2 production is often considered to be characteristic of T_H2 /M2 responses, as blockade of CCL2 has been shown to decrease production of T_H2 cytokines in animal models of infection [14]. Also, the production of CCL2 often promotes T_H2 -type cytokine production by activated T cells, most notably IL-4 [15]. However, CCL2 production has also been observed in the context of T_H1 /M1 inflammatory disorders, including inflammatory bowel disease [16], rheumatoid arthritis [17] and multiple sclerosis [18]. CCL2 has also been implicated in instances of chronic M1-type activation, as observed in macrophages from adipose tissue of patients with type 2 diabetes [19]. In the case of severe systemic inflammation, addition of exogenous CCL2 protects mice against peritonitis-induced mortality, whereas blockade of CCL2 (using antibodies) increases susceptibility [20]. These results suggest a complicated role for CCL2 in driving cytokine-specific immune responses and macrophage polarization.

The purpose of this study was to investigate the ability of CCL2 to promote classical vs. alternative activation of macrophages through assaying CCL2-mediated activation of these cells. Murine bone marrow-derived macrophages did not exhibit any M1 or M2-type gene expression in response to CCL2 treatment, suggesting that this chemokine alone does not drive macrophage polarization. However, when CCL2 pre-treated cells were subsequently

exposed to classical or alternative-activating stimuli, the CCL2-treated cells exhibited increased evidence of classical activation. This enhanced classical activation was observed when macrophages were treated with both inflammatory cytokine (IFN γ) and lipopolysaccharide (LPS). CCL2 pre-treated macrophages exhibited increased ERK1/2 phosphorylation, which correlated with a decrease in expression of the phosphatase Dusp6, a negative regulator of ERK signaling. In turn, decreases in Dusp6 expression correlated with increased expression of the micro-RNA miR-9, which was predicted to regulate Dusp6 mRNA based on *in silico* studies of mir-9 sequence specificity. These studies suggest that CCL2 may preferentially support classical activation of macrophages, in part via post-transcriptional control of negative regulators of signal transduction.

2. MATERIAL AND METHODS

Animals

8-to 12-week old female C57BL/6 mice at were purchased from Taconic (Hudson, NY). All mice were maintained in specific pathogen-free facilities in the Unit for Laboratory Animal Medicine at the University of Michigan. All experiments were approved by the University Committee on Use and Care of Animals at the University of Michigan.

Derivation of bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDMs) from single-cell suspensions of tibia and femur marrow were differentiated *in vitro* as previously described [21]. Briefly, murine bone marrow was cultured in RPMI 1640 (Lonza, Walkersville, MD) supplemented with 30% L-cell conditioned media, 20% Fetal Calf Serum and penicillin/streptomycin for a period of six days. Adherent BMDMs were harvested and replated in minimal media for a rest phase of 12–18 hours. Following this rest phase, BMDMs were pre-treated in certain conditions with CCL2 (R&D Systems, Minneapolis, MD) for a period of 12–18 hours, were indicated. Stimulations with IFN γ (Shenandoah Biotechnology, Warwick, PA), IL-4 (Shenandoah), and LPS (0111:B4, Sigma, St. Louis, MO) were performed following the rest and pretreatment phase. Reported endotoxin levels in the utilized recombinant cytokines are as follows: <0.01 EU/ μ g for CCL2 and <1 EU/ μ g for IFN γ and IL-4. The ERK1/2 inhibitor GDC-0994 (Selleck Chemicals, Houston, TX) was resuspended in DMSO and used at a final concentration of 50 nM in cell culture assays.

RNA isolation and qPCR

Total RNA was extracted from cultured cells using TRIzol (Life Technologies, Grand Island, NY), and reverse transcribed to cDNA using iScript (Bio-Rad, Hercules, CA) according to the manufacturer's protocols. Gene expression analysis was performed on an Applied Biosystems 7500 Real Time qPCR cycler. Primers for analysis of gene expression were obtained from Applied Biosystems (ThermoFisher Scientific, Waltham, MA). The primer sets were as follows: *Arg1*: Mm00475988_m1; *Ccl2*: Mm00441242_m1; *Ccr2*: Mm99999051_gH; *Dusp6*: Mm00518185_m1; *miR9*: 002231; *Nos2*: Mm00440502_m1; *Retnla*: Mm00445109_m1 *Rplp2*: Mm00782638_m1; *Tnfa*: Mm00443258_m1. Fold expression was calculated using the delta-delta Ct method, with RPLP2 serving as a housekeeping gene.

Microscopy

Cells cultured in Lab-Tek chambered slides (Electron Microscopy Sciences, Hatfield, PA) were fixed with methanol and stained using Diff-Quik (Siemens, Newark, DE). Images from light microscopy were captured using CellSens Dimension software (Olympus, Center Valley, PA).

Flow cytometry

BMDMs were stained in flow buffer (phosphate-buffered saline, 1% w/v bovine serum albumin, 0.05% sodium azide) with the following fluorescent antibodies at the indicated dilutions: PeCy7-F4/80 (eBioscience, San Diego, CA), 1:400; PE-IFN γ R (eBioscience), 1:200; PE-TLR4 (eBioscience), 1:200; PE-CCR2 (R&D Systems), 1:200; and PE-CCR7 (eBioscience), 1:200. Cells were then fixed in 4% paraformaldehyde and analyzed on a LSR II (BD Biosciences, San Jose, CA). Flow cytometry data was analyzed using FlowJo 9.6 (Tree Star, Ashland, OR).

Western Blot

Protein lysates obtained with cell lysis buffer (Cell Signaling Technology, Danvers, MA) were fractionated by SDS-PAGE (Life Technologies) and transferred to nitrocellulose membranes (iBlot, Life Technologies). After an overnight (minimum of 18 hours) incubation of a 1:1000 dilution of appropriate primary mAbs for p-ERK1/2 (Cell Signaling Technology), total ERK1/2 (Cell Signaling Technology), DUSP6 (Abcam, Cambridge, MA) or GAPDH (Santa Cruz Biotechnology, Dallas, TX) in Tris-buffered saline with 0.1% v/v Tween 20 (Cell Signaling Technology) and 5% w/v bovine serum albumin (Sigma-Aldrich, St. Louis, MO), the membranes were counterstained with a 1:5000 dilution of the appropriate peroxidase-conjugated secondary IgG (Cell Signaling Technology). Blots were visualized with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL) on a ChemiDoc XRS+ imager using Image Lab software (Bio-Rad).

Phosphoprotein detection by multiplex bead assay

Concentrations of phosphoproteins in cell lysates obtained with the manufacturer's cell lysis solution (Bio-Rad) were analyzed using a Luminex Bio-Plex 200 system (Bio-Rad) according to the manufacturer's protocol. Ratios of the relative detection levels of phospho- to total protein were used to generate values.

Statistical Analysis

Significance was calculated using repeated measures ANOVA when necessary, followed by *post hoc* tests for significance between experimental groups (e.g. Bonferroni or Sidak multiple comparison tests). For single-group analysis, two-tailed Student's *t*-tests were used to determine significance. In all cases, $p < 0.05$ were considered statistically significant. Data analysis was performed with GraphPad Prism v6.0b for Macintosh (GraphPad software, San Diego, CA, USA).

3. RESULTS

3.1. CCL2 stimulation promotes mild M1 phenotypes in macrophages

Due to the disparate nature of previously published studies on the role of CCL2 in macrophage polarization [14–18], we first sought to determine if CCL2 could drive polarization of bone marrow-derived macrophages (BMDMs) in a similar fashion as standard M1 (IFN γ) and M2 (IL-4) polarizing cytokines. BMDMs were treated with CCL2 or indicated polarizing cytokines for 6 hours, and lineage-specific marker gene expression was analyzed via qPCR. Unlike the strong polarizing cytokine IFN γ , CCL2 failed to drive the expression of the M1 marker gene *Tnfa* (Fig. 1A). In a similar fashion, CCL2 failed to drive expression of M2 marker gene *Retnla*, whereas IL-4 drove strong expression of this gene (Fig. 1B).

As this apparent inability of CCL2 to drive macrophage polarization may have been due to differences in polarizing signal strength between cytokines and chemokines, we next investigated the effects of CCL2 dosage and stimulation time on macrophage lineage-specific gene expression. In response to high doses of CCL2 (250 ng/ml), BMDMs exhibited a rapid burst of *Nos2* mRNA expression (2 hours), with levels rapidly returning to baseline levels by 6 hours (Fig. 1C). Interestingly, low doses of CCL2 (10 ng/ml) showed an increase in *Nos2* mRNA expression at 6 hours, but as with the high-dose CCL2 treatment, this expression returned to baseline levels at later timepoints (Fig. 1C). Interestingly, higher doses of CCL2 (100 and 250 ng/ml) drove enhanced expression of *Tnfa* mRNA at later timepoints of tissue culture, with a peak at 48 hours post-stimulation (Fig. 1D). Interestingly, the expression of *Arg1* mRNA was significantly decreased following 2 hours of CCL2 stimulation, however this significance was only observed for the 100 ng/ml dose of CCL2 (Fig. 1E). At timepoints past 2 hours, all culture conditions exhibited time-dependent decreases in *Arg1* expression regardless of CCL2 stimulation dose (Fig. 1E). Expression of the M2 marker gene *Retnla* was not observable in any of the CCL2 culture conditions at any of the indicated timepoints, indicating that CCL2 alone does not drive expression of *Retnla* (data not shown).

3.2 The CCL2/CCR2 axis is preferentially upregulated by M1 stimuli

Next, we sought to determine if CCL2 was preferentially expressed by macrophages following either M1 (IFN γ /LPS) or M2 (IL-4) stimuli, and if the addition of exogenous CCL2 would affect the expression patterns of either CCL2 or CCR2. BMDMs were stimulated with CCL2 and/or macrophage polarizing signals, and the expression of *Ccl2* was analyzed by qPCR. CCL2 treatment alone did not induce the expression of *Ccl2* by BMDMs (Fig 2A). However, M1 stimuli was able to induce expression of *Ccl2* mRNA (Fig. 2A), with the greatest increase observed in cells that were treated with the TLR agonist LPS either alone or in combination with chemokine/cytokine (Fig. 2A). Concurrent increases in *Ccr2* mRNA expression were observed in M1 stimulated macrophages, with the strongest increases observed in response to IFN γ stimulation, either alone or in combination with chemokine or TLR agonist (Fig. 2B). M2 activation (IL-4) was able to induce *Ccl2* mRNA expression to a similar level as observed with IFN γ stimulation alone (Fig. 2C); however statistically significant expression of *Ccl2* was only observed with combined stimulation

with exogenous IL-4 and CCL2 (Fig. 2C). Additionally, the relative expression of *Ccl2* mRNA was far below the levels observed for LPS-dependent M1 activation (approximately 10-fold induction for IL-4 alone vs. approximately 100-fold for LPS alone). Additionally, the expression of *Ccr2* was significantly decreased in IL-4 treated BMDMs (Fig. 2D), in contrast to the increase in *Ccr2* expression observed in M1 stimulated macrophages.

3.3 CCL2 stimulation does not drastically affect macrophage morphology in vitro

Resting BMDMs were treated with CCL2, M1 (IFN γ /LPS) or M2 (IL-4) stimuli for 24 hours, and were subsequently fixed and stained for morphological analysis via microscopy. Resting BMDMs displayed standard macrophage morphology, with sizeable nuclei and cytoplasm, and an elongated shape indicative of their focal attachment to the tissue culture well surface (Fig. 3A). Treatment with CCL2 did not drastically change this morphology; while some BMDMs appeared to become rounded as compared to resting BMDMs (approximately 30–40%), still others maintained the elongated, attached phenotype (60–70%) (Fig. 3B). In contrast, treatment with IFN γ /LPS resulted in significant changes to BMDM morphology, with the vast majority of the cell culture adopting a rounded cell shape and extensive endosomal formation in the cytoplasm (Fig. 3C). Treatment with IL-4 exacerbated the elongated & attached phenotype of the BMDMs, with the overwhelming majority of the cells extending pseudopodia in opposing directions (Fig. 3D). In each case, the standard M1 (IFN γ /LPS) and M2 (IL-4) treatments had a more pronounced effect on cellular morphology than CCL2.

3.4 Pretreatment of macrophages with CCL2 enhanced subsequent responses to M1 stimuli

As CCL2 treatment alone did not appear to effect M1 or M2-type activation in BMDMs, we next sought to determine if initial exposure to CCL2 could bias the response of these cells to secondary exposure to M1 or M2 stimuli. To test this, BMDMs were either rested in minimal media or pretreated with CCL2 for 12 hours; following pretreatment, cells were stimulated with either M1 (IFN γ /LPS) or M2 (IL-4) stimuli, and subsequent marker gene expression was analyzed. In response to M1 stimuli, M1 marker gene expression was significantly enhanced in CCL2 pre-treated macrophages that were subsequently challenged with both inflammatory cytokine (IFN γ) and TLR agonist (LPS) (Fig. 4A). Similar effects were observed for other pro-inflammatory genes associated with M1 activation, such as *Tnfa* (data not shown). Conversely, there was no effect of M2 marker gene expression in IL-4 stimulated macrophages that were pre-treated with CCL2 (Fig. 4B). The effects of CCL2 pre-treatment on the M1 marker gene expression in IFN γ /LPS-treated macrophages appeared to be dose-dependent, as increasing doses of CCL2 resulted in increasing proinflammatory cytokine expression in response to subsequent exposure to IFN γ /LPS, both for mRNA (Fig. 4C) and protein (Fig. 4D).

3.5 Pretreatment of macrophages with CCL2 did not enhance expression of M1-related surface receptors

One possible explanation for the enhanced M1 responsiveness of CCL2 pre-treated macrophages is enhanced expression of cell surface receptors involved with M1 signal transduction including TLR4 and IFN γ R. To investigate this possibility, BMDMs were

treated with CCL2 and analyzed by flow cytometry for the expression of M1 receptors and surface markers. Interestingly, despite the enhanced M1 marker gene expression of CCL2 pre-treated macrophages, these cells did not exhibit increased expression of either IFN γ R (Fig. 5A) or TLR4 (Fig. 5B) following CCL2 pretreatment. Surprisingly, surface levels of IFN γ R appeared slightly reduced as compared to untreated BMDMs (Fig. 5A); however, this result was not supported by analysis of IFN γ R mRNA expression by CCL2 pre-treated BMDMs, where expression levels were statistically similar to untreated BMDMs (data not shown). CCL2 stimulation also appeared to have no effect on surface expression of the cognate receptor CCR2 (Fig. 5C). The chemokine receptor CCR7 can be used as a cell surface marker for classical activation [22]; therefore, CCR7 expression on CCL2 pre-treated macrophages was also analyzed as a secondary measure of classical activation in these cells. CCL2 pre-treated cultures exhibited a slight increase in the number of CCR7+ macrophages (34%) as compared to untreated macrophages (30%), however there was no statistically significant difference in the average expression of CCR7 on CCL2 pre-treated cells as compared to untreated BMDMs (Fig. 5D). In a similar fashion as gene expression analysis, these results suggested that while CCL2 might bias macrophages towards classical activation, it did not appear to directly drive polarization in the absence of other factors, such as cytokines or TLR agonists.

3.6 Enhanced ERK1/2 phosphorylation in response to IFN γ /LPS stimulation in CCL2 pre-treated macrophages

As our previous experiments had suggested that M1-associated receptor expression was not significantly modulated by CCL2 pre-treatment (Fig. 5A/5B), we next determined if intracellular signal transduction through these receptors was enhanced in classically activated BMDMs following CCL2 pre-treatment. BMDMs were treated with CCL2 followed by IFN γ /LPS, and phosphorylation of receptor-associated signal transduction proteins was assayed by Western Blot. Of the major classical activation signal pathways studied, the strongest modulation was observed in the MyD88-MAP3K-AP-1 system, manifested as enhancement of p42/p44 (ERK1/2) phosphorylation following IFN γ /LPS stimulation (Fig. 6A). Following pretreatment with CCL2, BMDMs exhibited sustained ERK1/2 phosphorylation as compared to untreated BMDMs. To quantify the results from Western Blot, intracellular lysates from untreated and CCL2 pre-treated BMDMs were analyzed for ERK1/2 phosphorylation using a bead-based system (Luminex). Analysis of replicate BMDM cultures confirmed a statistically significant enhancement of ERK1/2 signaling in CCL2 pre-treated BMDMs as compared to untreated BMDMs at 60 minutes following IFN γ /LPS stimulation (Fig. 6B).

To test whether enhanced ERK1/2 signal transduction was playing a role in the enhanced expression of M1 marker gene expression, we utilized a pharmacological inhibitor of ERK1/2 for *in vitro* restimulation assays. BMDMs were pre-treated with CCL2 and restimulated with IFN γ /LPS as before, with the addition of replicate conditions treated with either an equivalent volume of vehicle (DMSO) or the ERK1/2 inhibitor GDC-0994 (50 nM). While the untreated & IFN γ /LPS restimulated BMDMs did not show any change in *Nos2* expression in response to this dose of ERK1/2 inhibitor, the CCL2 pre-treated BMDMs exhibited a significant reduction in *Nos2* expression (Fig. 6C). These results

suggest that M1 marker gene expression in CCL2 pre-treated BMDMs is more reliant on ERK1/2 signal transduction as compared to untreated BMDMs.

3.7 Down-regulation of the ERK1/2 phosphatase Dusp6 and up-regulation of the Dusp6-targeting miRNA miR-9 in CCL2 pre-treated macrophages

The enhanced ERK1/2 phosphorylation observed in CCL2 pre-treated BMDMs suggested that CCL2 signaling had a suppressive effect on negative regulators of ERK phosphorylation. The dual specificity phosphatase (DUSP) family of proteins can regulate MAPK signaling by removing phosphoserine/threonine and phosphotyrosine residues from proteins such as ERK1/2 [23]. Specifically, DUSP6 is known to directly regulate signal transduction through ERK1/2 via dephosphorylation [24]. To determine if DUSP6 expression was modulated by CCL2 expression, BMDMs were treated with increasing concentrations of CCL2 and *Dusp6* mRNA expression was analyzed by qPCR. CCL2 pre-treatment resulted in a decrease in *Dusp6* mRNA expression by BMDMs, with the suppressive effect enhanced in response to increased CCL2 concentrations (Fig. 7A). These results were confirmed further with DUSP6 protein analysis by Western Blot, whereby DUSP6 protein levels were observed to be decreased in CCL2 pre-treated BMDMs (Fig. 7B).

We next searched for possible mechanisms for CCL2-mediated downregulation of DUSP6 expression. Studies using pharmacological inhibition of proteasome function in CCL2 pre-treated BMDMs indicated no effect on DUSP6 downregulation (data not shown), suggesting that the reduction in DUSP6 expression observed in these cells is due to regulation of mRNA expression/stability rather than post-translational regulation of DUSP6 protein. We therefore focused on the possible role of micro-RNA (miRNA) in mediating CCL2-dependent regulation of DUSP6. An *in silico* analysis (TargetScan) of the “seed” sequence in the 3′ untranslated region (UTR) of the DUSP6 mRNA identified two possible high-probability miRNAs with specificity for DUSP6, miR-9 and miR-145 (Fig. 7C). To confirm these findings *in vitro*, BMDMs were pre-treated with CCL2 prior to secondary stimulation with IFN γ and/or LPS, and miRNA expression was analyzed via sequence-specific cDNA synthesis and miRNA qPCR. No statistically significant variation was observed in miR-145 expression by BMDMs regardless of treatment or stimulation (data not shown). In contrast, expression of miR-9 was significantly elevated in CCL2 pre-treated BMDMs as compared to untreated, suggesting that miR-9 may be participating in the downregulation of DUSP6 in CCL2 pre-treated BMDMs (Fig. 7D). These results suggest that CCL2 may drive enhanced ERK signaling through promotion of miR-9 expression levels in macrophages, resulting in decreased DUSP6 expression that would then drive enhanced ERK1/2 signal transduction due to the loss of regulatory phosphatase activity.

4. DISCUSSION

Cytokine stimulation is a key component of macrophage skewing, with IFN γ and IL-4 known to drive classical and alternative activation of macrophages, respectively [1]. TLR ligands such as bacterial cell wall components (LPS or peptidoglycan) can also participate in driving macrophage activation, with LPS being a standard *in vitro* stimulus for the

generation of classically activated macrophages [25]. Chemokine expression has been used in previously published studies as a biomarker for classical vs. alternative activation, and regulation of chemokine and receptor expression on macrophages has been used to delineate macrophage subpopulations *in vivo*. For example, expression of CCR2 on circulating monocytes has been proposed as a surface marker for “inflammatory” macrophages that express genes typical of classical activation [26]. However, the ability of chemokines like CCL2 to have a direct effect on macrophage activation (as seen with cytokines like IFN γ and IL-4) is less clear. The results of this study indicate that while CCL2 does not directly drive macrophage skewing, it does enhance classical activation responses and signal transduction strength via regulation of ERK1/2 phosphorylation. These results provide evidence for CCL2 as an important mediator of macrophage inflammatory responses.

In this proposed model, CCL2 stimulation via CCR2 results in the upregulation of miR-9, by an as-yet unknown mechanism that relies on signal transduction through CCR2. Once transcribed and processed, mature miR-9 can then target DUSP6 mRNA for degradation and/or translational silencing by targeting the silencing RNA machinery (i.e. RISC complex) to the 3' UTR of DUSP6 mRNAs. This results in a decrease in DUSP6 protein in macrophages that have responded to CCL2. This decrease in DUSP6 levels does not result in an overt phenotype in the absence of MAPK signal transduction, as resting macrophages do not exhibit baseline levels of ERK1/2 phosphorylation (Fig. 6A). This model would explain the apparent inability of CCL2 stimulation to promote macrophage activation in the absence of additional cytokines or TLR ligands. However, following secondary stimulus with MAPK-dependent signals, such as LPS (via TLR4), this decrease in DUSP6 correlates with enhanced ERK1/2 phosphorylation, presumably due to the decrease in regulatory phosphatase activity, increased pro-inflammatory signal transduction and enhancement of inflammatory gene transcription. This model suggests the possible use of DUSP6 and miR-9 as biomarkers for classical activation potential, as well as implicates CCL2 signal transduction as an important initial step driving the development of classically activated macrophages during microbial infections.

While chemokines have not historically been considered skewing signals to the same extent as cytokines or TLR ligands, the expression of chemokine ligands and receptors has been used to help delineate classical vs. alternatively-activated macrophages in inflamed tissues *in vivo*. For example, expression of CCR7 is often used as a marker for classical activation in both murine and human macrophages [22], while CCL17 expression by lung-resident macrophages is often observed in inflammatory diseases with a strong alternatively-activated macrophage component, such as allergic asthma [27]. The role of CCL2 in driving macrophage-specific immune responses is less clear, and is dependent on the disease context in which the chemokine is studied. Early investigations into the role of CCL2 suggested that it played a central role in leukocyte recruitment to the lungs in T-helper type 2 granulomatous lung inflammation, suggesting that CCL2 was involved with alternative activation [28, 29]. Paradoxically however, CCL2-deficient mice have been shown to exhibit increased expression of alternative activation genes, suggesting that CCL2 may have a negative regulatory role on macrophage alternative activation [30]. Further complicating the study of CCL2 is the vast number of inflammatory disorders that are affected by CCL2, encompassing immune responses that are characterized by classical (e.g. inflammatory

bowel disease) and alternatively-activated (e.g. allergic asthma) macrophage functions [7]. Clearly, the role of CCL2 in macrophage activation and polarization remains an important, unresolved area of investigation in cellular immunology.

Previous studies have identified an important role for CCL2 in inflammatory pathologies normally dominated by classical activation. For example, modulation of CCL2 *in vivo* (i.e. treatment with recombinant chemokine or blocking antibody) has significant effects on LPS-induced mortality in an animal model of endotoxemia [20]. Additionally, adipose tissue macrophages from animal models of type 2 diabetes (T2D) often exhibit enhanced CCL2 production, and CCL2 deficiency in these animals leads to the increased development of alternatively-activated macrophages in adipose tissue [30, 31]. The results of this study provide one possible explanation for the ability of CCL2 to help potentiate the classical activation of macrophages in these disparate disease models. Unlike the effects of certain cytokines that can direct lineage-specific gene expression (e.g. IL-4 dependent production of Fizz1), CCL2 appears to “prime” macrophage responses to inflammatory stimuli, resulting in enhanced classical activation.

The dual specificity phosphatase family of proteins (DUSPs) regulates ERK signal transduction through the removal of phosphate groups on target kinases [32]. As ERK signaling is central to numerous biological processes, from activation to differentiation and effector function of numerous cell types, DUSPs therefore can regulate a wide range of responses. In addition, aberrant DUSP expression and function can release ERK signaling from normal physiological levels; this process is hypothesized to be important in the development of many human cancers [33]. As ERK signal transduction is a central component of LPS-dependent activation of macrophages, it follows that DUSP expression/function can have a dramatic effect on macrophage activation. Indeed, DUSPs have been studied extensively in the context of cellular immunology, and animal models of DUSP deficiency have identified roles for specific DUSPs (i.e. DUSP1, DUSP2 and DUSP10) in regulating peripheral inflammatory responses [34]. The results of our present study provide evidence for DUSP6 as a critical regulator of classical activation in macrophages through its ability to regulate ERK signaling. Critically, CCL2 appears to potentiate the classical activation of macrophages via downregulation of DUSP6, correlating with enhanced ERK1/2 phosphorylation and increased proinflammatory gene expression in response to LPS. This suggests a novel role for DUSP6 as a specific negative regulator of classical activation in macrophages.

The decreased expression of DUSP6 observed in macrophages pre-treated with CCL2 suggests a possible mechanistic link between CCL2 signal transduction and regulation of DUSP6 expression. Preliminary studies focused on the possible involvement of proteasome-mediated degradation of DUSP6 protein following CCL2 stimulation; however, experiments using pharmacological inhibitors of proteasome function showed no effect on DUSP6 downregulation following CCL2 stimulation (data not shown). These results suggest that DUSP6 expression in CCL2 pre-treated macrophages requires regulation at the mRNA level rather than at the protein level. Regulation of mRNA translation in eukaryotic cells is often governed by the expression of micro-RNAs (miRNAs) that direct enzymatic complexes to the 3' untranslated region (UTR) of complementary mRNAs, effectively blocking

translation through cleavage/degradation of target mRNAs and/or physical inhibition of mRNA interactions with the ribosome. An *in silico* search was performed to determine if any previously identified mRNA species exhibited complementarity to the 3' UTR of DUSP6 mRNA. Two specific miRNA species were identified, namely miR-9 and miR-145, with predicted specificity for DUSP6 mRNA. Analysis of CCL2 pre-treated BMDMs indicated no significant modulation of miR-145 expression (data not shown), however miR-9 expression was increased following CCL2 stimulation. These results suggest that the downregulation of DUSP6 observed following CCL2 stimulation may be due to regulation of Dusp6 mRNA by miR-9, allowing for increased ERK signaling and enhanced classical activation of macrophages.

It is important to note that there remain significant gaps in our understanding of the impact of CCL2 on macrophage polarization. Most notably is the underlying mechanism governing the time-dependent effect of CCL2 on the responsiveness of macrophages to subsequent M1 stimuli. Our studies indicate that while CCL2 can enhance M1 marker gene expression, it does not appear to drive M1 polarization on its own in a fashion similar to TLR ligands and inflammatory cytokine (Fig.1). Additionally, while CCL2 pre-treated BMDMs exhibited enhanced ERK1/2 phosphorylation, this was primarily observed at later timepoints following IFN γ /LPS stimulation, and not necessarily at early timepoints (15 minutes post-stimulation) (Fig. 6). These results indicate that the ability of CCL2 to promote M1 responses may reside not only in a well defined range of concentrations, depending on the specific marker gene (as observed in Fig. 1C & 1D), but also in a small temporal window that may be susceptible to changes in timing and duration of the chemokine exposure. This may explain the lack of *Nos2* mRNA modulation in the untreated control groups in Fig. 6C, where BMDMs were subjected to multiple treatments of both inflammatory stimuli and/or pharmacological agents, in contrast to the more straightforward treatment protocol in earlier experiments. This is important to consider when placing these results in the context of tissue inflammatory processes *in vivo*, where monocytes are trafficking from the blood into inflamed tissues in response to chemokine gradients, including CCL2. Our report suggests that CCL2 helps to enhance M1 macrophage activation, although whether this enhancement occurs as a result of macrophage chemotaxis in response to CCL2, or as a result of macrophages becoming activated in a local tissue microenvironment that is rich in both M1 stimuli and CCL2, requires additional investigations to answer fully.

5. CONCLUSIONS

The CCL2-CCR2 chemokine axis participates in immune responses characterized by classical activation, and CCR2 expression has been used in previously published reports as a marker for “inflammatory” macrophages in tissues. Additionally, increased CCL2 levels have been observed in inflammatory environments with increased classical activation of macrophages, such as in adipose tissues of Type 2 Diabetes patients. The results of this study suggest that the CCL2-CCR2 chemokine axis serves to enhance ERK-dependent signal transduction, possibly through epigenetic regulation of DUSP6, a negative regulator of ERK1/2 phosphorylation. These findings provide a new conceptual role for chemokine stimulation in macrophage activation, whereby chemotactic factors can affect the magnitude of inflammatory responses through modulation of signal transduction pathway strength.

Acknowledgments

The authors would like to thank Linxuan Hao and Alex Dean for technical assistance, Robin Kunkel for assistance with graphic design and Judith Connett for critically reading the manuscript.

FUNDING SOURCES

This work was supported by NIH R01 HL031237 (to SLK) and K08 DK102357 (to KAG).

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HIGHLIGHTS

1. The C-C chemokine CCL2 enhances macrophage responses to pro-inflammatory stimuli.
2. CCL2 enhances ERK signal transduction in classically activated macrophages.
3. CCL2 suppresses macrophage expression of the ERK phosphatase DUSP6.
4. CCL2 increases macrophage expression of miR-9.

FIGURE 1.

Gene expression by BMDMs following stimulation with CCL2. A&B) Cells were treated with the indicated stimuli for 6 hours, and mRNA expression of A) *Tnfa* and B) *Retnla* was determined by qPCR. CCL2=100 ng/ml, IL-4=10ng/ml, IFN γ =10 U/ml. (*) = $p < 0.05$ vs. Control. C–E) BMDMs were treated with the indicated concentrations of CCL2 and mRNA expression was analyzed by qPCR. Results are representative of two independent experiments. For (C) and (D), (*) = $p < 0.05$ vs. all groups at the specific timepoint; For (E), (*) = $p < 0.05$ vs. 0 and 10 ng/ml CCL2.

FIGURE 2.

Regulation of the CCL2/CCR2 axis by extracellular signals. BMDMs were treated with the indicated stimuli for 6 hours, and mRNA expression of A&C) *Ccl2* and B&D) *Ccr2* was determined by qPCR. CCL2=100 ng/ml, IL-4=10ng/ml, IFN γ =10 U/ml, LPS=100ng/ml. Results are representative of three independent experiments. (*) = $p < 0.05$ vs. control.

FIGURE 3.

Effects of CCL2 stimulation on BMDM morphology *in vitro*. BMDMs were either A) left unstimulated, or treated with B) 100 ng/ml CCL2, C) 10 U/ml IFN γ + 100 ng/ml LPS or D) 10 ng/ml IL-4 for 24 hours in Lab-Tek 8-well chambered glass slides, with 2 replicate wells for each culture condition. Following incubation, cells were fixed with methanol and stained with Diff-Quik. A minimum of five distinct fields were observed in each replicate well via light microscopy. Representative images were captured using CellSens software. Images are representative of two independent experiments.

FIGURE 4.

Enhancement of classical activation gene expression in CCL2 pre-treated BMDMs. Following maturation in L-cell conditioned media, BMDMs were replated in either minimal media or media supplemented with 100 ng/ml CCL2. A&B) Following pre-treatment with CCL2 for 18 hours, BMDMs were treated with the indicated cytokines/TLR ligands (IFN γ =10 U/ml, LPS=100ng/ml, IL-4=10 ng/ml); 6 hours post-stimulation, mRNA was isolated for analysis by qPCR. Results are representative of four independent experiments. C) BMDMs were supplemented for 18 hours with varying concentrations of CCL2, followed by IFN γ /LPS for an additional 6 hours. mRNA was then isolated for analysis by qPCR. Fold expression levels were generated compared to untreated and unstimulated BMDMs that were cultured alongside the stimulated cells for the indicated time periods. Results are representative of three independent experiments. D) BMDMs were supplemented for 18 hours with varying concentrations of CCL2, followed by IFN γ /LPS for 24 hours. Supernatants from cultured BMDMs were then analyzed by multiplex bead assay (Luminex). Results are representative of two independent experiments. (*) = $p < 0.05$ vs Untreated (A&B) or 0 ng/ml CCL2 (C&D).

FIGURE 5.

Expression of activation markers on CCL2 pre-treated BMDMs. Following maturation in L-cell conditioned media, BMDMs were replated in either minimal media or media supplemented with 100 ng/ml CCL2. The following day, cells were treated with 10 U/ml IFN γ + 100 ng/ml LPS for 24 hours, and cells were fixed and stained for flow cytometry. Expression of A) IFN γ R, B) TLR4, C) CCR2 and D) CCR7 in PE-fluorescence was observed on F4/80+ BMDMs. Long dashed line: no PE antibody, Solid line: Untreated, Short dashed line: CCL2 pre-treated BMDMs. Results are representative of two independent experiments.

FIGURE 6.

Enhancement of ERK 1/2 signaling in CCL2 pre-treated BMDMs. A) Untreated or CCL2 pre-treated BMDMs were stimulated with 10 U/ml IFN γ + 100 ng/ml LPS for the indicated timepoints, cells were lysed and ERK1/2 phosphorylation was analyzed by SDS-PAGE electrophoresis and Western Blot. Results are representative of three independent experiments. B) Untreated or CCL2 pre-treated BMDMs were stimulated with IFN γ /LPS for 60 minutes, cells were lysed and ERK1/2 phosphorylation was quantitatively analyzed via Luminex bead assay. Results are representative of two independent experiments. (*) = $p < 0.05$ vs. Wild-type. C) Untreated or CCL2 pre-treated BMDMs were restimulated with IFN γ /LPS for 6 hours, with additional samples treated with either vehicle control (DMSO) or the ERK1/2 inhibitor GDC-0994 (50 nM). Expression of *Nos2* mRNA was analyzed via qPCR. (**) = $p < 0.01$ vs. Untreated.

FIGURE 7.

Regulation of DUSP6 and miR-9 expression in BMDMs by CCL2. A) Following maturation in L-cell conditioned media, BMDMs were treated with the indicated concentrations of CCL2 for 18 hours, and expression of *Dusp6* mRNA was analyzed via qPCR. Results are representative of two independent experiments. B) BMDMs were treated with the indicated concentrations of CCL2 for 18 hours, and DUSP6 protein expression was analyzed via SDS-PAGE electrophoresis and Western Blot. Results are representative of two independent experiments. C) Schematic representation of the DUSP6 3' UTR and putative matching seed regions for miR-9 and miR-145, adapted from an *in silico* study performed using TargetScanMouse6.2 (www.targetscan.org). D) Untreated or CCL2 pre-treated BMDMs were stimulated with the indicated cytokines/TLR ligands for 6 hours, and miR-9 expression was analyzed by miR-specific cDNA synthesis followed by qPCR. Results are representative of two independent experiments. (*) = $p < 0.05$ vs. unstimulated.