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Research paper

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 Toll-like 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.

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 (IFNγ). 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

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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 chemotactic/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 (IFNγ) 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

2.1. 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.

2.2. 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 h. Following this rest phase, BMDMs were pre-treated in certain conditions with CCL2 (R&D Systems, Minneapolis, MD) for a period of 12–18 h, were indicated. Stimulations with IFNγ (Shenandoah Biotechnology, Warwick, PA), IL-4 (Shenandoah), and LPS (0111:B4, Sigma, St. Louis, MO) were performed following the rest and pre-treatment phase. Reported endotoxin levels in the utilized recombinant cytokines are as follows: <0.01 EU/μg for CCL2 and <1 EU/μg for IFNγ 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.

2.3. 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.

2.4. 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).

2.5. 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: